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# Validation report for the new Test Guideline for the Bumble bee (Bombus spp.), Chronic Oral Toxicity Test (10-Day Feeding)

OECD Series on Testing and Assessment

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# Foreword

The Foreword was added to this publication by the OECD Secretariat.

This document describes the results of a validation exercise to support the new Test Guideline (TG) for the Bumble bee (Bombus spp.), Chronic Oral Toxicity Test (10-Day Feeding)

The new TG and validation report (VR) are the result of project 2.78 which was included in the work plan of the Test Guidelines Programme (TGP) in 2024, with Spain as the lead country.

The new TG describes a chronic oral toxicity test on adult worker bumble bees under laboratory conditions over an exposure period of 10 days. The test is based on the OECD TG 245 - honey bee 10-day chronic oral test and OECD TG 247 - bumble bee acute oral toxicity test. The test was validated by the ICPPR (International Commission for Plant Pollinator Relationships) ring-test group in 2017 with the buff-tailed bumble bee, Bombus terrestris, and in 2018 with B. terrestris and the common eastern bumble bee, B. impatiens.

The WNT dedicated Expert Group provided comments on the new TG and VR from September-October 2025.

The subsequent Working Party of National Coordinators of the Test Guidelines Programme (WNT) review took place from September – October 2025, and the WNT approved the new TG and this VR at its 38th meeting in April 2026.

This report was developed under the Working Party of the National Coordinators of the Test Guidelines.

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# Introduction

A decline in some pollinator species has led to stakeholders voicing concerns and regulatory bodies, specifically in the EU, responding by expanding pollinator risk assessment paradigms and testing approaches to include social (i.e., bumble bees) and solitary (e.g., mason bees) non-*Apis* bees (EFSA GD 2023). Because of the need to address long term effects on bumble bees, the International Commission for Plant-Pollinator Relationships (ICPPR) Non-*Apis* working group designed a test protocol and organized two ring tests to develop a first-tier chronic oral test for bumble bees (*Bombus* spp.) to characterize toxicity of plant protection products (PPP).

The new 10-day chronic oral test for bumble bees is based on the honey bee (*Apis mellifera*) adult 10-day chronic oral test guideline (OECD TG 245) and the bumble bee adult acute oral toxicity test guideline (OECD TG 247). The ring test with participant laboratories was conducted using dimethoate as test chemical. Laboratories that conducted the test with with Buff-tailed bumble bees *(B. terrestris*) used a formulated product [Dimethoate (EC 400)], while laboratories that conducted the test with Common Eastern bumble bees (*B. impatiens*) used technical grade active ingredient.

Two independent ring tests were conducted in 2017 and 2018 to summarize practical experience and to harmonize the testing procedure. During a first meeting on February 17, 2017, an international team of experts developed a protocol based on the experiences of the OECD TG 245 and OECD TG 247. In 2017, 10 laboratories from Europe took part in the first ring test. In 2018, 10 laboratories from Europe and 3 laboratories from the United States of America took part in this second ring test. European laboratories conducted the tests with *B. terrestris* while laboratories in the USA conducted the tests with *B. impatiens*.

In total 13 laboratories from 6 countries including one governmental institute, three industry laboratories, and 9 contract labs participated in this method evaluation in order to harmonize the current test procedures with the aim of the development of a Test Guideline for the evaluation of the chronic oral toxicity of PPP´s on adult bumble bees in the lab.

This 10-day chronic feeding test should be used to address a prolonged exposure phase and to determine toxicity endpoints like $LC_{50}$/$LDD_{50}$ (Lethal Concentration$_{50}$/Lethal Dietary Dose$_{50}$), LOEC/LOEDD (Lowest Observed Effect Concentration/Lowest Observed Effect Dietary Dose), and NOEC/NOEDD (No Observed Effect Concentration/No Observed Effect Dietary Dose), where possible. The LOEDD and NOEDD are also frequently interpreted by regulatory authorities as Lowest Observed Effect Daily Dose and No Observed Effect Daily Dose, respectively.

