Draft revised Guidance Document 75 describing the Honey Bee (Apis mellifera L.) Brood Test under Semi-Field Conditions

For first WNT-review by 24 January 2024

DRAFT 29 Nov 2023

Guidance Document on the Honey Bee (*Apis mellifera* L.) Brood Test under Semi-Field Conditions

Revised Draft Version: November 2023

ICPPR honey bee brood working group

Eds.: Abdulrahim Alkassab (Julius Kühn Institute), Markus Barth (BioChem Agrar GmbH), Christian Berg (FMC), Sigrun Bocksch (Eurofins), Kyongmi Chon (RDA), Jakob H. Eckert (Julius Kühn Institute), Lukas Jeker (Agroscope), Stefan Kimmel (Corteva Agriscience), Johannes Lückmann (RIFCON GmbH), Olivier Mamet (Testapi), Markus Persigehl (tier3 solutions GmbH), Rastislav Sabo (NRL UVLF), Christof Schneider (BASF), Thomas Steeger (US-EPA), Verena Tänzler (Bayer AG), Bettina Wenzel (IES Ltd.), Selwyn Wilkins (Fera).

Deleted: . The Honey Bee Brood Test is conducted

Deleted: semi-field conditions and enables the quantitative assessment of adverse effects of plant protection products on the development of the

Deleted: 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.

Deleted: 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In February 2006, the Secretariat circulated the initial draft Guidance Document to the WNT and to the Working Group on Pesticides, for comments.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.¶

DRAFT 29 Nov 2023

Table of contents

DRAFT 29 Nov 2023

1 INTRODUCTION

1. According to currently established decision-making schemes for the environmental risk assessment of pesticides and other chemicals a honey bee (*Apis mellifera* L.) brood test may be required if honey bee brood (defined as developing eggs, larvae and pupae) is potentially exposed and/or affected. The laboratory methods for acute (single dose) and chronic (repeated dose) tests with honey bee larvae are covered by OECD Test Guideline (TG) 237 and OECD Guidance Document (GD) 239, respectively. The following method can be used as a higher-tier semi-field study to further refine the understanding of the potential effects of pesticides and other chemicals on the development and performance of honey bee colonies.

DRAFT 29 Nov 2022

2 BACKGROUND

2. The purpose of this Guidance Document is to provide a semi-field test method for the quantitative assessment of adverse effects of pesticides and other chemicals on honey bee brood under more realistic exposure conditions and application procedures that are used for laboratory-based studies. The honey bee brood test is designed to assess the possible impact of pesticides and other chemicals on the development of the honey bee brood. The OECD GD 75 (2007) is intended for evaluating applications on highly bee attractive surrogate plants and is based on the studies of Oomen *et al.* (1992), Mühlen (1996), Tornier (1999), Schur *et al.* (2003) and European and Mediterranean Plant Protection Organization (EPPO) Guideline No. 170 (2010). The GD 75 has been updated based on the outcome of the analysis of the main endpoint "Brood Termination Rate" from Pistorius *et al.* (2012), Becker *et al.* (2015) and Szczesniak *et al.* (2018), recommendations of the European Food Safety Authority (EFSA) revised bee guidance document (2023), technical improvements (*i.e.*, digital brood assessments according to Jeker *et al.* (2011), Wang & Classen (2011)) and current experiences provided by the International Commission for Plant-Pollinator Relationships (ICPPR) bee brood group.

DRAFT 29 NOVEMBER 2023

3 SEQUENTIAL TESTING STRAT

3. The method described in this guidance document is designed to assess potential effects of pesticides and other chemicals on developing brood, and has been validated with honey bee brood, under semi-field (tunnel) conditions using a reference substance (*e.g.*, fenoxycarb; ethyl [2-(4-phenoxyphenoxy)-ethyl] carbamate (CAS No. 72490-01-8)) which is known to affect brood development. The aim of this test is to complement the sequential testing scheme with an improved test method under semi-field conditions and to produce quantitative data at the colony level that can be used for the evaluation of pesticides and other chemicals.

4. The Guidance Document is founded on the assumption that the most reliable risk assessment is based on data collected under conditions which closely resemble standard plant protection and bee-keeping practice; whereas laboratory tests are intended as lower-tier assessment tools which may be used to screen and/or identify specific acute or chronic effects on adult and developmental stages of honey bees.

5. Preliminary screening can be made by using *in vitro* bee brood-feeding (*e.g.*, OECD TG 237; OECD GD 239) and adult bee contact (OECD TG 214) and oral tests (OECD TG 213; OECD TG 245). Therefore, if any effects are detected in such laboratory tests, a higher-tier semi-field colony-level test as described in this Guidance Document might allow for a more quantitative assessment of the effects on brood within the honey bee colony.

6. As demonstrated by the use of the reference substance fenoxycarb, the methodology described in this guidance has proven effective in detecting direct effects on brood development and indirect effects on colony strength (*e.g.*, increased brood termination rate leading to reduced numbers of adult worker bees) as well.

DRAFT 29 July 2009

Deleted: FOREWORD 11¶
Introduction 13¶
Sequential testing strategy 13¶
Applicability of the test 14¶
Description of the test 15¶
Principle of the test 15¶
Experimental conditions 16¶
Design of the test 16¶
Preparation of the colonies 16¶
Test conditions 17¶
Application of treatments 17¶
Test product 17¶
Mode and time of application 17¶
Dosing 17¶
Assessments 17¶
Duration of the study 17¶
Meteorological data 17¶
Mortality of honey bees 18¶
Flight activity 19¶
Brood assessments 20¶
Evaluation of the test results 22¶
Brood termination-rate 23¶
Brood-index 23¶
Compensation-index 23¶
Statistical Analysis 24¶
Report 24¶
References 25¶
ANNEX I 27¶
Abbreviations 27¶
Glossary 27¶
¶ ...

Deleted: most

Deleted: normal

Deleted: ,

Deleted: might be considered convenient basic

Deleted: in addition

Deleted: clarify

Deleted: scientific issues. Field test results should be regarded as decisive when conclusions from ...

Deleted: 3.

Deleted: an

Deleted: test.

Deleted: a

Deleted: feeding test, or in

Deleted: qualitative tunnel or

Deleted: by Oomen *et al.* (1992), a 2nd tier brood test as described in the

Deleted: brood.

Deleted: Insegar (Fenoxycarb) potential effects on pupae and adult worker bees can be detected as well ...

Deleted: check of the brood effects might deliver an acceptable degree of reality as well as certainty

Deleted: 4. The method described in this guidance document was designed to assess the effects of plant ...

4 APPLICABILITY OF THE TEST

7. The test allows the assessment of data regarding potential effects of pesticides or other chemicals on colony performance in terms of honey bee brood as well as adult bee mortality, foraging activity, behaviour and overall colony development as a result of exposure to the test chemical applied to bee-attractive flowering crops. Pesticides and other chemicals of different types, and with different time and mode of application (*e.g.*, including, but not limited to seed treatment, application during night time, application before flowering) to which honey bees may be exposed, can be evaluated using this test method as long as the test chemical is transferred by foraging bees to the larvae/into the hive.

8. Compared to *in vitro* laboratory-based studies with individual honey bee larvae the method has the following advantages:

- The brood is developing in its natural environment inside the hive.
- The colonies are put into a realistic worst case exposure condition by the test design, in terms of contact exposure and ingestion of residues in pollen and nectar of treated plants.
- It is possible to evaluate the application of nearly all types of application scenarios (pre-flowering/full flowering), formulations and treatments (*i.e.*, sprays, wettable granules and powders, products for soil application and seed treatment). However, different application methods will require appropriate adaptation of the study design.
- It is possible to quantitatively evaluate the effects of pesticides and other chemicals to the bee brood and the corresponding changes in the colony within the hive comprising at least one complete bee brood cycle (*i.e.*, egg to adult bee emergence).
- The method (*i.e.*, detailed digital brood assessments) can also be transferred and used in higher tier field studies.

