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

**Cancels & replaces the same document of 15 July 2016**

**Guidance Document on Honey Bee (Apis mellifera) Larval Toxicity Test, Repeated Exposure**

**Series on Testing and Assessment**
**No. 239**

Following several questions raised after publication, the OECD would like to clarify that the validity criteria mentioned in paragraph 7 «across all replicates» should be understood as the average value across all replicates, a replicate being defined as the number of larvae originating from the same colony.

**OECD Environment, Health and Safety Publications**

**Series on Testing and Assessment**

**No. 239**

**Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure**

**IOMC**

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

**Environment Directorate**
**ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT**
Paris 2016
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**FOREWORD**

This document contains the Guidance Document for the Honey bee (*Apis mellifera*) larval toxicity test using repeated exposure. The project to develop this Guidance Document was submitted by France in 2011 and included into the TGP work plan in 2012 under project number 2.44.

The honey bee larval toxicity test using repeated exposure is based upon the single-dose test, i.e. TG 237 "Honey bees (*Apis mellifera*) Larval Toxicity Test, Single Exposure" which was published in 2013.

In 2014 a ring test of the repeated exposure methodology was performed, which resulted in a validation report in March 2015. The draft Guidance Document and validation report were subsequently discussed in a meeting of the Expert Group on Honeybee Toxicity Testing in April 2015.

The validation report and draft Guidance Document were amended based on the recommendations of the Expert group meeting and circulated to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) for review and commenting in July 2015. The resulting comments were incorporated into the Guidance Document, after which the Guidance Document was approved by the WNT at its 28th meeting in April 2016. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to declassification of the Guidance Document on 8 July 2016.

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

# 1 Guidance Document

## Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure

## INTRODUCTION

1.      This document describes a honey bee brood laboratory toxicity test using repeated doses. It is based on the OECD Test Guideline 237: Honey bees (*Apis mellifera*) larval toxicity test, single exposure (1) ring tested in seven European laboratories, which is itself based on a method developed in France (2) (3) (4), and on methods described in the Coloss Beebook (5) that provides useful guidance for honey bee testing, breeding honey bees, studying honey bee biology and understanding honey bee pests and pathogens.

2.      The Guidance Document addresses the requirements formulated by the United States, Canada, and Europe (6) (7) (8) to test the toxicity of chemicals on immature honey bees by feeding larvae with food added with the test chemical under laboratory conditions.  The present protocol involving repeated exposure of honey bees to chemicals has been evaluated by an international ring test (Annex 1) in order to confirm the acceptance criteria (see paragraph 7).

3.      The method aims at the determination of a No Observed Effect Concentration/Dose (NOEC/NOED) and, if data allows, $EC_{50}/ED_{50}$ and any $EC_x/ED_x$ (see Annex 1 for definitions) on day 22 (adult emergence) following a repeated exposure of larvae to a test chemical, particularly active ingredient or formulation (formulated product in case of low solubility of the active ingredient). The data should be used in an appropriate honey bee brood risk assessment scheme. The test methods on honey bee larval toxicity – single (1) and repeated exposure – complement the OECD TG 213 (9) and TG 214 (10) on young adult honey bees and should be seen as a lower tier screening test in the context of an overall risk assessment scheme for bees (6) (7).

## PRINCIPLE OF THE TEST

4.      On day 1 (D1) of the study, first instar (L1) synchronised larvae (*i.e.* larvae of the same age) are taken from the comb of three colonies and individually placed into 48 well-plates where they are fed with a standardized amount of artificial diet. From day 3 (D3) until day 6 (D6) of the test, the test chemical is administered daily to the larvae at a constant concentration equivalent to increasing test chemical doses per larva per day with the diet resulting in a cumulative dose on day 6 (for each treatment level) in a range of at least five increasing test concentrations, or at one concentration in case of a limit test. Mortality and other observations/abnormal effects are recorded daily from D4 to D8 and on D15 of the test, and emergence rate

should be recorded on D22. The NOEC/NOED and, if data allows, the $EC_{50}/ED_{50}$, and/or any $EC_x/ED_x$ are determined for adult emergences on D22.

## INFORMATION ON THE TEST CHEMICAL

5.        The water solubility, solubility in a solvent, and vapour pressure of the test chemical should be known. Useful information on the test chemical including its structural formula, purity, stability in water and light, and octanol-water partition coefficient ($K_{ow}$) should be reported. The physical appearance and source (batch, lot number) of the test chemical should be described. Guidance for testing substances with physical-chemical properties that make them difficult to test is provided in the OECD Guidance Document, series on Testing and Assessment, No. 23 (11).

## REFERENCE CHEMICAL

6.        The standard reference chemical (positive control) is selected based on the mode of action of the test chemical. It is technical grade dimethoate (CAS 60-51-5) or technical grade fenoxycarb (CAS 72490-01-8) if the test chemical is an insect growth regulator (IGR) with expected effects on the development of immature bees. A preliminary experiment/range-finding test may help to decide what is the most appropriate reference chemical (see paragraph 25). The reference chemical is tested to ensure that the test system and conditions are responsive and reliable at the constant concentration of 48 mg active ingredient (a.i.)/Kg of diet (i.e. 0.053 µg a.i./µL of diet) for dimethoate, or 0.320 mg a.i./Kg of diet (i.e. 0.35 ng a.i./µL of diet) for fenoxycarb. The table below indicates the amount of dimethoate and fenoxycarb which has to be added to the diet every day for one larva during the exposure period from D3 to D6, in order to maintain a constant concentration of the reference chemical.

| Day | D3 | D4 | D5 | D6 | Total amount/larva |
|---|---|---|---|---|---|
| Amount of dimethoate (µg) added to the diet | 1.06 | 1.58 | 2.11 | 2.64 | 7.39 |
| Amount of fenoxycarb (ng) added to the diet | 7.04 | 10.56 | 14.08 | 17.60 | 49.28 |

Note: Dimethoate can be directly dissolved into water. It is recommended dissolving fenoxycarb into 0.5% acetone.

## VALIDITY OF THE TEST

7.        For judging the acceptance and quality of data obtained with the repeated exposure test, the following performance criteria apply:

- In the control plate(s), cumulative larval mortality from D3 to D8 should be ≤15% across all replicates.

- In the control plate(s), the adult emergence rate on D22 should be ≥70% across all replicates  (see paragraph 2).

- Positive control: if the dimethoate is used, larval mortality should be ≥50% on D8 across all replicates;if  the fenoxycarb is used, the emergence rate should be ≤ 20% on D22 across all replicates.

## DESCRIPTION OF THE TEST

### Apparatus

8.        Larvae are reared in crystal polystyrene grafting cells (*e.g.* ref CNE/3, Nicoplast Society) having an internal diameter of 9 mm and a depth of 8 mm. The cells are initially sterilised, e.g. by immerging for 30 min in ethanol (e.g. 70%) or other sterilising solution, and then dried in a laminar-flow hood. Each cell is placed into a well of a 48-well plate. The top of the grafting cell is maintained at the level of the plate, e.g. by placing a piece of dental roll wetted with approximately 500 µL of the sterilising solution added with 15% weight/volume glycerol at the bottom of the wells (Figure 1). Note: covering *vs* uncovering the culture plates is left to the experimenter.
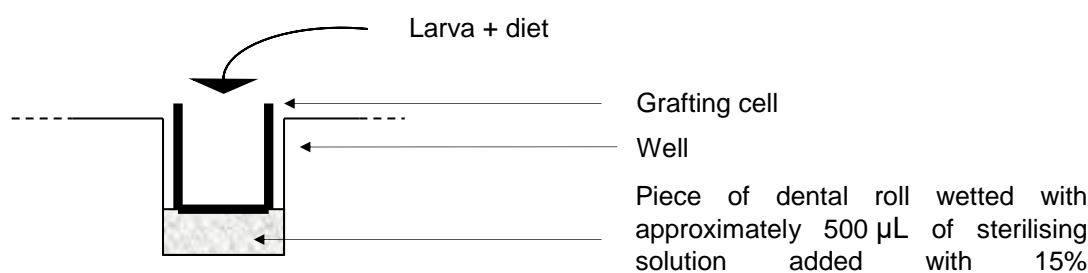


Figure 1: Larval cell in a tissue culture well

9.        The  culture plates are placed into a hermetic Plexiglas desiccator (*e.g.* Nalgene 5314-0120 or 5317-0180 according to the volume required) and kept at a relative humidity of 95% ± 5% adequate for larvae from D1 to D8 (e.g. humidity can be achieved with a dish filled with a potassium sulphate ($K_2SO_4$) saturated solution in order to keep a water-saturated atmosphere). The desiccator is placed into an incubator equipped with a forced air circulation system at 34-35 ºC) to equilibrate temperature around the desiccator for the duration of the test.

10.        On D8 (pre-pupae stage), the well-plates are transferred into a hermetic container at a relative humidity of 80% ± 5% adequate for pupae (e.g. humidity can be achieved with a dish filled with a saturated NaCl solution). The pieces of dental roll are removed from the wells. The container is then placed into an incubator at 34-35 ºC.