The test guideline informed by the results of these ring tests has the potential to be applied in multiple geographies because it was developed using two species of bumble bees and using two test chemical formats that reflect different regulatory expectations. The data and collected experiences by the ring test group allowed setting of validity criteria for the controls (i.e., untreated sucrose solution and acetone group) as well as for the test chemical treatment. Useful information on the preparation of the bumble bees and conduct of the test were obtained, enabling the set-up of a robust and standardized test guideline.

Dimethoate (formulated product or technical grade material), known as the toxic reference item for bee studies according to the OECD TGs 245 and 247, was used as a test chemical in this ring test. Five fixed concentrations of dimethoate for *B. terrestris* (0.2, 0.4, 0.8, 1.6 and 3.2 mg a.i./kg feeding solution) and 7 test concentrations for *Bombus impatiens* (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mg a.i./kg feeding solution) were used in the ring test to determine $LC_{50}$/$LDD_{50}$ and NOEC/NOEDD values. The rationale to include seven test levels for *B. impatiens*, including three lower test levels than those set for *B. terrestris* was based on indications of higher acute oral toxicity of dimethoate and the goal of identifying the NOEC/NOEDD values. One stock solution was prepared and used for the entire 10-day test period, but fresh feeding solutions were prepared at least every 4 days.

The stability of dimethoate in the stock solutions stored at 4 °C ± 2°C over a period of 10 days was assessed and confirmed analytically. Likewise, the lowest and highest feeding solutions sampled during one of the diet preparation timepoints were analyzed to verify the concentration of dimethoate.

Two control groups were included in the test: one untreated control (50 % sugar solution) and a solvent control (*i.e.*, 50 % sugar solution containing 5 % acetone). All bumble bees were fed either with treated or untreated 50 % sugar solution (feeding solution). Adult bumble bees (*Bombus* spp.) workers were used in the test. Laboratories participating in the ring test used their usual commercial bumble bee supplier.

A validity criterion of 15 % mortality at the end of the test (10 days following start of exposure) was set for both control groups taking into consideration the validity criterion set in the OECD TG 245.

In 2017, all participating laboratories met the survival validity criterion for the untreated control group and for the solvent control group (50 % sugar solution containing 5 % acetone).

In 2018, in 9 out of 10 participating laboratories testing *B. terrestris*, the validity criterion was met for the untreated control group and for the solvent control group. One laboratory (Lab8) testing *B. terrestris* was excluded because the validity criterion was not met in both controls (10-day mortality in the untreated control: 33.3 %;10-day mortality in the solvent control: 46.7%). The three laboratories testing *B. impatiens* met the validity criterion in both control groups.

For the test chemical treatment group, mean $LC_{50}$ and $LDD_{50}$ values could be calculated, as well as the NOEC and NOEDD levels.

The results gained in these tests provided an indication of the suitability and reproducibility of the method which could serve as a basis for an official test guideline.

# Information on the ring test group

In total, 13 laboratories from 6 countries participated in the ring test group: The names of the participating laboratories and contact details are given in the table below.