Limitations of the test:

- The test can be impacted by adverse climatic conditions; being conducted too late in season; or being conducted in a manner which is not consistent with good bee keeping practice (*e.g.*, interfering with Varroa mite (*Varroa destructor*) treatment procedures).
- Low daytime temperatures (*e.g.*, < 12°C) may limit foraging activity of the bees in the treated crop and thereby limit exposure to the test chemical.
- High daytime temperatures (*e.g.*, > 30°C) may reduce foraging activity and nectar secretion.
- High or low daytime temperatures may inhibit successful brood development and therefore put the endpoint of detailed brood assessment at risk.
- Adverse weather conditions (*e.g.*, precipitation) during the exposure period can affect exposure (*i.e.*, residue levels on plants) and bee foraging activity and should be avoided to the extent possible.
- Enclosure stress on colony under semi-field (tunnel) conditions may cause reduction in the number of bee brood (*i.e.*, caging effect).
- Stress resulting from experimental manipulations while measuring colony conditions may influence brood survival and colony behaviors.

DRAFT January 2022

Deleted: 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

Deleted: by

Deleted: contaminated

Deleted: tested according to

Deleted: substance

Deleted: taken up by the worker bees and

Deleted: . The method is suitable for all types of bee hives with movable frames, but not for the use of skeps

Deleted: 6.

Deleted: experiments

Deleted: brood

Deleted: shows some

Deleted: growing up

Deleted: in

Deleted: and is not disturbed by artificial test conditions

Deleted: bees and their brood

Deleted: an acceptable

Deleted: situation

Deleted: Because of the practical conditions of the test design

Deleted: will be possible,

Deleted: .

Moved (insertion) [1]: Limitations of the test:¶

Deleted: . Different

- High variability of the brood termination rate within control or treatment groups can occur.
- Testing of herbicides applied to flowering crops (*e.g.*, broad leaf herbicides) may reduce plant vitality and exposure to the bees.
- Crops not attractive to bees are not suitable for the test.
- Limited bloom duration/forage capacity within the tunnel typically limits the duration of exposure to 7 – 10 days.

DRAFT 29 Nov 2023

5 DESCRIPTION OF THE TEST

PRINCIPLE OF THE TEST

9. Small healthy honey bee colonies (*e.g.*, nucleus colonies or "nucs") are initially placed in the constructed enclosures (hereafter referred to as "tunnels") shortly before full flowering of the bee-attractive crop. For foliar applications at flowering, the honey bee hives are introduced into the tunnels a few days before the intended application and the exposure phase starts with the application day. The test chemical is then applied to the flowering crop (*e.g.*, either while bees are actively foraging or after daily bee flight or shortly before daily bee activity while bees are confined to their colony) after which the bees are allowed to forage within the tunnel; this is the "exposure phase" of the study. However, different modes of application require appropriate adaptation of the study design. For pre-flowering applications or seed treatment scenarios, the honey bee hives are introduced a few days before the application of the reference item and control, but exposure to the test item starts with the placement of the hives in the tunnels.

Following exposure phase of the bees in the tunnel during flowering of the crop (*e.g.*, at least 7 days after application of the test chemical), the hives are then placed outside the tunnel to a monitoring site for the remainder of the study and are free to forage under full-field conditions; this is referred to as the post-exposure "monitoring phase" of the study.

There should be no mass-flowering crops in the vicinity of the monitoring site. Information on the landscape surrounding the monitoring site can be provided in the raw data as support (*e.g.*, via geographic/agricultural landscape internet portals). Assessments are conducted several times over a period of at least 4 weeks after the initial brood evaluation. Results are evaluated by comparing the treated colonies with the water-treated control colonies (negative control) and with the reference substance-treated (positive control) colonies. Each brood cycle is 21 days and it is possible to monitor the colonies for multiple brood cycles. Protocols should specify the number of brood cycles that will be evaluated during the post-exposure phase of the study.

DRAFT 09 NOV 2023

Deleted: ¶
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.

Moved up [1]

Deleted: 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

Deleted: colonies

Deleted: initially placed in tunnel tents (herein after named "tunnels") shortly before full flowering of the crop,

Deleted: test chemical.

Deleted: for the period of

Deleted: .

Deleted: product

Deleted: remaining

Deleted: 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

Deleted: assessment

Deleted: chemical

Deleted: ¶
¶
¶
<object><object><object><object><object><object><object>¶
<object><object><object><object><object><object><object>¶
¶
¶
<object>¶
¶
¶
<object><object><object><object>¶
¶

10

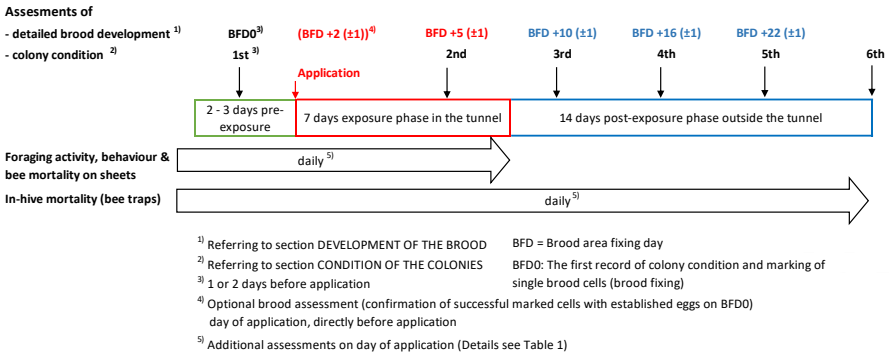

Figure 1. Example schedule of a bee brood study and foliar (spraying) application scenario

EXPERIMENTAL DESIGN

10. Worker honey bees of a queen-right colony forage in a tunnel containing a bee-attractive flowering crop treated with either the test chemical, water-treated negative control (except in seed treatment studies), or suitable reference substance-treated positive control.

Treatment groups	• test chemical treatment group(s) • water or formulation blank group (negative control) • reference substance treatment group (positive control)
Application rates	• Test chemical: normally applied at the highest labelled single application rate. • Untreated control group: tap water volume according to good agricultural practice (GAP) recommendation (*e.g.*, 200-400 L/ha). If a solvent or adjuvant is used in the preparation of the test material, then a solvent/adjuvant control should be used in addition as well. • Reference substance treatment group (*e.g.*, fenoxycarb at 300 g a.s./ha); other active substances with known properties of an insect growth regulator (IGR) may be used as a reference substance, but sufficient dosing and corresponding effects on brood (*i.e.*, larvae and pupae) and brood termination rate (BTR) need to be demonstrated. • Additional reference substance treatment group (*e.g.*, dimethoate at 400 g a.s./ha) may be included to detect other non-related brood treatment effects (*e.g.*, adult mortality). All spray applications should be made using the same water volume (where alternative modes of application are being investigated, such as seed or soil treatments, this is only applicable for the control and reference substance).
Replicates	It is suggested to run the test with at least four replicates; However, where possible larger numbers of replicates improve the ability of the study to detect/document treatment effects. As an option, if two reference substance groups are included, the replicate number of each reference group may be reduced (*e.g.*, to three tunnels per reference substance group instead of four). Additional replicates may be included for the collection of residue samples for assessment of exposure. These additional replicates should not be used for effects assessments.

Deleted: : Timescale

Deleted: 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

Deleted: 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.

Deleted: <#>Experimental conditions¶
10.

Deleted: small

Deleted: are forced to

Deleted: an

Deleted: . 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

Deleted: in the

Deleted: tunnels has to be done at the same time period as…

Duration	Observation of the brood development may cover one or multiple brood cycles if deemed appropriate. The time schedule of the brood assessment days (see **Table 4**) was chosen to check the bee brood at different expected stages during the development with the assumption that worker bees typically require 21 days for one brood cycle and to emerge as adults. If a second brood cycle is evaluated, a new batch of eggs and larvae should be marked at BFD+21 of the first brood cycle or after end of exposure in the tunnels (*e.g.*, BFD+10).

PREPARATION OF THE COLONIES

11. Apparently healthy honey bee colonies should be used for the test. All colonies used in the test should be produced at the same time. Honey bee queens should be the same age, in a reproductive phase, and preferably not more than 2 years old (*i.e.*, queens from the previous bee season). Sister-queens should be used if possible. Initial colony strength should depend on regional and seasonal conditions and based on the available crop area per tunnel but should be equal and comparable across all tunnels. Studies conducted in central-Europe indicated that a tunnel area of ≥ 60 m² Phacelia (*Phacelia tanacetifolia*) and a colony size of about 6,000 to 8,000 adult worker bees per colony is suitable (Pistorius *et al.* (2012), Becker *et al.* (2015) and Szczesniak *et al.* (2018)).