11.        On D15 (pupae stage), each plate is transferred into an emergence box, e.g. a crystal polypropylene box (e.g. 11 x 15 x 12 cm) with a cover aerated e.g. with wire gauze/mesh. Emerging bees are fed with syrup/sucrose solution dispensed *ad libitum*, using bird feeders, syringes or any other suitable tool (see Figure 2). The boxes are transferred into an incubator at 34-35°C with a relative humidity within the range 50 - 80% .
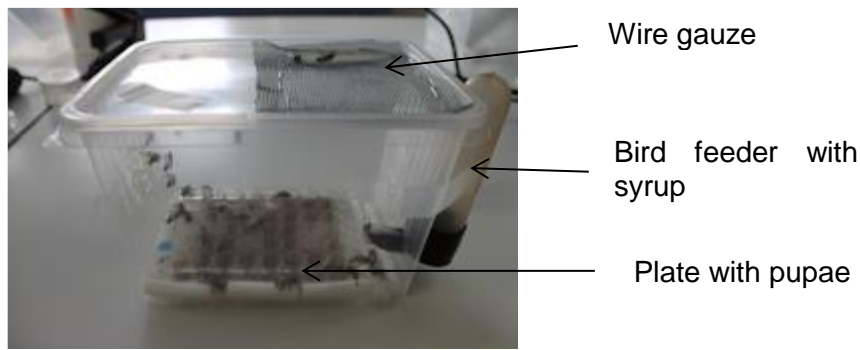
Figure 2: Example of an emergence box

## Test organisms

### *Origin of the larvae*

12.     Larvae are collected from three different colonies, each one representing a replicate (see paragraph 21). Colonies should be adequately fed, all in the same healthy conditions (*i.e.* as far as possible disease- and parasite-free), with known history and physiological status. It is recommended checking bee colonies for signs of variability and test performance before starting the season by conducting a pre-test with a representative number of larvae per potential test colony.

13.     Tests are conducted during the egg laying period of the queen. In case of sanitary treatment (*e.g.* mite or disease treatment measures), the date of application to the colony and the product identity are reported. No treatment with chemicals – such as antibiotics, anti-varroa, etc. – is allowed within the four weeks preceding the start of the test; healthy honey bees in the same healthy conditions should be used.

14.     On D-3 (pre-phase of the test, see Figure 5), in order to ensure the production of larvae from three colonies, the queens of a minimum of three colonies are confined in their own colony in an exclusion cage containing an empty comb or a comb with emerging worker brood and empty cells (Figure 3). The exclusion cage is placed close to the combs containing brood. On D-2 (pre-phase of the test, see Figure 5), maximum 30 hours after encaging, the queen is released from the cage, after checking the presence of freshly laid eggs. Depending on the fertility of the queen, it is recommended reducing the isolation time in order to minimise the variability in egg ages. The comb containing the eggs is left in the cage, near the brood, during the incubation phase and until hatching (D1).
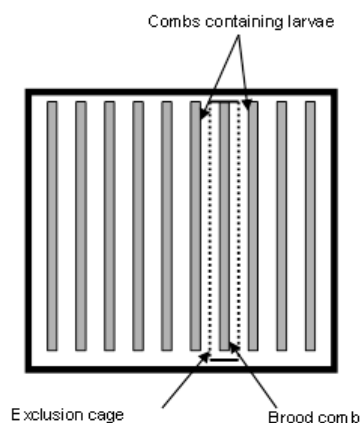
Figure 3: Cross section of a hive with an excluder cage

## Preparation of rearing material

### *Larval food*

15. The food is composed of the three following diets, adapted to the needs of the larvae at different stages of development:

- Diet A (D1): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose.

- Diet B (D3): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.

- Diet C (from D4 to D6): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.

Note: The diets A, B and C prepared in this way have a density of about 1.1 mg/µL (e.g. 20 µL diet correspond to 22 mg diet).

16. If some aggregates remain in sugar solutions they have to be completely dissolved before mixing with the royal jelly. A "fresh royal jelly" is a royal jelly collected during the preceding 12 months; it is divided into aliquots (e.g. of approximately 5 g) in order to avoid defrosting the whole batch at each test, and stored in a freezer at ≤-10°C. Commercial sources of royal jelly might be acceptable if it can be shown that their performance compare to historical data within the testing facility, e.g. mortality does not exceed 15% during the larval period. It is recommended conducting a multi-residues analysis of each royal jelly batch in order to verify the absence of contaminants (mainly antibiotics and insecticides). Note: The analytical verification of low traces of contaminants is not necessary provided that the validity criteria are met.

17. The diets, prepared freshly prior to each test, can be stored in a fridge at ≤ +5°C (but not frozen) during the whole duration of the test. The diets may be prepared in advance and stored deep-frozen (between -18ºC and -25ºC) until use (and if needed subsequently stored in a fridge at ≤ +5°C during the performance of the test).

## Test solutions

18.      The test chemical is normally dissolved or dispersed in deionised water. For poorly soluble chemicals a solvent (e.g. acetone) may be used to prepare the stock solution. In such a case, a solvent-control – diet with the same volume of solvent as used for the treatments – is tested in addition to the regular water diet-control. The volume of the organic solvent, if used, should be kept as low as possible, and in any case it should not exceed 2% of the diet final volume during the exposure period D3 to D6. The stock solution should be prepared freshly at each feeding day unless the stability of the test chemical has been demonstrated in former studies.

19.      Dilutions of the stock solutions into the series of five test solutions are made preferably with deionised water – or solvent for poorly soluble substances, preferably just before administration to the larvae, using disposable pipette tips equipped with a filter. The volume of the test solution mixed into the diet should not exceed 10% of the final volume if water is used to dissolve the test chemical (e.g. 2 μL of test solution for a final diet volume of 20 μL on D3) or 2% maximum if an organic solvent is required (e.g. 0.4 µL of test solution for a final diet volume of 20 μL on D3). The overall composition of the diet as stated in paragraph 15 should be observed, i.e. the total volume of water/solvent used for preparing the test chemical solution should be taken into account for diet preparation. The test solution should be mixed into the diet in a manner that will result in even distribution of the test chemical throughout the diet (e.g. using ultrasonic dispersion).

20.      A sample of the solution used to prepare the diet with the highest and lowest concentrations will be stored in a freezer at ≤-20°C in order to be further checked for analytical determination of the actual concentration of the test chemical. If required, the test chemical concentration may be measured in the diet Nevertheless, one should be aware that the analytical measurement of the test chemical concentration in the diet is more difficult because of the presence of royal jelly. Any difficulties with analytics and their justification should be reported in the test report.

## PROCEDURE

## Conditions of exposure

21.      The experimental unit is the individual cell containing a larva. A minimum of twelve larvae from each of three colonies are allocated, on the same plate, to each treatment level and to the control(s) and toxic reference chemical. For each test, the following treatments and control(s) are used:

-      control without solvent (minimum 12 larvae X 3 colonies = 36 larvae minimum);

-      control with solvent if necessary (minimum 12 larvae X 3 colonies = 36 larvae minimum);

-      at least five treatments, *i.e.* five increasing test concentrations (each containing a minimum 12 larvae X 3 colonies = 36 larvae minimum per treatment) in a geometric series, spaced by a factor not exceeding three, and covering the NOEC/NOED or the $EC_{50}/ED_{50}$ or any $EC_x/ED_x$; alternatively, when a limit test is performed (see paragraph 25), a dose of 100 µg a.i. (or test chemical)/larva (equivalent to 650 mg a.i. (or test chemical)/kg of diet) or the maximum achievable solubility, whichever is lower, may be tested;

-      a reference chemical, dimethoate 48 mg/Kg of diet or fenoxycarb 0.32 mg/Kg of diet (minimum 12 larvae X 3 colonies = 36 larvae minimum).

22.      A total of seven to eight (if solvent is used) well-plates are used per test. Each group of a minimum of 12 larvae from each of the three colonies is considered a replicate for a given treatment level and identified as such on the microplate.

23.      The plates are kept under dark conditions for the duration of the test. During the test, the temperature in the incubator is kept between 34ºC and 35ºC. Temporary deviations are allowed, however temperature should not drop below 23°C or go above 40°C, and these deviations should not last, as far as possible, more than 30 minutes once every 24 hour.

## Range-finding test

24.      In order to determine the NOEC/NOED or the $EC_{50}/ED_{50}$ range, it is recommended that a preliminary experiment be conducted with concentrations of the test chemical varying according to a geometrical ratio from 5 to 10. This preliminary experiment will also provide information about the mode of action of the test chemical that will help select the reference chemical (see paragraph 6).

## Limit test

25.      In some cases (e.g. when a test chemical is expected to be of low toxicity or is poorly soluble) a limit test may be performed using 100 µg a.i. or test chemical/larva (equivalent to 650 mg a.i. or test chemical/Kg of diet) or the maximum achievable solubility for poorly soluble chemicals, whichever is lower, in order to demonstrate that no effect is observed at this concentration level. Three replicates of a minimum of twelve larvae from three different colonies are used for the limit dose tested, as well as the relevant control(s), and the use of the reference chemical. If statistically significant effects occur compared to the control, a full study should be conducted.

## Collection of larvae

26.      On D1, the comb containing first instar larvae (Figure 5) is carried from the hive to the laboratory in an insulated container in order to avoid temperature variation, and maintained at ambient temperature (not below 20ºC). It is then introduced into a (switched off) laminar-flow hood or under other clean conditions for grafting. In order to avoid bias due to possible heterogeneity of the larvae, it is recommended selecting newly hatched larvae that have not yet formed a "C" shape and/or using larvae lying in (cloudy) royal jelly, and allocating larvae randomly to the plates for each colony. A minimum of twelve larvae from each of the three replicate colonies is needed on D3 – the day of the first administration of the test chemical treatment. To ensure that all larvae are alive before the first administration of the treated diet on D3, the test may be initiated with larvae in excess in order to replace dead and non-suitable larvae (e.g. too small) on D3.