| Laboratory | Responsible person | Address and contact details |
|---|---|---|
| Lab 1 | Annette Kling | Eurofins Agroscience Services EcoChem GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany<br>Email: Annette.Kling@as.eurofinseu.com |
| Lab 2 | Kristin Amsel | BioChem agrar GmbH,<br>Kupferstraße 6, 04827 Machern/ OT Gerichshain, Germany<br>Email: Kristin.Amsel@biochemagrar.de |
| Lab 3 | Eugenia Soler | Eurofins Trialcamp, S.L.U.<br>Av. Antic Regne de Valencia, 25<br>46290 Alcàsser-Valencia<br>Email: EugeniaSoler@as.eurofinseu.com |
| Lab 4 | Nicole Hanewald | BASF SE,<br>APD/EE - Li425, Speyerer Strasse 2,<br>67117 Limburgerhof, Germany<br>Email: nicole.hanewald@basf.com |
| Lab 5 | Dominika Chwiesko | IBACON GmbH, Institut für Biologische Analytik und Consulting IBACON GmbH, Arheilger Weg 17, D-64380 Rossdorf, Germany<br>Email: Dominika.Chwiesko@ibacon.com |
| Lab 6 | Stephen Vinall | Mambo-Tox<br>Science Park Limited<br>2 Venture Road<br>Chilworth Southampton<br>Hampshire, SO16 7NP, United Kingdom<br>Email: stephen.vinall@mambo-tox.co.uk |
| Lab 7 | Nina Exeler | Bayer, Crop Science Division, Environmental Safety Testing, Alfred-Nobel-Str. 50, 40789 Monheim am Rhein, Germany<br>Email: nina.exeler@bayer.com |
| Lab 8 | Emma Wright<br>Selwyn Wilkins | Centre for Chemical Safety and Stewardship<br>The Food and Environment Research Agency Sand Hutton, York YO41 1LZ, UK<br>Email: selwyn.wilkins@fera.gsi.gov.uk<br>Email: Selwyn.Wilkins@fera.co.uk |
| Lab 9 | Arnaud Zicot | Syntech Research<br>Inc and SynTech Research Laboratory Services LLC<br>17745 South Metcalf Avenue<br>Stilwell<br>KS, USA 66085<br>Email: azicot@syntechresearch.com |
| Lab 10 | Bettina Hodapp (formerly also involved Stefan Kimmel) | Innovative Environmental Services (IES) Ltd, Benkenstrasse 260, 4108 Witterswil, Switzerland<br>Email: b.hodapp@ies-ltd.ch |
| Lab 11 | Ana Cabrera | Bayer CropScience LP<br>2 T.W. Alexander Drive, Research Triangle Park, NC 27709, USA<br>Current address: 700 Chesterfield Parkway W., Chesterfield, MO 63017, USA |

| | | |
|---|---|---|
| | Statistical analysis:<br>Allen Olmstead | Email: ana.cabrera@bayer.com<br><br>Bayer CropScience LP<br>700 Chesterfield Parkway W., Chesterfield, MO 63017, USA<br>Email: allen.olmstead@bayer.com |
| Lab 12 | David Lehmann | United States Environmental Protection Agency<br>Center for Public Health and Environmental Assessment, Health and Environmental Effects Division<br>Research Triangle Park, NC 27711, USA.<br>Email: lehmann.david@epa.gov |
| Lab 13 | Mike Patnaude | Smithers Viscient LLC<br>790 Main St., Wareham, MA 02571, USA.<br>Email: mpatnaude@smithers.com |

To avoid any conflict of interest associated with disclosure of the names of laboratories that participated in this ring test, all names were anonymized for data analysis and results reporting using the following format: “Lab X", where “Lab" stands for “laboratory" and “X" indicates the number of that laboratory (out of 13 participants).

# Time schedule

| Activity | Scheduled date |
|---|---|
| Pre-Ring Test Meeting | February 17, 2017 at the University of Wageningen, Netherlands |
| Experimental completion | Latest end of October 2017 |
| Ring Test Meeting | February 28, 2018 at FERA Fera Science Ltd., Sand Hutton, York |
| Experimental completion | Latest end of October 2018 |

# Materials and methods

## Test chemical and Control groups

Commercially available formulation of Dimethoate (EC 400) was used for European laboratories. Laboratories based in the US used technical grade dimethoate.

In the control group, the bees received untreated 50 % (w/v) aqueous sugar solution *ad libitum.* An additional control group was fed *ad libitum* with 50 % (w/v) aqueous sugar solution containing 5 % acetone. The solvent (acetone) control group was included in the ring tests to address potential future needs with other test chemicals with low solubility in the 50 % (w/v) aqueous sugar solution. Dimethoate, technical grade active ingredient (purity 99.5% - 99.8%) and formulation EC 400, are water soluble and did not need acetone to get into solution.