12. Daily bee mortality (*i.e.*, adults and brood), adult bee number, and the number of brood cells within each of the colonies should be as homogeneous as practically possible at study start. Moreover, colonies should consist of at least 2-3 brood combs (depending on the bee hive type) and all brood stages (*i.e.*, eggs, larvae and pupae [capped cells]), should be present in each colony. The colonies should contain enough pollen and nectar/honey to guarantee adequate food reserve to avoid starvation and to maintain brood rearing activity inside the tunnels. All colonies should be well balanced with regard to food stores, number of brood cells and adult bee strength before the start of exposure. This should be achieved at least one week before introduction of the colonies to the tunnels. To reduce variability, it is recommended to prepare a surplus of colonies and select the most suitable ones based on the collected data before brood area fixing day (BFD) 0 (see section BROOD ASSESSMENTS). If colonies differ in size or background mortality levels, colony strength should be uniformly distributed among the treatment groups.

13. Bees should be free of clear clinical signs of bee diseases (*i.e.*, viral, fungal, bacterial) and parasites. Medical treatments against pests and pathogens within 4 weeks before the start of the test should be avoided as far as practicable. If medical treatment (*e.g.*, varroa treatment) of the colonies is necessary, all colonies should be treated equally and at the same time. The rationale for a medical treatment should be clearly articulated in the study report and be consistent with local best beekeeping practices.

14. For a good acclimatisation, the colonies should be set-up in the tunnels shortly before full flowering (BBCH 61-63; Meier (2018)) of the crop and at least two days before application. Depending on the type of bee hive used, dead bees should be removed from the bottom of the hives after set-up in the tunnels. The colonies should be exposed to the treated crop in the tunnels for a period of at least 7 days after the application. Adaptations can be made according to application scenarios and weather conditions.

15. Avoid supplemental feeding during the exposure phase of the study (tunnel phase). If feeding (*e.g.*, supplemental sugar and/or protein) of the colonies is necessary after the exposure phase, all colonies should be treated equally (*i.e.*, the same source and amount of offered food) and at the same time. The rationale to provide supplemental food should be clearly articulated in the study report and be consistent with local best beekeeping practices.

Deleted: chemical tunnels, in order to ensure

Deleted: conditions (weather conditions, flight

Deleted:) for application for a direct comparability of the treatments

Deleted: ***<#>Design of the test***¶
11. Each test should include 3

DRAFT 09 May 2023

TEST SET-UP

16. Tunnels are placed on the crops before flowering (BBCH ≤ 60) a few days before experimental start (see **Figure 2**). Tunnels with a minimum size of 60 m^2 area of treated crop should be used. All tunnels within a study should cover then same area and have the same dimensions in terms of length, width and height. The test crop should be attractive to honey bees as a source of both nectar and pollen. Suitable crops include but are not limited to Phacelia (*Phacelia tanacetifolia*), mustard (*Sinapis* sp.) and oilseed rape (*Brassica napus*). The test crop should be planted and maintained according to the recommendations for GAP to guarantee a sufficient plant density. Irrigation during growth and drip irrigation during flowering inside the tunnels is recommended, when necessary, to guarantee sufficient nectar flow during the exposure-phase.

17. During the whole testing period the colonies should be supplied with fresh water. A water source should be placed into each tunnel as a water supply for the bees. Water sources for bees within the tunnel should contain floating aids to prevent drowning and should be removed from the tunnel during application of the test chemical and reference substance to prevent contamination. Direct over spraying of the hives or bee traps should be avoided.

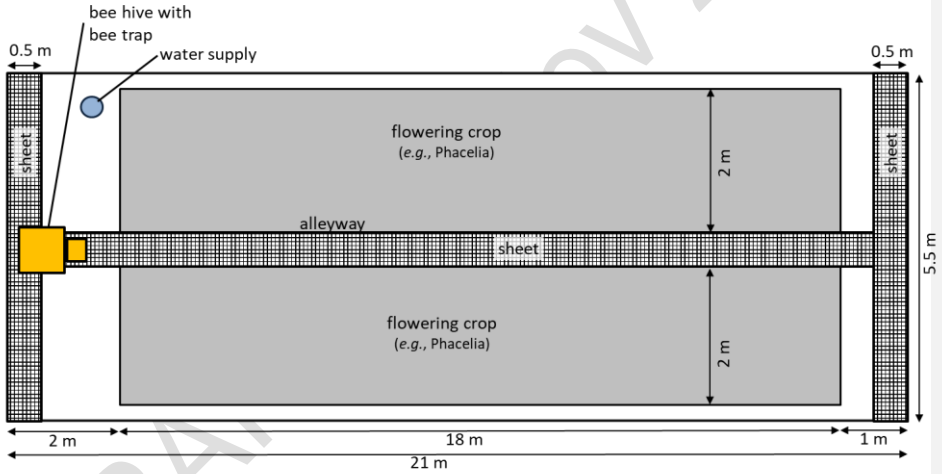

Figure 2. Example sketch of tunnel set-up

18. The gauze covering the exterior of each tunnel should have a maximum mesh size of 3 mm. The tunnels should be separated from one another by at least 2 meters and 2 meters to the field borders. Each tunnel should be subdivided in the middle by a cleared alleyway, which serves as a walkway for conducting the application and as a means of observing dead/debilitated adult bees. Additionally, at the front and back sides of each tunnel the plants should be removed, and the bare ground covered with sheets for a similar purpose. Total sheet area should be the same for all tunnels.

DRAFT Nov 2025

APPLICATION OF TREATMENTS

TEST CHEMICAL

19. Typically, the use of formulated products is preferred. However, this may be modified if appropriate for the objectives of the study. Adaptions should be described in detail in the study plan and study report.

MODE AND TIME OF APPLICATION

20. Typically, the products should be applied at the time of full flowering of the crop (*e.g.*, BBCH 63-65) during the daytime, during full bee flight and foraging activity (*e.g.*, for Phacelia, an average of ≥ 5 foraging bees/m^2 per tunnel should be counted at a given time (*e.g.* see par. 27-29), to ensure that the bees and colonies are exposed. However, this may be modified if appropriate for the objectives of the study (*e.g.*, when testing systemic compounds applied pre-flowering, seed dressings, spray and soil-applied products), or application prior to or after bee flight (*e.g.*, twilight or when bees are manually confined to colony).

21. The treatments (negative control, reference substance, test chemical) should be applied with appropriate equipment (*e.g.*, calibrated boom sprayer) according to good agricultural practice. Spraying of the tunnel's covering gauze should be avoided.

22. The application of the different treatment groups should be carried out as reasonably possible to ensure the same conditions (*i.e.*, weather conditions, foraging activity) for application. If a high number of treatments/replicates is required, the use of additional spraying equipment should be considered.

23. The wind speed should not exceed 3 m/sec measured outside the tunnels. There should not be any rainfall before directly sprayed applications have dried (*e.g.*, for at least 2 h after application).

ASSESSMENTS

DURATION OF THE STUDY

24. Pre-application period (colony acclimatisation period) should be at least two full days. The total observation period of the colonies following application is at least 28 days (7-day exposure period; 21 day post-exposure monitoring phase); as an option post-exposure monitoring may extend for one or more brood cycles (*e.g.*, 42 day post-exposure phase (second brood cycle)).

METEOROLOGICAL DATA

25. 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)
- Optional: cloudiness as an additional parameter to relate to changes in foraging activity

MORTALITY OF THE HONEY BEES

26. The assessments of the number of dead bees should be carried out at approximately the same time of day, preferably in the morning (**Table 1**). Mortality of honey bees should be assessed on sheets suitable for the collection of bees (*e.g.*, linen sheets) which are spread out at the front, middle and back of

DRAFT 29 Nov 2022

Deleted: :¶
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 or[...]

Deleted: exposure of the brood and to prevent that larvae feed on uncontaminated food. The ratio brood to food (nect[...]

Deleted: nozzles

Deleted:

Deleted: 19. Application

Deleted: normally

Deleted: performed at the time of full flowering of

Deleted: crop and during full bee flight or, when

Deleted: (e.g. for testing of residual or delayed action), in accordance with

Deleted: intended

Deleted: pattern of the product (normally late morning).

Deleted: 2

Deleted: tunnel.

Deleted: ***<#>Dosing***¶[...]

Deleted: . Based on pre-application data, information should be available on when high flight activity should[...]

Deleted: ***<#>Meteorological data***¶
22.

Deleted:);

Deleted:);

Deleted:);

Deleted: <#>wind speed (only during application inside and outside the tunnel);¶[...]

Deleted: .

Deleted: .