27.      Alternatively, this randomised allocation of larvae may be done on D3, just before the administration of the first chemical treatment.

## Grafting and feeding of larvae

28.      The diet is warmed in the incubator before use. The grafting is performed preferably on a warming plate maintained at 34-35°C. The micropipettes used to place the diet into the cells are equipped with disposable tips or a multi-stepper pipette can be used. On day 1 (D1), a volume of 20 µL of diet A is dropped into each cell, and one larva is delicately collected from the comb and transferred to each cell, on the surface of the diet, using a grafting tool or a wetted paintbrush (e.g. No. 3/0). When a plate is completed with a

minimum of 12 larvae from each colony, it is placed, as far as possible in a single layer, into the hermetic container, which has previously been placed into a ventilated incubator at 34-35°C (Figure 4), and as close as possible within that range for the duration of the test.



Figure 4: larvae incubation device

29.     All larvae are fed once a day, except on D2, preferably on a warming plate that should not be warmed above 35 °C, with a sterilised transparent pipette tip with filter (to avoid contamination) or a multi stepper pipette following the schedule of Figure 5, in particular the volume of diet provided to individual larva is adapted on a daily basis. Care should be taken to avoid touching and drowning the larvae when feeding them. Food is placed close to the larva, along the wall of the grafting cell. Additional food should be added to the cell even if the previous allocation has not been totally consumed. The presence of uneaten food on D8 should be qualitatively recorded.

## Repeated administration of the chemical in the test solution

30.     On D3, a minimum of twelve well-fed larvae from each of the three colonies are selected. From D3 to D6, larvae are fed with diets (diet B at D3, and diet C from D4 to D6) containing the test solution at the suitable concentration. The mixing of the test solution with the diet is performed just before administration, unless the stability of the test chemical in the diet has been demonstrated and is reported. From D3 to D6, for each treatment, a different micropipette tip with filter or a multi-stepper pipette is used to administer the diet containing the treatment in order to avoid contamination.
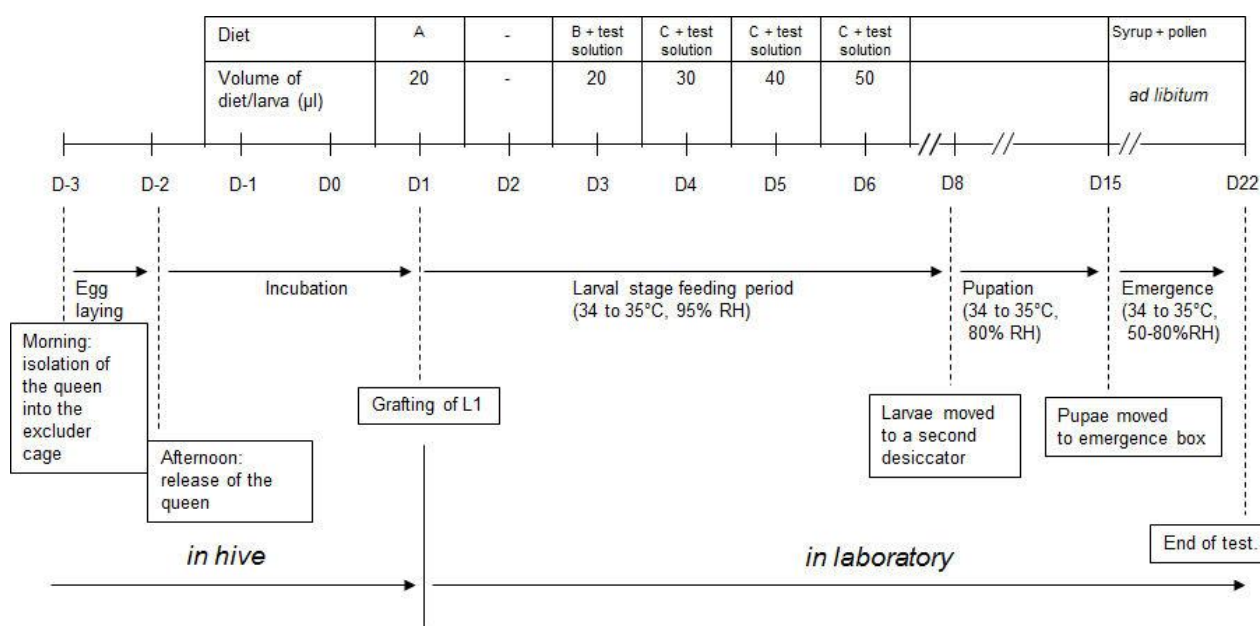
| Diet | A | - | B + test solution | C + test solution | C + test solution | C + test solution | | Syrup + pollen |
|---|---|---|---|---|---|---|---|---|
| Volume of diet/larva (µl) | 20 | - | 20 | 30 | 40 | 50 | | *ad libitum* |

| D-3 | D-2 | D-1 | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D8 | D15 | D22 |

Egg laying

Incubation

Larval stage feeding period (34 to 35°C, 95% RH)

Pupation (34 to 35°C, 80% RH)

Emergence (34 to 35°C, 50-80%RH)

Morning: isolation of the queen into the excluder cage

Afternoon: release of the queen

Grafting of L1

Larvae moved to a second desiccator

Pupae moved to emergence box

*in hive*

*in laboratory*

End of test.

Figure 5: Schematic representation of the important steps of the larval repeated exposure toxicity test (D=day; RH=relative humidity)

## Termination of the test

31.    On D22, the number of emerged adults and non-emerged bees are counted (see paragraph 33) and the test is terminated by freezing the plates at ≤ -10ºC, or preferably ≤ -80ºC or by using other humane methods.

## Observations

32.    Following the first chemical exposure on D3, mortalities are checked and recorded at the time of feeding from D4 to D8 and on D15. An immobile larva or a larva which does not react to the contact of the grafting tool or paintbrush, or which does not show signs of respiration under a stereomicroscope is noted as dead. On D15, larvae that have not transformed into pupae are recorded as dead (see Annex 2) and removed. Hatched adults that show a normal development – i.e. alive adult bees and dead adults which have left their cell – are recorded on D22.

33.    At the feeding time, dead larvae are systematically removed for sanitary reasons.  The number of emerged bees and non-emerged bees (pupal mortality) are counted on D22. [Note: empty cells indicate emerged bees; in rare occasions bees can re-enter cells]. Adult emergence rate is calculated in percentage by comparing the number of bees emerged on D22 to the number of larvae on D3 when dosing starts. The pupal mortality is calculated in percentage by comparing the number pupae failed to emerge, including those bees without emergence on D22 and dead pupae removed during pupa stage – from D8 to D22 – to the number of bees entering pre-pupa stage on D8. The larvae mortality is calculated in percentage by comparing the number of bees died during larvae stage – from D3 to D8 – to the number of larvae on D3 when dosing starts.

34.    Other observations, e.g. larval appearance and size, behaviour, morphological differences and any other adverse effects after emergence (in comparison with controls) should be recorded qualitatively. Bees showing severely impaired behaviour or other severe effect after emergence that suggest bees are in pain

should be euthanised immediately using the most humane method. The presence of uneaten food on D8 should be qualitatively recorded.

# DATA AND REPORTING

## Data and statistical analysis

35.      Data are summarised (e.g. in a tabular form), showing for each treatment group, as well as control and reference chemical groups, the number of larvae used, mortalities and adverse effects: larval mortalities from D3 to D8, pupal mortalities from D8 to D15 and emergence rate on D22. Data are analysed using appropriate statistical methods according to the OECD Guidance Document No. 54 (12).

### NOEC/NOED estimation

36.      The NOEC/NOED is determined on D22 for adult emergence (see Annex 2 for definitions). In case no effects are detected at all test concentrations/doses the NOEC/NOED will be considered to be higher or equal to the highest concentration tested. If, in a limit test, the effect at the tested concentration/dose is not statistically significantly different from the control, it should be indicated that the NOEC/NOED is higher or equal to the concentration/dose tested.

### $EC_{50}/ED_{50}/EC_x/ED_x$ estimation

37.      If data allows an $EC_{50}/ED_{50}$ and any $EC_x/ED_x$, including the associated lower and upper confidence limits, is/are calculated for emerged adult bees on D22 (see Annex 2 for definitions).

### Test report

38.      The test report should include the following:

### Test chemical:

-        Physical nature and relevant physical-chemical properties;

-        Chemical identification data, including purity of the active ingredient and, if testing a formulated product, the composition of the formulation;

### Test species:

- Source, species and sub-species of honey bee, supplier of source (if known) and the culture conditions used;

- Health condition of the hive used in the test and colony treatment history since the last winter preceding the performance of the test.

### Test conditions:

-        Place and date of the test;

- Description of the test system: type of well-plates used, information on the components of the diet (e.g. source of the royal jelly, yeast extract, etc.), number of larvae per treatment level and controls, solvent and concentrations used (if any), test concentrations used for the test chemical;

- Incubation conditions: temperature (mean, standard deviation, minimum and maximum values) and relative humidity.

*Results:*

- The number and percentage of bees considered dead at each treatment level, control(s) and toxic reference chemical (dimethoate or fenoxycarb);

- The nominal test concentrations used and measured concentration in the stock solution. The measured concentration should be within 20% of nominal.