## Test Organism / Test System

| | |
|---|---|
| Species | *Bombus* spp. (*Bombus terrestris* L., *Bombus impatiens* Cresson [Hymenoptera, Apidae]) |
| Life stage | Adult worker bumble bees of unknown age (colonies to be used within one week counted from the date of receipt in the testing laboratory) |
| Source | Commercial supplier |
| Hives | Only hives that appeared to be adequately fed, healthy and as far as possible disease-free and queen-right with known history and physiological status were used for bumble bee collection. |
| Collection of the bumble bees | Bumble bees were collected within one week counted from the date of receipt in the testing laboratory. Bumble bees were collected from their colony under red light (NOT anesthetized) in single housing cages (commonly known as nicot cages in Europe, and as queen hatching/hair roller cages in the USA). To enhance repeatability and comparability between studies, outliers in terms of size (very small as well as very large individuals) were identified by visual inspection and were excluded from the experiment. Individuals included in the experiment were transferred to individual test cages and their weight was measured. To have enough bumble bee workers for the assignment to the treatment and control groups, the weight range was targeted |

| | |
|---|---|
| | at 30 % variability around the mean bumble bee weight. Selected bumble bees were randomly assigned to one of the test groups (treatment or controls). |
| Acclimatization | In all cases bumble bees were acclimatized to the test conditions for at least 12 h prior the start of the test (initial feeding). During this time bumble bees were supplied with untreated 50% (w/v) aqueous sucrose solution *ad libitum*. As moribund bumble bees may occur, these were removed from consideration and replaced by healthy bumble bees before starting the test. |

## Test Design

| | |
|---|---|
| Test design | Dose response test |
| Exposure time | 10 days |
| Number of treatment groups | 2 control groups (untreated and solvent control containing 5 % acetone), 5 concentrations of dimethoate were tested for B. terrestris and 7 concentrations of dimethoate were tested for B. impatiens |
| Bumble bees per treatment group | 30, single housing in nicot (a.k.a. hair roller) cages |

## Test Cages

| | |
|---|---|
| Test cages | Bumble bees were kept individually in cages – “single housing” (APPENDIX A). Single housing prevents hierarchy fights (among the queen-less bumble bee workers) potentially introducing mortality and it allows a precise assessment of consumption and affected or non-affected bumble bees. Furthermore, bumble bees do not share food via trophallaxis and need to be fed individually. The cages were easy to clean or disposable and passively ventilated. |

## Test Conditions

| | |
|---|---|
| Temperature | 25 ± 2 °C |
| Relative humidity | 60 ± 10% |
| Exposure to light | Constant darkness except during the exchange of feeders and assessments. |
| Ventilation | The cages allowed passive ventilation. |
| Feeding | During acclimatization: 50 % aqueous sugar solution *ad libitum.*<br>During test period: treated or untreated feeding solutions *ad libitum.* |
| Specific documentation | Temperature and humidity were recorded continuously with appropriate, calibrated equipment. |

(Short-term deviations (< 2 hours) from the recommended temperature and humidity ranges are unavoidable and should not affect the integrity or outcome of the study).

## Feeding Solutions

Feeding solutions

Feeding solutions for the control, solvent control and test chemical treatments were prepared with 50 % (w/v) sugar solution. The bumble bees were fed *ad libitum* with a 50 % (w/v) sugar solution containing either:

- 50 % (w/v) sugar solution (untreated control group),
- 5 % acetone as solvent (solvent control group).
- the test chemical at 5 concentrations (*B. terrestris*) or 7 concentrations (*B. impatiens*) (test chemical treatment group)

The treated and untreated feeding solutions were offered using plastic syringes or other suitable devices as feeders. Every day the feeders were replaced by a new one with fresh treated or untreated food.

## Analytical verification

Feeding solution and stock solution

The target concentrations of dimethoate in the stock solutions and in the feeding solutions (lowest and the highest concentrations at preparation and at maximum storage period) were verified analytically.