Deleted: in front of the hives and

the tunnels. Before the start of the test, paths are laid down in each tunnel by removing of the plants and by smoothing the bare ground. Subsequently, the path is covered with the aforementioned linen/plastic sheets in order to facilitate the collection of the dead bees in the tunnels. All hives should also be equipped with a dead bee trap (Illies *et al.* (2002)) at the colony entrance to facilitate counting of dead bees. The assessments will be done according to the schedule listed in **Table 1**. The number of dead bees should be separated into adult worker bees, males (drones), sum of worker bee larvae/pupae and drone larvae/pupae (in rare cases where larvae are found they will be counted together with the pupae for worker bees and drones).

Table 1. Honey bee (*Apis mellifera*) mortality assessment schedule

Schedule	Assessments*
over at least two days before application	once a day at the same time of the day
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
up to day +28[1)] after application (out of the tunnels; only in bee traps)	once a day at the same time of the day

* Remark: At each evaluation day the dead bees should be counted and removed.

[1)] Additional assessments for a second brood cycle up to day +42 and to cover a third brood cycle up to day +63

FORAGING ACTIVITY

27. Foraging is defined as bees that are actively foraging on flowers to collect nectar or pollen, not just flying over the crop.

28. Adult bee foraging activity should be recorded on a 1 m² area, at 3 different places in each tunnel according to the schedule summarized in **Table 2**. At each assessment time the number of bees that are foraging on flowering plants will be counted for a short time period (snap-shot; depending on the crop for example at least 10-15 seconds in Phacelia) per selected area. Any abnormal adult bee activity (*e.g.*, lethargy, loss of coordination, excessive self-grooming, convulsions) should be recorded.

Table 2. Honey bee (*Apis mellifera*) foraging assessment schedule

Schedule	Assessments
over at least two days before daytime application	once a day at the same time of the day
on the day of daytime application	• shortly before application • 2 times during the first hour following application • 2 h after application • 4 h after application • 6 h after application
on the days following application	once a day at the same time of the day
during the exposure period in the tunnels	once a day at the same time of the day

DRAFT 29 Nov 2023

Deleted: will be created
Deleted: will be
Deleted: (area covered approx, 8 m²)
Deleted: crop area. Additionally the dead bees will be noted and counted in the
Deleted: traps which are fixed
Deleted: of the hives.
Deleted: time table presented
Deleted: ,
Deleted: males.
Moved (insertion) [2]: ¶ Table
Deleted: ¶
Deleted Cells
Deleted: ← 4m →
Deleted Cells
Deleted Cells
Moved (insertion) [3]: once a day at the same time of the day
Deleted: 🖶
Moved (insertion) [4]: in the evening after daily flight activity of the bees
Deleted: ← 10m →
Deleted: ¶
Moved (insertion) [5]: (out of the tunnels; only in bee traps)
Deleted: ¶
Deleted: = Linen Sheets

FLIGHT/HIVE ACTIVITY

29. Additional information may also be collected on the flight activity and hive activity of the bees.

30. Flight activity is defined as bees flying over the crop, but not actively engaged in foraging.

Adult bee flight activity can be recorded at the same time and at the same locations as described for foraging activity above. At each assessment time the number of bees that are seen to fly across the observation area will be counted for a short time period (snapshot; depending on the crop for example at least 10-15 seconds in Phacelia) per selected area.

31. Hive activity at the entrance is recorded (*e.g.*, the number of bees entering and exiting the hive over a one-minute period). This assessment can be made at a similar time as the foraging assessments. This measurement will give an indication of the general activity of the hives and can be used as supporting information and may give an indication to repellence (*i.e.*, many bees may be flying, but no foraging activity).

BEHAVIOURAL ABNORMALITIES

32. Observations on behaviour (*e.g.*, lethargy, erratic movement, excessive self-grooming, loss of coordination, convulsions) of the bees should be assessed quantitatively, if appropriate and possible.

33. Observations of behavioural abnormalities are conducted during the assessments of mortality and foraging activity.

34. Sub-lethal effects such as signs of toxicity or any abnormal behaviour at the hive entrance or on the plants in comparison to the negative control may be described and recorded accordingly, if appropriate and possible (see APPENDIX I).

BROOD ASSESSMENTS

CONDITION OF THE COLONIES

35. The condition of the colonies will be assessed once before the application and five times after the application according to the schedule in **Table 3 and Figure 1**.

Table 3. Honey bee (*Apis mellifera*) colony condition assessments

Assessment days
• 1-2 days befor
• + 5 days (± 1
• + 10 days (± 1 day) after BFD0
• + 16 days (± 1 day) after BFD0
• + 22 days (± 1 day) after BFD0
• + 28[1)] days (± 1 day) after BFD0

[1)] Additional assessments for a second brood cycle +35 days and +42 days and to cover a third cycle +49 days +56 and +63 days

DRAFT 29 Nov 2023

Moved (insertion) [6]: ¶
Table

Deleted: ◘ = 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¶

Deleted: Time of the test ...

Deleted: once a day at the same time of the day in the morning

Deleted Cells

Deleted: on the day of application

Deleted Cells

Moved up [4]

Deleted: • shortly

Deleted:
• 2 h after application
•

Deleted: during exposure period in the tunnels

Moved up [3]

Deleted: in the morning

Deleted: once a day at the same time of the day in the morning

Deleted Cells

Moved up [5]

Deleted: up to

Deleted: +28

Deleted: BFD

Deleted: * 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.

Moved up [2]

Deleted: 2: Evaluation of flight activity¶
Time of the test ...

36. For the condition of the colonies, the following parameters are assessed in order to record effects of the test chemical:

- Strength of the colony (through estimation of percent/comb area/sub-area covered with adult bees);
- Presence of a healthy egg-laying queen;
- Comb areas (percent/sub-areas) with pollen and nectar;
- Comb areas (percent/sub-areas) containing eggs, larvae and capped cells;
- Any noted signs of diseases.

37. Colony strength in terms of the number of adult bees, amount of brood and food provisions should be recorded in a quantified manner (*e.g.*, weight, sub-area or percentage of bees/brood or food on both sides of each frame; see Imdorf & Gerig (1999) and Imdorf *et al.* (1987)). Bees on the walls (*i.e.*, interior side boards), the bottom board and the lid/cover should also be estimated. Other methods are possible and should be reported, if used.

DEVELOPMENT OF THE BROOD

38. Observation of brood development may cover one or multiple brood cycles if deemed appropriate. The time schedule of the brood assessment days (**Table 4**) was chosen to check the bee brood at different expected stages during the development with the assumption that worker bees typically require 21 days to complete a brood cycle and to emerge as adults. In case a second brood cycle is observed, a new batch of eggs and larvae may be marked at BFD+21 of the first brood cycle or after end of exposure in the tunnels (*e.g.*, BFD+10). Prior to test chemical application (spray application), the development stage of brood within the colony is initially assessed and is referred to as brood area fixing day (BFD) 0. The application in the tunnels is performed 2-3 day (± 1 day) after the initial brood fixing day. Subsequent observations are made through the course of the exposure phase to determine how brood are developing relative to what is typically expected.

Table 4: Honey bee (*Apis mellifera*) brood development assessment schedule

Assessment day	**Determined brood stage in marked cells**
BFD0 (2-3 days ± 1 day) before application	Egg
Optional: BFD +2 day ± 1 (day of application before treatment application)	Confirm identification of viable egg on BFD0
Assessment day	**Expected brood stage in marked cells**
+ 5 days (± 1 day) after BFD0	young to old larvae
+ 10 days (± 1 day) after BFD0	capped cells
+ 16 days (± 1 day) after BFD0	capped cells shortly before emergence
+ 22 days (± 1 day) after BFD0	empty cells or egg containing cells
Assessment day of additional brood cycles (see paragraph 38)	
*BFD +10/+21	Egg
+ 5 days (± 1 day) after BFD+10/+21	young to old larvae
+ 10 days (± 1 day) after BFD+10/+21	capped cells
+ 16 days (± 1 day) after BFD+10/+21	capped cells shortly before emergence
+ 22 days (± 1 day) after BFD+10/+21	empty cells or egg containing cells

Because of biological variation and uncertainty at time of egg mapping on BFD0 (*e.g.*, an accelerated or delayed development) there may be minor changes in the development pattern. Much of the available software allows for manual adjustment for slower/accelerated development.