- The mortality from D3 to D8, on D15 and emergence rate on D22, NOEC/NOED and/or $EC_{50}/ED_{50}$, and/or any $EC_x/ED_x$ for adult emergence on D22, and a graph of the fitted model, the slope of the concentration-response curve and its corresponding 95% confidence limits and the criteria for goodness of fit; statistical/mathematical procedures used for the determination of the NOEC/NOED and $EC_{50}/EC_x$ if appropriate;

- Other observations, including the presence of uneaten food at the end of the feeding period (D8);

- Explanation for any deviation from the test method and whether these deviations affected the results;

Any difficulties with analytics and their justifications.

## LITERATURE

(1)      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 doi: 10.1787/9789264203723-en

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**ANNEX 1**

**Validation report**
**Results of an international ring test of the honey bee (*Apis mellifera*) larval toxicity test, repeated exposure**

Compiled by

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17 April 2015

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# 1. Introduction

## 1.1 Background

A new test for assessing effects of chemicals on honey bee larvae and using a laboratory rearing protocol described by Aupinel *et al.* (2005) was presented to the International Commission for Plant Bee Relationship (ICPBR) Symposium in York in 2005. This test allows for assessing short and long term effects of chemicals/pesticides on honey bee workers after an acute or repeated exposure during larval stage under laboratory conditions. During the ICPBR symposium, it was decided to conduct a ring test for validation.

A ring test was conducted in 2008 (Aupinel *et al.*, 2009) using acute single exposure conditions and measuring an endpoint on D7, i.e. at the end of the larval stage. The test method was submitted to the OECD in 2011 and adopted as an OECD Guideline for the testing of chemicals in 2013: TG 237, Honey bee (*Apis mellifera*) toxicity test, single exposure.

A ring test of the repeated exposure protocol was performed during spring and summer 2014; the ring test results are presented in this report. The ring test conditions were as follows:

- Larvae from D3 to D6 were exposed in order to avoid mortality due to grafting and to facilitate larvae randomisation.
- Two chemicals were used: dimethoate and fenoxycarb. The rationale for this choice was justified by the difference of mode of action of these chemicals. An effect on larvae can be expected with dimethoate, while an effect on pupae would be observed with fenoxycarb.
- Each participant had to purchase the two chemicals. A high purity level (>98%) was required.
- Adult emergence rate is measured on D22. An Effect Concentration for 50% effect ($EC_{50}$) will be calculated for dimethoate, a No Observed Effect Concentration (NOEC) determined for fenoxycarb.
- Data loggers placed into the desiccators (close to the plates) are used in order to record variations of temperature and relative humidity during the test. It was recognised that these parameters are the main factors likely to influence larvae sensitivity to an insecticide, and that they must be controlled with a high frequency close to the plates to ensure that the test is conducted under the required conditions or that deviation can be identified.

## 1.2 Participating laboratories

18 laboratories expressed interest in participating in the ring test; 14 of them performed trials. One laboratory did not comply with an important requirement which was using data loggers close to the plate, so their results were not taken into account. In total, 13 laboratories from five countries participated in the ring test, coming from governmental institutions, universities, chemical industries and contract laboratories: Australia (one), Austria (one), France (two laboratories), Germany (six), Italy (two), and Switzerland (one). They are listed below.

Ecotox Services Australasia, **Australia** – *Amandine Vincent*

University of Graz, **Austria** – *Ulrike Riessberger-Gallé*

INRA Le Magneraud, **France** – *Pierrick Aupinel* [Project leader and organiser of the ring test], *Dominique Fortini, Carole Moreau-Vauzelle*

ANSES, **France** – *Nicolas Cougoule, Frank Schurr, Marie-Pierre Chauzat*

LAVES Institute für Bienenkunde Cell**, Germany** – *Martina Janke*

BAYER Crop Science AG, **Germany** – *David Gladbach*, *Dunja Przygoda*

BASF SE, **Germany** – *Nicole Hanevald, Stephanie Royer*

Eurofins Agroscience Services EcoChem GmbH, **Germany** – *Jakob Eckert*

BioChem agrar GmbH, **Germany** – *Katharina Kleebaum, Markus Barth*

Ibacon GmbH, **Germany** – *Alexander Ehmke, Stephan Schmitzer*

Biotecnologie BT S.r.l., **Italy**, *Monica Colli*

CRA-API, **Italy** – *Piotr Medrzycky*

IES, **Switzerland,** *Stefan Kimmel*

### 1.3 Work performed before the ring test

Dimethoate and fenoxycarb had already been tested under repeated exposure conditions with an exposure from D1 to D6 (Aupinel *et al.*, 2007). Considering that, in this method, exposure starts on D3, preliminary tests were performed in INRA (Institut National de la Recherche Agronomique, France) in order to define the respective test concentrations range for dimethoate and fenoxycarb, for assessing an $EC_{50}$ and a NOEC on adult emergence rate.

Based on the results of these tests, it was decided to use a concentration range of 20 µg.kg$^{-1}$ to 302 µg.kg$^{-1}$ of diet for fenoxycarb, and 3 mg.kg$^{-1}$ to 48 mg.kg$^{-1}$ of diet for dimethoate, with a spacing factor of two for both chemicals.

These preliminary trials led to define concentrations of dimethoate and fenoxycarb in positive controls, and expected effects on mortality on D8 (minimum 50%) and emergence rate on D22 (maximum 20%) respectively in acceptance criteria. One of the objectives of this ring test was also to check/confirm these values.

## 2. Results

### 2.1 Experimental design

The detailed protocol was provided to each participant before the ring test. It is based on the draft test method which was presented to the OECD in February 2014.

First instar larvae were collected from three hives and reared in individual cells until the third day with pure diet (diet without test chemical). On D3, larvae were randomised and fed with spiked diet until D6, except control samples fed with pure diet. An acetone control sample was added for fenoxycarb. At this stage, a minimum of 12 larvae per hive and per treatment was required. On D8, at the pre-pupal stage, larva were removed from their container regulated at 34.5 ± 0.5°C, 95 ± 5% of relative humidity (RH) and transferred into another one regulated at 34.5 ± 0.5°C, and 80 ± 5% RH. On D15, each plate was transferred into an emergence box placed into an incubator regulated at 34-35 °C and a minimum of 50% RH. Mortalities were checked each day from D4 to D8, at D15 and D22. Emerged adults, dead or alive, were recorded on D22.

### 2.2 Dimethoate

11 laboratories performed 13 dimethoate tests (Table 1) (laboratories No. 11 and 12 did not perform any dimethoate test).

Control larval mortality (water) on D8 ranged from 0.0% to 13.9% and thus was lower than 15% which is the maximum admitted in the acceptance criteria. The emergence rate on D22 exceeded 70% except for the tests No. 6 and 12 with values at 66.7% and 69.0% respectively, which is below the minimum value required in the acceptance criteria. The measured concentrations of the stock solutions did not differ from more than 20% of the nominal concentration and were in the 0.80-1.20 interval (except the Laboratory No. 12 with a measured concentration of 0.62 mg/ml). The laboratory No. 8 did not provide any sample for analysis.

According to the acceptance criteria, two laboratories (No. 6 and 12) did not meet all the criteria. It was decided to keep these results in our analysis in order to estimate the incidence of such deviations.

The corrected emergence rates from the initial number of larvae on D3 were calculated using the Abbott formula (Abbot, 1925). The $EC_{50}$ values were determined from the linear regression equation which provides the corrected emergence rate from the log of tested concentrations, computed with Minitab 15 (Annex 3). $EC_{50}$ values were from 4.72 to 8.14 for 11 of the 13 tests (Table 1, Figure 2). The highest value was for the test 7 with an $EC_{50}$ at 14.14 mg.$Kg^{-1}$, and the lowest one for the test 9 with an $EC_{50}$ at 2.92 mg.$Kg^{-1}$. It can be noted that the test 7 was performed with slightly lower temperatures than requested values (from 33.7°C±0.4°C to 34.1°C±0.1°C instead of 34.5°C±0.5°C), and relative humidity for the Test No. 9 was much lower and variable compared to requirements (69.1%±14.3% instead of 95%±5%).