39. The development of the bee brood in individual marked cells will be observed by photographing the combs and using digital imagery coupled with image analysis software (Höferlin *et al.* (2013), Jeker *et*

Deleted: will be
Deleted:),
Deleted: ,
Deleted: ,
Deleted: ,
Deleted: 28. The coverage
Deleted: a comb is estimated assuming that a comb is covered by 120
Deleted: per 100 cm² if
Deleted: are sitting very close to
Deleted: other (
Deleted: ., 1987) The estimations will be done for all combs (both sides) in each hive.
Deleted: in the study record
Deleted:
Deleted: 29. The assessment of the areas containing brood and food will be done by estimating subareas of 100 cm². Afterwards the number of cells per brood stage/food stock is calculated assuming that 100 cm² 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*
Deleted: ¶ 30.
Deleted: in order
Deleted: (see Table 4). The application in the tunnels
Deleted: days (±
Deleted: BFD
Deleted: : Assessment of the
Deleted: of the bee brood
Deleted: egg
Deleted: BFD
Deleted:
Deleted: (±
Deleted:
Deleted: (±
Deleted:
Deleted: (±
Deleted:
Deleted:
Deleted: hatch
Deleted: (±
Deleted:
Deleted: ¶
Deleted: acetate sheets.

al. (2011), Pistorius *et al.* (2012), Kleinhenz *et al.* (2014), Wang & Classen (2011)). Digital photography reduces the time a comb is outside the hive ("off-hive-time") during assessments and therefore reduces the stress for the whole honey bee colony. At the initial assessment before the application (BFD0) one (or more) brood combs are taken out of each colony to select areas with at least 200 cells containing eggs. A digital photograph/image of the comb is taken. After saving the file on a computer, at least 200 cells containing eggs will be selected. The selection of monitored eggs should be done before the next BFD (= BFD +5 ± 1 day). For each subsequent brood assessment (BFD +n), the selected brood combs from each hive are rephotographed and the file saved. The development of the eggs is followed at the subsequent assessment dates. The content of the selected cells, (*i.e.*, respective brood stage, as being filled with food or being empty) is identified and marked using different numbers, symbols, colours or letters. To ease later evaluation, the recorded growth stages are transformed into numerical values ranging from 0 (expected brood stage not present and thus development regarded as terminated) to 5 (empty after emergence or again filled with eggs or young larvae or food after undisrupted development). The schedule of the detailed brood assessment dates is chosen in order to record the bee brood at different expected stages during its development. However, other methods could be used and described in the study report (*e.g.*, acetate sheet method as described in Appendix II).

40. For the evaluation of the different brood stages of single marked cells, the recorded growth stages are transformed into values using the following proposed classifications:

0: termination of the development (*e.g.*, empty, 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 emergence or again filled with brood (eggs and small larvae)

N: cell containing nectar

P: cell containing pollen

41. Cells filled with nectar and pollen after the termination of the brood in the respective cell (counted 0) may be identified by an "N" and "P" in the following assessments.

42. 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. There is an increase of the brood index during the following assessment if normal development of the brood occurs. This increase is caused by the development from eggs to larval stages, then to the pupae, and finally to the adult, emerged adult bee and so on due to the rising numbers which are assigned to each of the developmental stages.

BROOD TERMINATION RATE

43. The brood termination rate (BTR) is the percentage of brood cells that do not successfully perform the transition from eggs to emerged adult worker bees.

BROOD COMPENSATION INDEX

44. The brood compensation index is a measure of the number of terminated brood cells that were subsequently refilled with brood.

DRAFT 29 Nov 2021

Deleted: (= BFD

Deleted: comb will be

Deleted: 100

Deleted: first acetate sheet is used as a positioning device for all sequent assessments. The sheet

Deleted: fixed with needles on the wooden frame and the position on the frame will be

Deleted: . This procedure allows placing sequent sheets exactly in the same position on each of the following observation days. The position of the first 10 ...

Deleted: sheets.

Deleted: growth stage

Deleted: each cell will be noted on the acetate sheet. ...

Deleted: .: photos and measurement on the frame for ...

Moved up [6]

Deleted: 32. The acetate sheet is removed between ...

Deleted: **5: Coding of the brood stages**¶ ...

Deleted: transferred

Deleted: counting from 0 to 5 and listed in tables a ...

Deleted:

Deleted: .

Deleted:);

Deleted:

Deleted:

Deleted: –

Deleted:

Deleted: –

Deleted:

Deleted:

Deleted: hatching

Deleted:

Deleted:

Deleted: 34.

Deleted: ; the respective cells have to be excluded fro ...

Deleted: 35.

Deleted: An

Deleted: (see paragraph 40)

Deleted: can be observed, if a

Deleted: is presumed

Deleted: furthermore

Deleted: brood

Deleted: Details of the evaluation of the results are ...

Deleted: <#>Evaluation of the test results¶ ...

BROOD INDEX

45. The brood index is used as an indicator of bee brood development of the originally mapped eggs, where cells are classified from 0 to 5 (0=empty; 1=egg; 2=young larvae; 3=old larvae; 4=capped brood; 5=empty after hatching or filled again with new brood).

VERIFICATION OF EXPOSURE

46. In addition to the verification of exposure through the assessment of foraging bees, in case of spray applications, the sprayed concentration and residue in flowers should be verified analytically. If this is done, analysis should be conducted on samples of:

- The spraying solution itself. The measured concentration should be in a range of recoveries relative to nominal concentration (*e.g.*, 80-120%) or in the range specified in current guidelines (*e.g.*, SANTE/2020/12830, Rev.1 24/02/2021).
- The treated crop: open flowers from the upper part of crop canopy can be collected for analysis.
- Flowers will be collected in all tunnels of control and test chemical treatment. Treated flowers should be collected on the day of application as soon as it is practicable after the spray solution has dried (in case of a daytime application). In case of an evening application/pre-flowering application, samples should be collected when bees start to forage on the treated crop for the first time (= start of exposure). It is also possible to collect additional residue samples throughout the test to estimate a depletion (dissipation) curve for the compound. The number and timing of additional crop samples will depend on the expected stability of the test chemical.
- Other types of applications (*e.g.*, seed treatment) may require adaptations to the verification of the exposure.
- Optional: Residue collection (pollen/nectar), extra tunnels/replicates needed.

VALIDITY CRITERIA

47. The test is considered valid if the following conditions are fulfilled:

- a statistically significant effect of the reference substance should be detected/demonstrated on the response variable of interest (*e.g.*, a statistically significant increase in brood termination rate for fenoxycarb or mortality of pupae and/or larvae and/or adults);
- exposure of colonies to the test chemical should be demonstrated (*e.g.*, via residue analysis (see section VERIFICATION OF EXPOSURE) and assessment of foraging activity.

48. Further consideration should be given regarding the variability of brood termination in the control treatment. Ideally, control group brood termination rates should be ≤ 30%. Nevertheless, the evaluation of historical data (Pistorius *et al.* (2012), Becker *et al.* (2015), Szczesniak *et al.* (2018)), showed that, despite improvements to the test design, variability within the respective studies cannot be completely excluded with a high proportion of replicates with control BTR ≥ 30%.

EVALUATION OF THE TEST RESULTS

49. The evaluation of the results will be done by comparing the results in the test chemical treatment to the water treated (negative) control and to the reference substance treatment(s) pre- and post-application with respect to:

Deleted: chemical

Deleted: , and furthermore by comparing the

Deleted: data regarding

DRAFT 29 NOV 2023

- Brood development
- Brood Termination Rate
- Brood Index
- Brood Compensation Index
- Daily and overall adult worker bee mortality (in the dead bee traps and on the linen sheets)
- Daily and overall pupae mortality (including dead larvae)
- Adult bee foraging activity in the crop
- Condition of the colonies:
  - presence of a queen (*i.e.*, presence of eggs or visual detection of the queen)
  - amount of brood (*i.e.*, number of cells containing eggs, larvae or pupae (capped cells))
  - amount of food provisions (*i.e.*, number of cells containing pollen or nectar/honey)
  - colony strength (*i.e.*, number of bees per colony)

The test results allow further calculations such as:

BROOD TERMINATION RATE

50. Based on the Brood Termination Rate (BTR) the failure of originally marked individual eggs to develop successfully into larvae, pupae and adults is quantitatively assessed. For the calculation of the BTR the observed cells are split into 2 categories:

1. 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.
2. 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.

Because of biological variances and uncertainty at time of egg mapping at BFD0 (*e.g.*, an accelerated or delayed development) there may be minor changes in the development pattern. Much of the available software allows for manual adjustment for slower/accelerated development.

51. 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 BTR reported as a percent (%).

BROOD COMPENSATION INDEX

52. The Brood Compensation Index is an indicator for recovery of the colony and will also be calculated for each assessment day and colony (see **Table 5** for schedule). The cells are classified from 1 to 5, 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 to obtain the average compensation index.

Table 5. Honey bee (*Apis mellifera*) Brood Compensation Index assessment schedule

Assessment Day	*Expected Brood Index*
1 day (± 1 day) before application (=BFD0)	1

Deleted: (number of dead adult bees,

Deleted: and

Deleted: flight

Deleted: condition

Deleted: of the colonies (through estimation of comb area covered with bees)¶
brood development¶
average brood areas

Deleted: hive

Deleted: <#>detailed brood assessment in single cells¶
37.

Deleted: ***<#>Brood termination-rate***¶
38.

Deleted: brood termination-rate

Deleted: or larvae

Deleted: brood termination-rate

Deleted: →

Deleted: →

Deleted: 39.

Deleted: brood termination-rate in %.

Deleted: ***<#>Brood-index***¶
40. The brood-index

DRAFT 29 Nov 2023

Optional: 2 days (± 1 day) after BFD0, day of application (=BFD +2)	1*, 2**
+ 5 days (± 1 day) after BFD0 (= BFD +5)	2 to 3
+ 10 days (± 1 day) after BFD0 (= BFD +10)	3 to 4
+ 16 days (± 1 day) after BFD0 (= BFD +16)	4
+ 22 days (± 1 day) after BFD0 (= BFD +22)	5

* old egg in vertical position; ** freshly hatched young larva (L1)
Because of biological variances and uncertainty at time of egg mapping on BFD0 (*e.g.*, an accelerated or delayed development) there may be minor changes in the development pattern. Much of the available software allows for manual adjustment for slower/accelerated development.

BROOD INDEX

53. 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 BFD0 up to BFD +22. The cells are classified from 1 to 5, if the cells contain the expected brood stage at the different assessment days. If a cell does not contain the expected brood stage, is empty, or food is stored in the cell during BFD +5 to +16, the cell has to be classified as 0 at that assessment day and also on the following days, irrespective whether the cell is filled again with brood. 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 to obtain the average brood index.

CONDITION OF THE COLONIES

54. Colony strength, brood and food of the colonies are quantified as the percentage or covered area of bees/brood or food on each side of the frame. The resulting values are converted into absolute numbers taking the total number of bees or cells per unit/comb side into consideration. Mean values and standard deviations per colony are calculated for each treatment group and BFD.

STATISTICAL ANALYSIS

55. Data should be statistically analysed using suitable methods, if appropriate. For example, it is recommended to follow OECD No. 54. (2006) Current Approaches in the Statistical Analysis of Ecotoxicity Data. If statistical analysis is not used, this should be justified.

56. As statistical analysis should normally be performed using appropriate methods, the following proposals are considered as recommendations only and other methods may be used if appropriate.

57. The measurement endpoints for statistical evaluation should be mortality (daily and overall number of dead adult bees and larvae/pupae), overall foraging activity (number of foraging bees/m^2), Brood Termination Rate, Brood Index and Brood Compensation Index, whereas other measurement endpoints (*e.g.*, behavioural endpoints) may not be suitable for statistical evaluation.

58. Based on the test results for normal distribution and variance of homogeneity suitable test (pairwise or multiple) should be used to evaluate the data appropriately. For pre-application data two-sided tests could be used, while for post-application data one-sided tests are preferable.

59. Specific statistical analyses for bee trials under semi-field conditions are still under development and could be considered on a case-by-case basis.

Deleted: brood-index

Deleted: BFD 0

Deleted: as described in paragraph 33

Deleted: (see Table 4)

Deleted: counted 0 (see Table 5)

Deleted: 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. …

Deleted: in order

Deleted: compensation-

Deleted: ***<#>Statistical Analysis***¶
42.

Deleted: analysis

Deleted: in

Deleted: and field

Deleted: . In general it is recommended to follow the OECD guidelines (OECD, 2006).

DRAFT 28 Nov 2023

REPORT

60. The report should contain at least the following data:

- the objective of the study
- a description of the test chemical (*i.e.*, the physical/chemical properties and any additional data needed for the identification of the test chemical)
- the experimental design, including description of the tunnels
- the test conditions
- the health status and source of the colonies
- a description of all methods and procedures used
- the experimental findings/test results (*i.e.*, exposure data, mortality, foraging activity, condition of the colonies and bee brood development)
- meteorological data
- test duration and performance of the test
- a summary and conclusion of the results obtained
- a description of the most relevant operations, calculations, and statistical analyses that were performed on the data presented
- a description of all circumstances that may have influenced the quality and integrity of the results
- tabular and graphic presentation of results
- biological and statistical relevance of the observed effects
- statistical methods used
- any deviations from the study protocol

ACKNOWLEDGEMENTS

The editors would also like to acknowledge the valuable contribution made to this guidance document by former members: Roland Becker (BASF), Hervé Giffard (Testapi), Jens Pistorius (Julius Kühn Institute), Stephan Schmitzer (ibacon) and Bronia Szczesniak (Eurofins).

DRAFT 29 Nov 2023

Deleted: <#>Report¶

Deleted: the

Deleted: further

Deleted: ,

Deleted: day of

Deleted: preparation

Deleted: colonies,

Deleted: ,

Moved down [7]

Deleted: <#>,¶

Moved down [8]

Deleted: tunnels,¶
description of the test design,¶

Deleted: ,¶

Deleted: flight

Moved (insertion) [7]: meteorological data

Deleted: Tabular

Moved (insertion) [8]: test duration and performance of the test¶

Deleted:

Deleted: Ecological significance

Deleted:

Deleted: <#>Population recovery (observed or inferred), with a discussion of relevance to natural recovery processes ¶
Statistical

Deleted: REFERENCES¶

Aupinel P, Fortini D, Michaud B, Marolleau F, Tasei J N, Odoux J F (2005): A larval in vitro rearing method to assess effects of pesticides on honey bee brood, Meeting of the ICP-BR Bee Protection Group, 9th International Symposium Hazards of Pesticides to Bees, October 12-14, 2005, York, UK¶

Czoppelt C., 1993.- Effects of fenoxycarb and pyriproxifen on post-embryonic development of honeybees, *Apis mellifera* L. Evaluation of toxicity by an in vitro test.- *Proceedings of the Fifth International Symposium on the Hazards of Pesticides to Bees*, October 26-28, Wageningen, The Netherlands, Appendix 7.¶

6 LITERATURE

Becker, R. Lückmann, J. Pistorius, J. (2015): Effectiveness of method improvements of OECD GD 75 – Evaluation by the ICP-PR Bee Brood Working Group. Julius-Kühn-Archiv, 450, 83-92

EFSA (2023): Revised guidance on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees). EFSA Journal 2023; 21(5):7989, 133 pp https://doi.org/10.2903/j.efsa.2023.7989

Höferlin B., Höferlin M., Kleinhenz M., Bargen H. (2013): Automatic Analysis of Apis mellifera comb photos and brood development. Association of Institutes for Bee Research, Report of the 60th Seminar in Würzburg. Apidologie, vol. 44

Illies I., Mühlen W., Dücker G., Sachser N. (2002): The influence of different bee traps on under taking behaviour of the honey bee (Apis mellifera) and development of a new trap. Apidologie, 33, 315 -326

Imdorf A., Gerig L. (1999): Lehrgang zur Erfassung der Volksstärke, Schweizerisches Zentrum für Bienenforschung. [Course in Determination of Colony Strength]

Imdorf A., Bühlmann G., Kilchenmann V., Wille H. (1987): Überprüfung der Schätzmethode zur Ermittlungder Brutfläche und der Anzahl Arbeiterinnen in freifliegenden Bienenvölkern. Apidologie 18 (2), 137 – 146

Jeker L., Meschberger T., Candolfi M., Magyar J. P. (2011): Digital image analysis as a tool to improve the assessment and evaluation of brood development in higher tier honey bee studies. Workshop of AG Bienenschutz on Improvement of Bee brood studies, Julius Kühn-Institut (JKI) February 23th, 2011