No influence of bee strain on the values of $EC_{50}$ is noted (Figure 1, KW test, p=0.721).
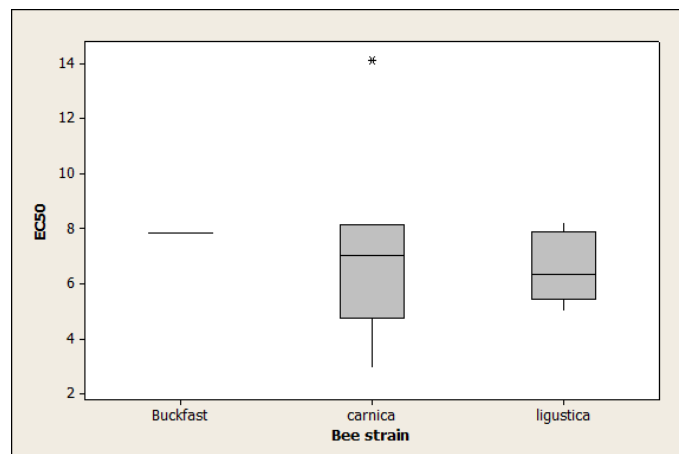


*Figure 1: EC50 depending on bee strain*

The $EC_{50}$ confidence intervals (CI) (calculated with the formula extracted from Zar (1999) have been obtained for the Tests No. 9 and No. 13. Low $R^2$ values explain that CI could not be calculated for the Tests No. 2, 6 and 8. In spite of a high value of $R^2$ for the Test No. 9, the CI could not be obtained due to the fact that $EC_{50}$ was closed to the lower concentration tested.

| lab | test | Bee strain | Dimethoate Purity (%) | EC$_{50}$[1] (mg.kg$^{-1}$) EC$_{50}$ 95% confidence interval | R$^2$ | Acceptance Criteria 0.8<[C]<1.2 Measured [C] (mg/ml) | M<15% Control mortality D8 (%) | E>70% Control emergence rate D22 (%) | D1-D8 Control T°C mean (Sd) | RH% mean (Sd) | D1 or D3-D8 Treated T°C mean (Sd) | RH% mean (Sd) | D8-D15 Control T°C mean (Sd) | RH% mean (Sd) | D8-D15 Treated T°C mean (Sd) | RH% mean (Sd) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | ligustica | 98.5 | **6.30** *4.20-7.31* | 96.8 | 0.81 | 2.1 | 91.7 | 35 (0.1) | 90.2 (3.8) | 34.4 (0.3) | 92.3 (5.3) | 34 (0.1) | 73.6 (6.4) | 34 (0.1) | 75.7 (1.3) |
| 2 | 2 | Buckfast | 99.5 | **7.85** *-* | 61.6 | 0.96 | 8.3 | 70.1 | 34.1 (0.3) | 91.2 (5.1) | 34.9 (0.2) | 91.9 (4.2) | 34.6 (0.2) | 89.7 (5.6) | 34.6 (0.1) | 84.8 (3.6) |
| 3 | 3 | carnica | 99.8 | **6.12** *0.84-15.01* | 82.1 | 1.083 | 2.6 | 82.1 | 34.7 (0.1) | 97.0 (0.9) | 34.7 (0.14) | 97.0 (0.9) | 34.6 (0.2) | 89.6 (2.3) | 34.6 (0.2) | 89.6 (2.3) |
| 4 | 4 | carnica | 98.0 | **7.56** *6.68-8.44* | 99.0 | 1.1 | 6.3 | 81.2 | 34.7 (0.3) | 94.3 (6.7) | 34.7 (0.1) | 99.0 (3.6) | 34.8 (0.1) | 79.7 (3.5) | 34.8 (0.1) | 79.7 (3.5) |
| 5 | 5 | carnica | 99.8 | **7.52** *5.46-10.12* | 94.1 | 1 | 11.1 | 80.6 | 34.5 (0.5) | 95.0 (3.0) | 34.5 (0.5) | 95.0 (3.0) | 34.5 (0.5) | 80.0 (15.0) | 34.5 (0.5) | 80.0 (15.0) |
| 5 | 6 | carnica | 99.8 | **5.82** *-* | 52.4 | 1 | 13.9 | 66.7 | 34.5 (0.5) | 95.0 (3.0) | 34.5 (0.5) | 95.0 (3.0) | 34.5 (0.5) | 80.0 (15.0) | 34.5 (0.5) | 80.0 (15.0) |
| 6 | 7 | carnica | 99.8 | **14.14** *7.90-24.42* | 87.1 | 0.99 | 0 | 76.2 | 33.9 (0.1) | 99.7 (2.1) | 34.1 (0.1) | 99.6 (2.9) | 33.7 (0.4) | 87.8 (4.2) | 33.9 (0.4) | 83.9 (4.9) |
| 7 | 8 | carnica | 98.5 | **7.06** *-* | 60.3 | 1.1 | 2.1 | 89.6 | 34.8 (0.5) | 96.4 (5.4) | 34.8 (0.5) | 96.4 (5.4) | 34.8 (0.5) | 81.6 (5.3) | 34.8 (0.5) | 81.6 (5.3) |
| 8 | 9 | carnica | 98.5 | **2.92** *-* | 83.4 | * | 8.3 | 77.8 | 35.0 (0.1) | 69.1 (14.3) | 35.0 (0.1) | 69.1 (14.3) | 34.5 (0.04) | 61.7 (2.3) | 34.5 (0.04) | 61.7 (2.3) |
| 9 | 10 | carnica | 98.5 | **8.14** *2.79-12.20* | 96.0 | 0.96 | 12.5 | 79.1 | 34.4 (0.3) | 99.5 (3.7) | 34.4 (0.3) | 99.5 (3.7) | 34.9 (0.1) | 99.7 (2.7) | 34.9 (0.1) | 99.7 (2.7) |

| 9 | 11 | carnica | 98.5 | **4.72** 3.71-6.0 | 85.1 | 1.1 | 11 | 79.1 | 34.4 (0.3) | 99.0 (0.2) | 34.4 (0.3) | 99.0 (5.2) | 34.9 (0.2) | 91.3 (4.5) | 34.9 (0.2) | 91.3 (4.5) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **10** | 12 | ligustica | 98.0 | **7.98** 4.52-12.10 | 89.4 | 0.62 | 12.5 | 69 | 35.2 (0.3) | 88.5 (4.4) | 35.2 (0.3) | 88.5 (4.4) | ** | ** | ** | ** |
| **13** | 13 | ligustica | 99.5 | **5.01** 2.29-6.29 | 90.0 | 0.97 | 2.8 | 72.2 | 34.6 (0.5) | 98.5 (2.3) | 34.6 (0.5) | 98.5 (2.3) | 34.6 (0.5) | 96.6 (2.1) | 34.6 (0.5) | 96.6 (2.1) |

*Table 1: Dimethoate test results*

*: no sample sent;
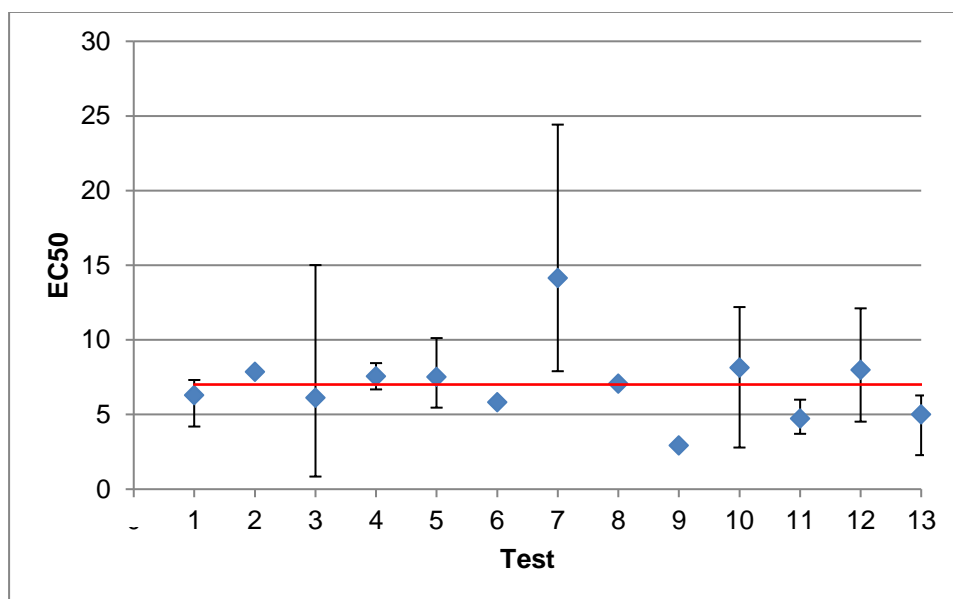**: not informed
(1): calculated with the nominal value

*Figure 2: EC$_{50}$ values (±95% CI when calculation was possible) for each test (the red line indicates the mean value: 7.01mg.Kg$^{-1}$)*

### 2.3 Fenoxycarb

11 laboratories performed 13 fenoxycarb tests (Table 2) (laboratories 10 and 13 did not perform any fenoxycarb test).

Larval mortality control (water and solvent) on D8 ranged from 0.0% to 14.6% and thus was lower than the minimum required of 15% as indicated in the acceptance criteria. The emergence rate on D22 exceeded 70% except for the Test No. 4 for the water control and the Test No. 6 for the solvent control with values of 69.4% and 58.3% respectively. The measured concentrations of the S1 solutions (1/100 stock solution) did not differ from more than 20% of the nominal concentration and ranged in an interval of 57-86, except for the Tests No. 9 and 13 with measured concentration at 53 µg.ml$^{-1}$ and 97 µg.ml$^{-1}$ respectively. The laboratory No. 8 did not provide any sample for analysis, and the sample provided by the laboratory No. 9 was damaged during shipment.