Kleinhenz M., Höferlin B., Höferlin M., Bargen H. (2014): HDR-Fotografie und automatische Auswertung von Honigbienen-Brutwaben (Apis mellifera) mit der HiveAnalyzer Software [HDR-photography and automatic analysis of honeybee brood combs (Apis mellifera) using the HiveAnalyzer software]. 59. Deutsche Pflanzenschutztagung, Freiburg. Julius-Kühn-Archiv, No. 447, p. 376

Meier U. (2018): Growth stages of mono- and dicotyledonous plants: BBCH Monograph. https://doi.org/10.5073/20180906-074619

Mühlen W. (1996): Implication of the IGR Alsystin WP 25 on the Development of Honeybee Colonies under Field and Semi-field Conditions. Hazards of Pesticides to Bees – 6th International Symposium of the ICP-BR Bee Protection Group, in Braunschweig, Appendix 16, 8 pages

OECD (2006): Environment Health and Safety Publications, Series on Testing and Assessment, No. 54: Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application

OECD (1998): Test No. 213: Honeybees, Acute Oral Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264070165-en

OECD (1998): Test No. 214: Honeybees, Acute Contact Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264070189-en

OECD (2013): Test No. 237: Honey Bee (Apis mellifera) Larval Toxicity Test, Single Exposure, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264203723-en

Deleted: ,
Deleted: ,
Deleted: ,
Deleted: undertaking
Deleted:
Deleted: ,
Deleted: ,
Deleted: ,
Deleted: Ermittlung der
Deleted: 2006.
Deleted: .

DRAFT 29 Nov 2023

OECD (2017): Test No. 245: Honey Bee (Apis mellifera L.), Chronic Oral Toxicity Test (10-Day Feeding), OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264284081-en

OECD (2021): Series on Testing and Assessment No. 239: Guidance Document on Honey Bee (Apis mellifera) Larval Toxicity Test, Repeated Exposure, OECD Environment, Health and Safety Publications, ENV/JM/MONO(2016)34

OEPP/EPPO (2001): Guideline for the efficacy evaluation of plant protection products – Side effects on honeybees. QEPP/EPPO, PP 1/170 (3) update 2000, 19 – 23

Oomen P. A., De Reuijter A., Van Der Steen J. (1992): Method for honeybee brood feeding tests with insect growth-regulating insecticides. OEPP/EPPO Bulletin 22, 613 – 616

Pistorius J., Becker R., Lückmann J., Schur A., Barth M., Jeker L., Schmitzer S., Von der Ohe W. (2012): Effectiveness of method improvements to reduce variability of brood termination rate in honey bee brood studies under semi-field conditions. Julius-Kühn-Archiv, 437, 115-120

Rembold H., Czoppelt C. (1982): The influence of synthetic and botanical insect growth regulators on the development of honeybee larvae in vitro. Proceedings of the 2nd Symposium of the Harmonisation of Methods for Testing the Toxicity of Pesticides to Bees, Hohenheim, Germany, Appendix 7

Schur A., Tornier I., Brasse D., Mühlen W., Von Der Ohe W., Wallner K., Wehling M. (2003): Honey bee brood ring-test in 2002: method for the assessment of side-effects of plant protection products on the honey bee brood under semi-field conditions. Bulletin of Insectology, 56 (1), 1 – 7

Szczesniak B., Pilling E., Bocksch S., Becker R., Lückmann J. (2018): Variability of brood termination rates in reference to validity criteria and limited effectiveness of method improvement in honeybee semi-field studies (OECD GD 75). Julius-Kühn-Archiv, 462, 111-115

Tornier I. (1999): Side effects of an insect growth regulator on bumble-bee and honey-bee. Hazards of Pesticides to Bees – 7th International Symposium of the ICP-BR, Bee Protection Group, 299, Avignon

Wang M., Classen C. (2011): Automated evaluation of honey bee brood trials using digital image processing according to OECD 75, OOMEN (1992) and beyond. Poster presentation at 32nd SETAC North America, 13 -17 November 2011, Boston/Massachusetts

Deleted: PA,

Deleted: ,

Deleted: .,

Deleted: .-

Deleted: .-

Deleted: on

Deleted: Universität

Deleted: .

Deleted: ,

Deleted: ,

Deleted: ,

Deleted: ,

Deleted: ,

Deleted: ,

Deleted:

Deleted: .

Deleted: Wittmann D and Engels W. (1980): Development of test systems for insecticide effects on honeybee larvae. ICPB-Symposium on the harmonisation of methods for testing the toxicity of pesticides to bees, 23 – 25 September 1980, Wageningen, The Netherlands, Appendix 5, 10 pages¶
Wittmann D., 1981.- Besitmmung der LC50 von Dimilin WP25 für Bienenbrut mit einem neuen Apis-Larven Test.- Zeitschrift für angewandte Entomologie, 92: 165-172.¶
Wittmann D., 1982.- Entwicklung von Testverfahren und Experimente zur Beurteilung von Insektizid-Wirkung auf Bienenlarven.- Dissertation, Eberhard-Karls-Universität, Tübingen.¶
ANNEX I¶

DRAFT 23 Nov 2023

7 APPENDICES

APPENDIX I

Sub-lethal effects may be described according to the following categories (other specifications and/or observations may be possible):

SGB = Selection by guard bees = guard bees attacking, fighting off and/or preventing returning bees from entering the hive

ICL = Intensive cleaning = the bee is cleaning/grooming itself by using middle or hind legs

FwL = Flying without landing = bee inspecting different flowers or many bees flying quickly over the crop without landing and foraging.

Clu = Clustering = clustering at the bee hive entrance = assemblage of a large number of bees at the hive entrance ("bee beard") (estimate number of bees)

Cr = cramping = crouched/curved posture (spasms/convulsions); muscle, entire body or abdomen contracting, not motionless

LP = locomotion problems = uncoordinated movements and/or bee walks on two or four legs instead of six and drag the other legs which appear to be paralysed. The bee may walk on the ground, roll on its side, then set off in another direction, spinning, show uncoordinated wing movements, etc.

Tr = trembling = vibrating movements of only some body parts (*e.g.*, leg or antennae)

IA = inactive = bee is motionless: does not walk or forage, does not clean itself, not cramping (see above) nor little movements of body parts or breathing, may start moving after touching (not cold or moisture impaired bees), showing lethargy, apathy

Ha = hanging bees = the bee is hanging on to the plant/flower with one of two legs (may be motionless or cleaning itself)

DRAFT 29 Nov 2023

APPENDIX II

Brood Development Assessment Using Acetate Sheets

It is highly recommended that digital photography combined with a validated piece of image analysis software specifically designed for such brood monitoring studies is used (*e.g.*, Höferlin *et al.* (2013), Jeker *et al.* (2011), Pistorius *et al.* (2012), Kleinhenz *et al.* (2014), Wang & Classen (2011)). However, if this method is unavailable, it is still possible to assess brood development by using acetate sheets to map the brood throughout the test period and performing manual calculation of the measurement endpoints.

Method for brood development assessment

On BFD0, frames with the appropriate age brood are selected as per the methods described in the main body of the Guidance Document.

1. The frame should be labelled appropriately with the hive number, test group, study number, etc.
2. The frames should be carefully transported to an area where the cell marking will take place. Ideally, this would be in a laboratory if possible, or if in the field, a tent or vehicle for example. This will help reduce 'interference' from flying bees and would keep the frames out of direct sunlight/wind which may adversely affect the brood.
3. A clear acetate sheet is pinned to the top bar of the brood frame so that it covers the comb surface. The top of the acetate sheet should be marked with the hive number using an indelible marker. The sheet should also be labelled with the age group, the test group identification, the frame side (*i.e.*, A or B), and frame number (if more than one frame is used per hive).
4. The brood stage (*e.g.*, egg or larvae) to be marked or assessed are identified by circling each cell on the acetate sheet with an indelible marker pen. If brood of different ages is to be tracked, then a different colour and acetate sheet should be used for each age group (see **Table A1**).
5. To aid assessment at subsequent BFDs at the end of each row on the frame, the number of the last cell counted should be marked for easy reference. Where rows are not obvious or uniform it may be necessary to number the cells individually.
6. After marking out the cells, the acetate sheet is removed and the pins re-affixed in their original holes in the top bar. To aid relocation of the acetate sheet at subsequent BFD assessments, it helps to circle the pins/pin holes with an indelible pen in case the pins become dislodged when returned to the colony.
7. At each of the subsequent BFD assessments, the original acetate map is repositioned over the frame using the same pinholes in both the acetate sheet and the brood frame so that the map is located accurately.
8. The cell contents and the condition of each cell are recorded on appropriate forms. It is recommended to read the brood cells from left to right, top to bottom so that they are always recorded in the same order. Mark the acetate sheet as suggested in **Table A1** over any cell which is empty or in which the larva, or pupa, is obviously dead or replaced, and record this on the form against the appropriate cell number using the appropriate code. If using a light source to help see into the cells, ensure that only a cold source illumination is used so no heat damage to the brood occurs.
9. For the different brood stages, when assessing single cells, the following symbols and colours presented in **Table A1** are suggested.

DRAFT 23 May 2023

Table A1: Suggested coding of the brood stage Symbol/Colour

Cell Contents	Colour/Symbol
Eggs	O Blue
Young larvae (L1 – L2)	O Green
Old larvae (L3 – L5)	O Red
Pupae (capped cells)	O Black
Nectar	X Blue
Pollen	X Green
Dead larvae/pupae	⊕ Black
Empty	X Black

10. For the evaluation of the different brood stages of single marked cells, the recorded growth stages are converted into values from 0 to 5 as listed below:

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

Cells filled with nectar and pollen after the termination of the brood in the respective cell (originally counted as 0) may identified by an “N” and “P” in the subsequent BFD assessments; the respective cells will be excluded from further calculations, but will be included in the overall evaluation in the end.

Calculations can then be made for Brood Termination Rate (BTR), Brood Index (BI) and Compensation Index (CI).

A) Brood termination-rate

Brood Termination Rate is a quantitative assessment based on the failure of individual eggs or larvae to develop. For the calculation of the BTR the observed cells are split into 2 categories:

I. **Successful development:** The bee brood in the observed cell reached the expected brood stage for each of the BFDs. **Allocated 1 for calculation of brood termination rate**

II. **Terminated bee brood development:** The bee brood in the observed cell did not reach the expected brood stage at one of BFDs, or was removed and replaced. **Allocated 0 for calculation of brood termination rate.**

$$Brood\ Termination\ Rate = \frac{\text{Number of "0" Terminated Cells} \times 100}{\text{Total Number of "0" and "1" Cells Oberseved}}$$

B) Brood-index

The brood-index is an indicator of the bee brood development and facilitates a comparison between different treatments. The cells are classified from 1 to 5 as described above if the cells contained the *expected* brood stage at the different assessment days. If a cell does not contain the expected brood stage, is empty, or food is stored in the cell before the brood should have emerged, the cell is assigned a value of 0 at that assessment day and also on the following days, irrespective of whether the cell is laid in again.

DRAFT 29 Nov 2023

The brood index is calculated for each assessment day and colony:

$$Brood\ Index = \frac{\text{Sum of Cell Classifications}}{\text{Total Number of Cells Oberseved}}$$

C) Compensation-index

The compensation-index is an indicator for recovery of the colony. The cells are classified from 1 to 5 as above, solely based on the identified growth stage on each assessment day.

$$Compensation\ Index = \frac{\text{Sum of Cell Classifications}}{\text{Total Number of Cells Oberseved}}$$

DRAFT 29 Nov 2023

APPENDIX III

Abbreviations

a.s.	Active Substance
BBCH	System for a uniform coding of phenologically similar growth stages of all mono- and dicotyledonous plant species. The abbreviation derives from Biologische Bundesanstalt, Bundessortenamt and CHemical industry.
BFD	Brood area Fixing Day
BTR	Brood Termination Rate
EFSA	European Food Safety Authority
EPPO	European and Mediterranean Plant Protection Organization
GAP	Good Agricultural Practice
GD	Guidance Document
ha	Hectare
ICPPR	International Commission for Plant-Pollinator Relationships
IGR	Insect Growth Regulator
L1-5	Larval Stage 1-5
OECD	Organization for Economic Co-operation and Development
PEC	Predicted Environmental Concentration
TER	Toxicity/Exposure-Ratio
TG	Test Guideline

Deleted:

Deleted: ¶

Deleted:

Deleted: ¶

Deleted:

Deleted: ¶

Deleted:

Deleted: ¶

¶

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.***

DRAFT 29 Nov 2023

1516 **APPENDIX IV**

1517 ***Definitions***

Brood area fixing day (BFD)	Day where the first detailed brood assessment is performed, and the brood area is fixed (*i.e.*, egg marking for the acetate sheet method or photographing the brood frames) for all following brood assessment days. Usually, the brood area fixing day is performed before application of the test item and considered as BFD0.
Brood compensation-index (BCI)	The brood compensation index is a measure of the number of terminated brood cells that were subsequently refilled with brood.
Brood-index (BI)	The brood index is used as an indicator of bee brood development of the originally mapped eggs, where cells are classified from 0 to 5 (0=empty; 1=egg; 2=young larvae; 3=old larvae; 4=capped brood; 5=empty after hatching or filled again with new brood).
Brood termination-rate (BTR)	The brood termination rate is the percentage of brood cells that do not successfully perform the transition from eggs to emerged adult worker bees.
Caging effect	Enclosure stress on honey bee colonies under semi-field (tunnel) conditions which may cause a reduction in the number of bee brood.
Colony Strength	Number of adult bees in one honey bee hive (= colony). The initial colony strength before start of the test should be adapted to the available crop area per tunnel. Ideally, the colony strength should be equal and comparable across all tunnel replicates.
Complete brood cycle	Honey bee life cycle from egg to adult emergence usually 21 days ± 1
Condition of the colonies	The condition of the colonies reflects the colony strength, which includes quantifying the number of adult bees, overall food reserves (*i.e.*, comb cells containing pollen and nectar) and the number of brood (*i.e.*, eggs, pupae and capped cells) stored on each side of each frame in one colony. The resulting values are converted into absolute numbers considering the total number of bees or cells per unit/comb side.
Daytime application	Application of a treatment group (*e.g.*, test item, negative control and positive control) during daytime when honey bees are actively foraging on the crop. Depending on factors like *i.e.*, weather, crop condition, foraging activity (≥ 5 bees per m^2); daytime application can take place early in the morning or later during the day.
Dead bee trap	Boxes positioned at hive entries to determine dead or disabled honey bees, pupae and larvae that were discarded from the colony. Based on the cleaning behaviour of the honey bees, dead or disabled bees are dragged out of the hives by so called house cleaning bees. Dead/disabled bees within the trap can then in turn be counted and removed afterwards.
Evening application	Usually, application of only the test item treatment group after bee flight in the (late) evening. Start of exposure is, therefore, the following day when bees start foraging on the treated crop for the first time.
Exposure-phase (= tunnel-phase)	Start of foraging activity on the treated flowering crop inside the tunnels until end of the tunnel phase (*e.g.*, 7 days after application of the test chemical).
Flight activity	Honey bees flying over the crop, but not actively foraging for pollen or/and nectar.
Foraging activity	Honey bees actively foraging for food supply (*i.e.*, nectar and pollen) from blooming crops.
Health Status	Colonies will be checked and should be free of clear clinical signs of bee diseases (*i.e.*, viral, fungal, bacterial). Medical treatments against pests and pathogens within 4 weeks before the start of the test should be avoided as far as practicable. If medical treatments (*e.g.*, varroa treatments) of the colonies is necessary, all colonies should be treated equally and at the same time. The rationale for a medical treatment should be clearly articulated in the study report and be consistent with local best beekeeping practices.
Honey bee brood	All honey bee brood stages: eggs, larvae and pupae (capped brood).
negative-control	Water-treated crop also referred as control colonies
Nucleus or 'nuc' colonies	Smaller sized honey bee colonies (in numbers of adult bees and brood cells) compared to commercially used honey bee colonies.

DRAFT 2nd TM 2023

off-hive-time	Considers the time comb(s) are extracted and therefore outside their hive and without coverage of adult bees (and subject to ambient environmental conditions) during the digital brood assessments.
Pre-application period	Also called colony acclimatization period. Time period of honey bee colonies to acclimatize to the enclosures (tunnels) after set-up. Ideally, the pre-application period should consist of at least two full days.
positive-control	Colonies in reference substance-treated (*e.g.*, fenoxycarb) crop also referred to as reference colonies.
Post-exposure or monitoring phase	Following exposure-phase of the bees in the tunnel during flowering of the crop (*e.g.*, at least 7 days after application of the test chemical), the honey bee hives are placed outside the tunnel to a monitoring site for the remainder of the study and are free to forage under full-field conditions.

1518

DRAFT 29 Nov 2023