According to the acceptance criteria, nine tests were valid, and four tests (No. 4, 6, 9 and 13) did not meet all the criteria. We kept them in our analysis in order to estimate the incidence of these deviations on the results.

| lab | test | Bee strain | Fenoxy carb Purity (%) | NOEC µg.Kg⁻¹ | Acceptance Criteria 57<[C]<86 Measured [C] (µg.ml⁻¹) | M<15% Control Mortality D8 (%) Water Solvent | E>70% Control Emergence rate D22 (%) Water Solvent | D1-D8 Control T°C mean (Sd) | RH% mean (Sd) | D1 or D3-D8 Treated T°C mean (Sd) | RH% mean (Sd) | D8-D15 Control T°C mean (Sd) | RH% mean (Sd) | D8-D15 Treated T°C mean (Sd) | RH% mean (Sd) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 1 | ligustica | 99.5 | **40** | 84 | 2.1 6.3 | 90.0 85.0 | 35.0 (0.1) | 92.5 (4.7) | 34.3 (0.5) | 96.6 (3.8) | 34.5 (0.1) | 75.3 (2.9) | 33.9 (0.2) | 79.7 (5.7) |
| **1** | 2 | ligustica | 99.5 | **40** | 80 | 12.5 4.2 | 88.0 88.0 | 34.5 (0.2) | 94.8 (4.8) | * | * | 33.1 (0.1) | 77.7 (3.9) | * | * |
| **2** | 3 | Buckfast | 99.5 | **<20** | 86 | 6.3 14.6 | 75.0 75.0 | 34.7 (0.3) | 92.2 (5.4) | 34.6 (0.3) | 95.7 (5.9) | ** | ** | ** | ** |
| **3** | 4 | carnica | 99.5 | **40** | 63 | 5.6 8.3 | 69.4 83.3 | 34.7 (0.2) | 98.4 (1.8) | 34.7 (0.2) | 98.4 (1.8) | 34.7 (0.2) | 76.8 (1.0) | 34.7 (0.2) | 76.8 (1.0) |
| **4** | 5 | carnica | 99.4 | **40** | 75 | 2.1 0.0 | 91.7 93.8 | 34.7 (0.1) | 98.8 (3.9) | 34.8 (0.3) | 94.7 (6.2) | 34.9 (0.1) | 81.4 (4.3) | 34.9 (0.1) | 81.4 (4.3) |
| **5** | 6 | carnica | 99.3 | **40** | 85 | 0.0 2.8 | 75.0 58.3 | 34.5 (0.5) | 95.0 (3.0) | 34.5 (0.5) | 95.0 (0.3) | 34.5 (0.5) | 80.0 (0.15) | 34.5 (0.5) | 80.0 (0.15) |
| **5** | 7 | carnica | 99.3 | **40** | 82 | 8.3 11.1 | 77.8 72.2 | 34.5 (0.5) | 95.0 (3.0) | 34.5 (0.5) | 95.0 (3.0) | 34.5 (0.5) | 80.0 (1.5) | 34.5 (0.5) | 80.0 (1.5) |
| **6** | 8 | carnica | 99.5 | **40** | 84 | 0.0 2.4 | 76.2 71.4 | 33.9 (0.1) | 99.7 (2.1) | 34.2 (0.1) | 99.3 (3.8) | 33.7 (0.4) | 87.8 (4.2) | 33.9 (0.4) | 89.4 (4.5) |
| **7** | 9 | carnica | 98.5 | **40** | 53 | 4.2 2.1 | 87.5 81.5 | 34.9 (0.5) | 96.3 (5.4) | 34.9 (0.5) | 96.3 (5.4) | 34.9 (0.5) | 81.6 (5.3) | 34.9 (0.5) | 81.6 (5.3) |
| **8** | 10 | carnica | 99.5 | **<20** | **** | 8.3 2.8 | 77.8 88.9 | 35.0 (0.1) | 69.1 (14.3) | 35.0 (0.1) | 69.1 (14.3) | 34.5 (0.04) | 61.7 (2.3) | 34.5 (0.04) | 61.7 (2.3) |
| **9** | 11 | carnica | 98.5 | **40** | ***** | 0.0 0.0 | 72.9 72.9 | 34.4 (0.3) | 99.4 (4.3) | 34.4 (0.3) | 99.4 (4.3) | 34.9 (0.2) | 99.3 (3.8) | 34.9 (0.2) | 99.3 (3.8) |
| **11** | 12 | *** | 99.9 | **<20** | 82 | 0.0 5.6 | 72.7 86.7 | 34.7 (0.3) | 93.6 (6.5) | 34.7 (0.3) | 93.6 (6.5) | 34.7 (0.3) | 93.6 (6.5) | 34.7 (0.3) | 93.6 (6.5) |

| 12 | 13 | ligustica | 99.5 | 40 | 97 | 0.0 2.8 | 94.4 94.4 | 34.0 (0.4) | 99.0 (7.8) | 34 (0.4) | 99.0 (7.8) | 34.0 (0.4) | 99.0 (7.8) | 34 (0.4) | 99.0 (7.8) |

*Table 2: Fenoxycarb test results*

*\*: absence of data logger (lack of equipment);*

*\*\*: problem with data logger; \*\*\*: not informed;*

*\*\*\*\*: no sample sent;*

*\*\*\*\*\*: sample damaged during shipment*

Comparison between water and solvent controls by Chi-square test did not show any significant differences between these treatments (Table 3). The emergence rate value recorded on acetone control could then be used to determine NOEC which is the highest concentration of fenoxycarb which does not induce significant difference of emergence rate compared to the emergence rate observed with the solvent control. A Chi-square test was used to make these comparisons.

| Test | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **water control** | **89.58%** | **87.50%** | **75.00%** | **69.44%** | **91.67%** | **75.00%** | **77.78%** | **76.19%** | **87.50%** | **77.78%** | **72.92%** | **66.70%** | **94.44%** |
| **acetone control** | 85.42% | 87.50% | 75.00% | 83.33% | 93.75% | 58.33% | 72.22% | 71.43% | 81.25% | 88.89% | 72.92% | 72.20% | 94.44% |
| **20 µg.Kg$^{-1}$** | **91.67%** | **91.67%** | 45.83% | **58.33%** | **91.67%** | **61.11%** | **69.44%** | **80.95%** | **89.58%** | 47.22% | **68.75%** | 36.10% | **88.89%** |
| **40 µg.Kg$^{-1}$** | **89.58%** | **91.67%** | 37.50% | **72.22%** | **87.50%** | **72.22%** | **55.56%** | **66.67%** | **85.42%** | 36.11% | **62.50%** | 44.40% | **88.89%** |
| **80 µg.Kg$^{-1}$** | 54.17% | 47.92% | 33.33% | 36.11% | 77.08% | 19.44% | 2.78% | 14.29% | 39.58% | 36.11% | 12.50% | 33.30% | 47.22% |
| **160 µg.Kg$^{-1}$** | 0.00% | 0.00% | 25.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 2.08% | 27.78% | 2.08% | 0.00% | 0.00% |
| **320 µg.Kg$^{-1}$** | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |

*Table 3: emergence rates observed on D22 for each test (1 to 13) according to fenoxycarb concentrations (in bold type: emergence rate values that no significantly differ from acetone control)*

10 tests lead to determine a NOEC of 40 µg.Kg$^{-1}$ for fenoxycarb (Tables 2, 3). We noted on test No. 10 that the NOEC could not be defined in this range of concentrations. Relative humidity conditions applied for this test are the same low and variable conditions (69.1% ±14.3% instead of 95%±5%) than those applied by this laboratory for dimethoate test.

## 2.4 Additional results

### 2.4.1 Mortality on D22

It was clearly indicated that the main endpoint to record is the number of emerged bees, dead or alive, on D22. However, total mortality on D22 was also required in the protocol as an additional observation. Adult mortality on D22 could be relevant when some compounds could induce early mortality, just after emergence. In this case, only considering the emergence rate could undervalue the toxicity of a compound. This observation was recorded by a few laboratories considering that most of emerged bees were alive on D22.

Two laboratories (No. 1 and 2) observed that the total mortality on D22 was superior to the total non-emerged bees, which means that some emerged bees were dead on D22. The $LD_{50}$ based on the total mortality on D22 were 7.16 mg.Kg$^{-1}$ and 3.90 mg.Kg$^{-1}$ for laboratories No. 1 and 2 respectively, while $EC_{50}$ were 6.30 mg.Kg$^{-1}$ and 7.85 mg.Kg$^{-1}$ for the same laboratories. Even if $LD_{50}$ and $EC_{50}$ are of the same order of magnitude for laboratory No. 1, it should be noted that the $EC_{50}$ which is twice higher than $LD_{50}$ in the experiment performed by the laboratory No. 2. In this case, $EC_{50}$ seems to undervalue the toxicity of dimethoate.

Taking into account mortality instead of emergence did not change the NOEC value for fenoxycarb.

### 2.4.2 Colony effect

Independent observations according to larvae colony origin were performed by three laboratories (No. 4, 7 and 11).

A significant difference of mortality between colonies is observed in one case in the dimethoate trial performed by laboratory No. 4 at the concentration of 3 mg.Kg$^{-1}$. The high $R^2$ value (99.0) indicates that this slight difference does not seem to influence the relation between concentration and effect.
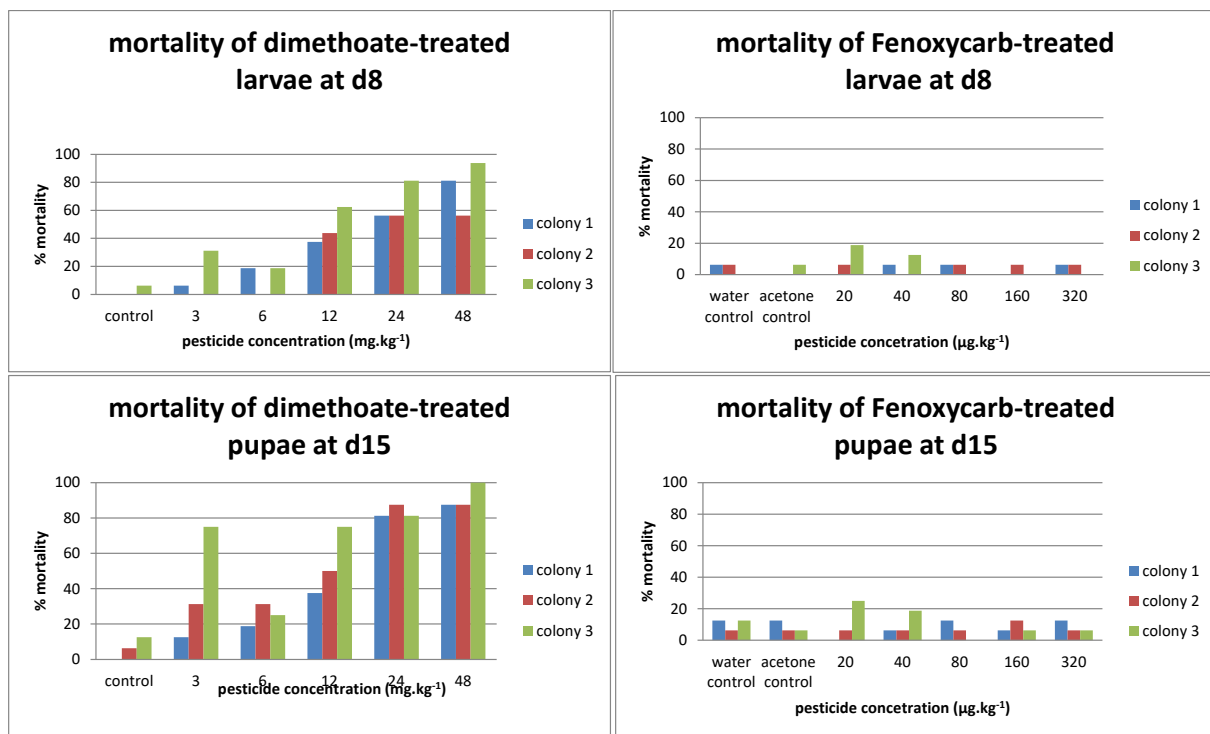
*Figure 2: Mortalities for dimethoate and fenoxycarb treated larvae recorded on D8 and D15 by lab 7.*

In the trials performed by laboratory No. 7, it can be noted that one colony was specially characterised by various mortality levels at D8 and D15 (Figure 2). The NOEC recorded for fenoxycarb by this laboratory is equal to 40 µg.Kg[-1], not different from values noted by most of the laboratories, in spite of high mortalities for the concentrations of 20 and 40 µg.Kg[-1]. We can then assess that this colony does not affect significantly the NOEC value. On the contrary, it seems clear that mortality recorded at the lower concentration of dimethoate influences the emergence rate for this concentration, and then the linearity level of the relation between concentration and emergence (Figure 3).
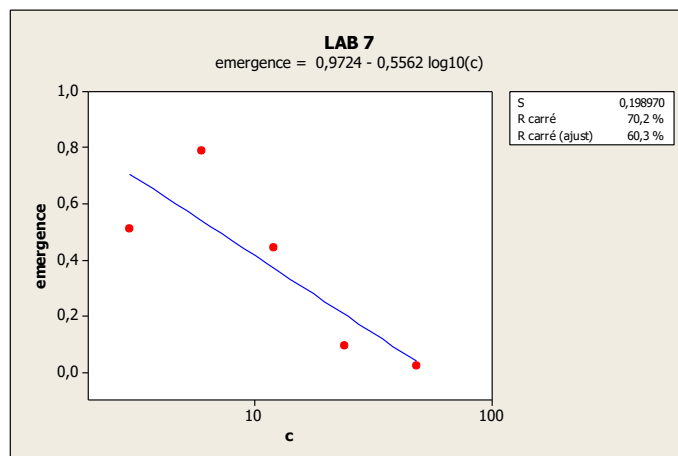


Figure 3: Effect of dimethoate concentration on emergence rate on D22

In the fenoxycarb test performed by the laboratory No. 11, one colony showed a higher mortality value which can explain a low emergence rate and no possibility to assess a NOEC value.

### 2.4.3 Recommended concentrations in control samples

The recommended concentrations in control samples determined from the preliminary trials were:

- 40 mg a.i./Kg of diet for dimethoate
- 250 ng a.i./Kg of diet for fenoxycarb

In the dimethoate positive control, the larval mortality should be ≥ 50% on D8. In the fenoxycarb positive control, the emergence rate should be ≤ 20% on D22.
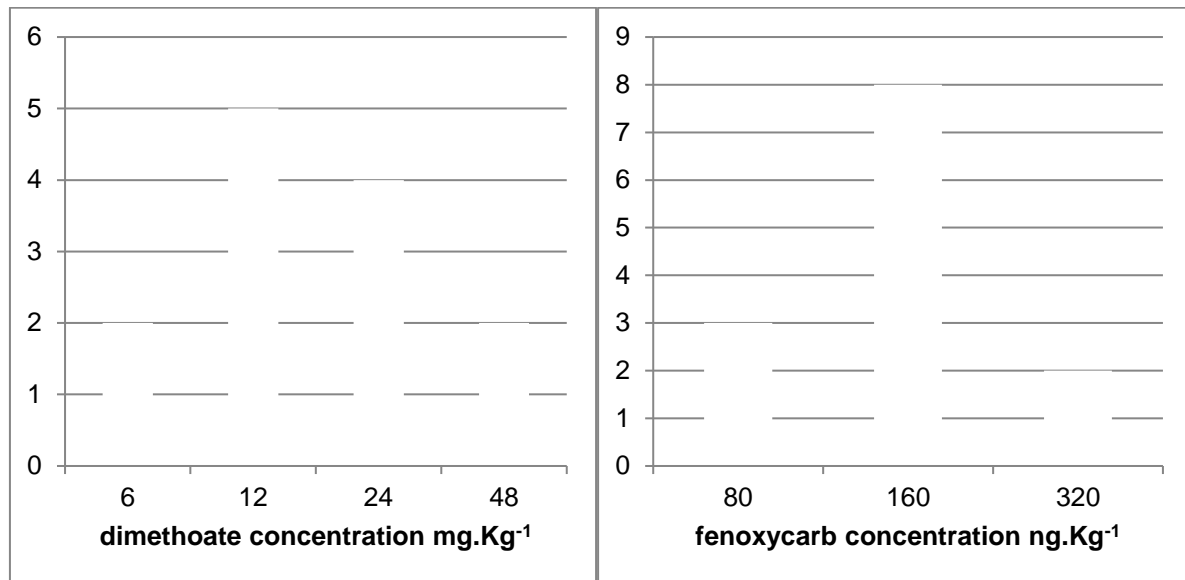


*Figure 4: number of test according to the lowest dimethoate concentration leading to a minimum of 50% mortality on D8*

*Figure 5: number of test according to the lowest fenoxycarb concentration leading to a maximum of 20% emergence on D22*

A concentration of 48 mg.Kg$^{-1}$ of dimethoate was necessary to reach a minimum of 50% mortality on
D8 (Figure 4), and a concentration of 320 ng.Kg$^{-1}$ of fenoxycarb was necessary to have an emergence rate lower than 20% on D22. See Figure 5 for two trials out of 13.

## 3. Discussion

The $EC_{50}$ values obtained from 11 of the 13 dimethoate tests ranged from 4.72mg.Kg$^{-1}$ to 8.14 mg.Kg$^{-1}$ and thus show a good level of agreement, with values differing by less than a factor of two from the mean. Medrzycki et al (2010) revealed that a difference of 2°C below the optimal temperature rearing conditions may induce a $LD_{50}$ (48h) 28 times higher. Consequently, it is realistic to explain that the rearing temperature applied in the test No. 7 that differ from 0.5°C below the optimal may lead to an $EC_{50}$ value two times higher than the mean. The $EC_{50}$ value calculated from test 9 is clearly lower than the mean, and the relative humidity conditions applied

in this test were really low and variable compared to the required conditions. If it is admitted that a slight temperature difference may influence larvae sensitivity to an insecticide, it is highly probable that sub optimal relative humidity conditions in the range of 25% lower than optimal values could operate as a stress factor resulting to increase sensitivity of larvae to the insecticide. It also has to be noted that laboratory 8 which performed this test did not send any sample of the stock solution of dimethoate for analysis in accordance with the protocol, which leads to wonder if another cause could not be involved to explain such difference.

10 of the 13 fenoxycarb tests provide a NOEC value at 40 $\mu$g.Kg$^{-1}$. One of the three remaining tests was run by the laboratory No. 8 with the same low and variable relative humidity conditions applied in dimethoate test, and the absence of a control sample for concentration analysis. This result reinforces the hypothesis of the influence of sub optimal relative humidity conditions on larvae sensitivity to a pesticide. In one of the remaining two tests (test No. 12), the mortality comparison between colonies shows one colony with significant higher mortality rate leading to a low emergence rate, and affecting general emergence rate observed on the whole plate. This may explain that in this test no NOEC value could be determined.

Some tests did not completely meet all the acceptance criteria and were in marge of some required values. For dimethoate, control emergence on D22 was less than 4% lower than the required minimum for the test No. 6. In the test No. 12, the control emergence on D22 was 1% lower than the required minimum, and the measured concentration was 0.18 mg/ml lower than the required minimum. In spite of these differences, these tests were considered acceptable with EC$_{50}$ values showing a good level of agreement.

Emergence rates were not those expected for fenoxycarb tests 4 and 6 (control water and control solvent respectively) as well as measured concentrations for tests 9 and 12. However, the obtained NOEC value of 40 $\mu$g.Kg$^{-1}$ is in accordance with most of the other tests.

On the opposite, deviations of relative humidity or temperature seem to affect the sensitivity of larvae to insecticides leading to invalid tests which do not meet these elementary requirements. This analysis supports the use of data loggers in order to get precise control conditions close to the plates, and the use of desiccators with salt solutions in order to assure controlled and regular climatic conditions.

Some results can be affected by a colony effect. This suggests that additional observations on D15 could be useful in order to check relative homogeneity of mortality between colonies. In order to avoid undervaluing a toxic effect, it should be recommended recording mortality on D22.

Based on the expected effects in positive control samples, the recommended concentrations for dimethoate and fenoxycarb should be 48 mg.Kg$^{-1}$ and 320 ng.Kg$^{-1}$ respectively, instead of 40 mg.Kg$^{-1}$ and 250 ng.Kg$^{-1}$ as it was recommended in the last version of the test method.

## 4. References

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Zar, J. H., 1999, Biostatistical analysis, Prentice Hall, 4th Edition.

**Appendix**

### Regression analysis

### Test 1, Lab 1

```
L'équation de régression est
E = 1,007 - 0,6342 log10(c)

S = 0,0632020   R carré = 96,8 %   R carré (ajust) = 95,8 %


Analyse de variance

              Somme des
Source       DL    carrés        CM       F      P
Régression    1   0,364463   0,364463   91,24  0,002
Erreur        3   0,011983   0,003994
Total         4   0,376446
```

### Test 2, Lab 2

```
L'équation de régression est
E = 0,9284 - 0,4787 log10(c)

S = 0,207903   R carré = 61,6 %   R carré (ajust) = 48,8 %

Analyse de variance

              Somme des
Source       DL    carrés        CM       F      P
Régression    1   0,207699   0,207699   4,81   0,116
Erreur        3   0,129671   0,043224
Total         4   0,337370
```

### Test 3, Lab 3

```
L'équation de régression est
E = 1,072 - 0,7267 log10(c)

S = 0,186455   R carré = 82,1 %   R carré (ajust) = 76,1 %

Analyse de variance

              Somme des
Source       DL    carrés        CM       F      P
Régression    1   0,478516   0,478516   13,76  0,034
Erreur        3   0,104297   0,034766
Total         4   0,582812
```

**Test 4, Lab 4**

```
L'équation de régression est
E = 1,398 - 1,022 log10(c)

S = 0,0405420   R carré = 99,3 %   R carré (ajust) = 99,0 %

Analyse de variance

                Somme des
Source      DL    carrés        CM        F       P
Régression   1   0,473373   0,473373   288,00   0,003
Erreur       2   0,003287   0,001644
Total        3   0,476660
```

**Test 5, Lab 5**

```
L'équation de régression est
E = 1,514 - 1,157 log10(c)

S = 0,110936   R carré = 96,1 %   R carré (ajust) = 94,1 %

Analyse de variance

                Somme des
Source      DL    carrés        CM       F        P
Régression   1   0,606480   0,606480   49,28   0,020
Erreur       2   0,024614   0,012307
Total        3   0,631094
```

**Test 6, Lab 5**

```
L'équation de régression est
E = 1,231 - 0,9551 log10(c)

S = 0,309990   R carré = 68,3 %   R carré (ajust) = 52,4 %

Analyse de variance

                Somme des
Source      DL    carrés        CM      F       P
Régression   1   0,413281   0,413281   4,30   0,174
Erreur       2   0,192187   0,096094
Total        3   0,605469
```

**Test 7, Lab 6**

```
L'équation de régression est
E = 1,599 - 0,9551 log10(c)

S = 0,171543   R carré = 90,4 %   R carré (ajust) = 87,1 %

Analyse de variance

                Somme des
Source      DL    carrés        CM       F        P
Régression   1   0,826562   0,826562   28,09   0,013
Erreur       3   0,088281   0,029427
Total        4   0,914844
```

### Test 8, Lab 7

```
L'équation de régression est
E = 0,9724 - 0,5562 log10(c)

S = 0,198970   R carré = 70,2 %   R carré (ajust) = 60,3 %

Analyse de variance

              Somme des
Source     DL   carrés        CM      F      P
Régression  1   0,280368  0,280368  7,08  0,076
Erreur      3   0,118767  0,039589
Total       4   0,399135
```

### Test 9, Lab 8

```
L'équation de régression est
E = 0,7872 - 0,6169 log10(c)

S = 0,103510   R carré = 88,9 %   R carré (ajust) = 83,4 %

Analyse de variance

              Somme des
Source     DL   carrés        CM       F       P
Régression  1   0,172449  0,172449  16,10  0,057
Erreur      2   0,021429  0,010714
Total       3   0,193878
```

### Test 10, Lab 9

```
L'équation de régression est
E = 1,511 - 1,110 log10(c)

S = 0,0878727   R carré = 97,3 %   R carré (ajust) = 96,0 %

Analyse de variance

              Somme des
Source     DL   carrés        CM       F       P
Régression  1   0,558483  0,558483  72,33  0,014
Erreur      2   0,015443  0,007722
Total       3   0,573927
```

### Test 11, Lab 9

```
L'équation de régression est
E = 0,8419 - 0,5070 log10(c)

S = 0,0989324   R carré = 88,8 %   R carré (ajust) = 85,1 %

Analyse de variance

              Somme des
Source     DL   carrés        CM       F       P
Régression  1   0,232964  0,232964  23,80  0,016
Erreur      3   0,029363  0,009788
Total       4   0,262327
```

### Test 12, Lab 10

```
L'équation de régression est
E = 1,163 - 0,7349 log10(c)


S = 0,118531   R carré = 92,1 %   R carré (ajust) = 89,4 %


Analyse de variance

             Somme des
Source    DL    carrés        CM        F       P
Régression  1  0,489348   0,489348   34,83   0,010
Erreur      3  0,042149   0,014050
Total       4  0,531497
```

## Test 13, lab 13

```
L'équation de régression est
E = 0,9112 - 0,5877 log10(c)


S = 0,0920938   R carré = 92,5 %   R carré (ajust) = 90,0 %


Analyse de variance

             Somme des
Source    DL    carrés        CM        F       P
Régression  1  0,313018   0,313018   36,91   0,009
Erreur      3  0,025444   0,008481
Total       4  0,338462
```

# 2 Annex 2

## DEFINITIONS

**EC$_x$/ED$_x$:** (Effect concentration/dose for x% effect) is the concentration/dose that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC$_{50}$ is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

**Lowest observed effect concentration/dose (LOEC/LOED)** is the lowest tested concentration/dose of a test chemical at which the chemical is observed to have a statistically significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC/LOED. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC/LOED (and hence the NOEC/NOED) has been selected.
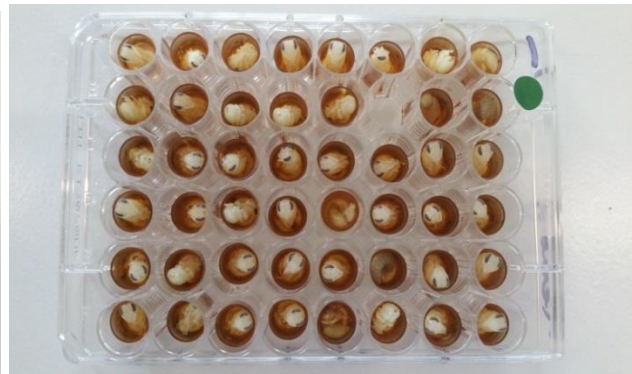
**No observed effect concentration/dose (NOEC/NOED)** is the test concentration/dose immediately below the LOEC/LOED, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.
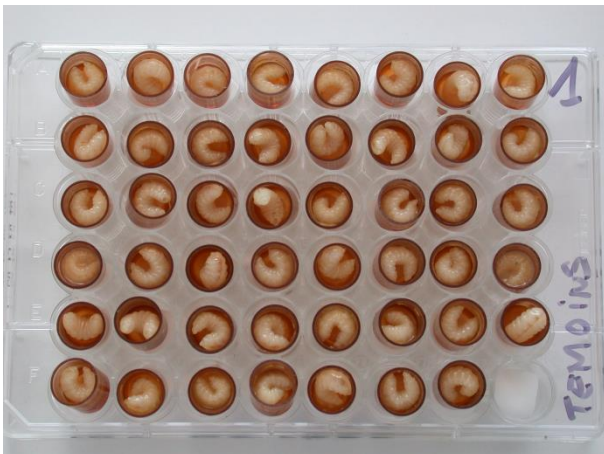
# 3 ANNEX 3

## PHOTOS OF PRE-PUPAE (D8) AND PUPAE (D15)
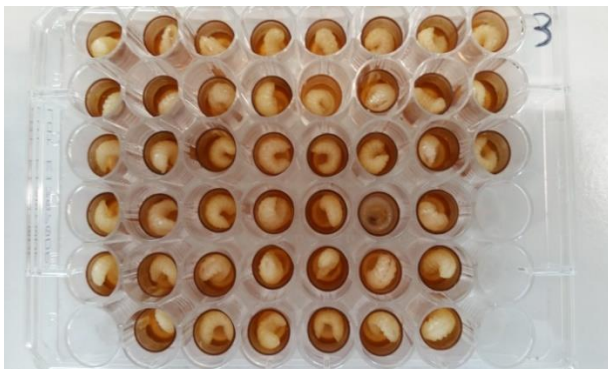


**D8**



**D15 (control Group)**



**D8**



**D15 (treatment Group)**