## Application

### *Preparation of the Control and Test Chemical Feeding Solutions*

Preparation of the control feeding solution

The feeding solution for the control group was prepared with 50 % (w/v) aqueous sugar solution.

For the additional solvent control, acetone was added to the 50 % (w/v) aqueous sugar solution to reach a final concentration of 5 %.

The control feeding solutions were prepared at least every 4 days and were stored in the refrigerator at approx. 4 °C.

Preparation of the test chemical feeding solution(s)

A stock solution of the test chemical* was prepared once for the entire test period and stored in the refrigerator at 4 ± 4 °C for up to 10 days. Water was used as the solvent.

The test chemical feeding solutions were prepared out of the stock solution at least every 4 days and were stored in the refrigerator at 4°C ± 4°C.

*[dimethoate is stable in sugar solution over a period of 10 days when stored in the refrigerator at 4 °C ± 4 °C; Internal BASF SE analytical

report (March 25, 2014) and IBACON analytical report (79371136A, March 19, 2014)]

### *Application Details*

| | |
|---|---|
| Concentration levels | 0.2, 0.4, 0.8, 1.6 and 3.2 mg a.i. (dimethoate)/kg feeding solution for *B. terrestris*<br><br>0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mg a.i. (dimethoate)/kg feeding solution for *B. impatiens* |
| Dose levels | The exact dose per bee was calculated at the end of the test after determination of the actual food uptake. |
| Application mode | The treated and untreated feeding solutions were offered to the test organisms via feeders introduced into each test unit (*e.g.,* plastic syringes, approx. 3.5 mL). The tip of each syringe was removed so that the bees had access to the feeding solution.<br><br>Amount of feeding solution<br><br>The quantity of food offered to each cage exceeded the amount actually needed by the bumble bees in order to guarantee *ad libitum* feeding. |
| Application procedure | Every 24 h (± 2 h in relation to the 1st application) the feeders containing the different control and test chemical feeding solutions were replaced by fresh ones (one application interval). The treated and untreated feeding solutions were offered *ad libitum* to each cage in feeders. The feeders were weighed daily before introduction into the cages and after the feeding interval. |
| Food consumptions | The amount of feeding solution consumed daily per bumble bee was determined by weighing the individual feeders before and after feeding, using calibrated equipment. |

### *Time Schedule*

| Time in relation to the first feeding | Action |
|---|---|
| Day - 7 to day -1 | Delivery of bumble bee hives |
| Day - 1 to day 0 | Collection of bumble bees and acclimatization<br>Weighing of bumble bees |
| Day 0 = 1st Application | Preparation of stock solution(s), preparation of feeding solution(s), weighing of feeders; 1st application |
| Day 1 to day 9 (± 2 h in relation to 1st Application) | Assessment of mortality and behavioral abnormalities; preparation of feeding solutions (at least every four days), daily replacement of feeding solutions by fresh ones, determination of consumed feeding solutions by weighing the feeders. |
| Day 10 (± 2 h in relation to 1st Application) | Assessment of mortality and behavioral abnormalities;<br>determination of consumed feeding solutions by weighing the feeders. Test termination. |

## Assessments

### *Mortality, Behavioral Abnormalities and Food Consumption*

| | |
|---|---|
| Mortality | Mortality was assessed and recorded daily at about the same time of the day (every 24 h ± 2h), for a period of 10 days starting 24 ± 2 hours after start of the test period (initial feeding) until test end. |
| Behavioral abnormalities (sublethal effects) | Behavioral abnormalities such as symptoms of poisoning or any abnormal behavior in comparison to the control were recorded according to the following categories:<br>u = unaffected, exhibit inconspicuous behavior<br>m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation; *e.g.,* light or blowing; bees may recover but usually die),<br>a = affected (bees still upright and attempting to walk but showing signs of reduced coordination),<br>Behavioral abnormalities were assessed and recorded daily at about the same time of the day (every 24 h ± 2h), for a period of 10 days and starting 24 ± 2 hours after start of the test period (initial feeding). |
| Food consumption | Food consumption was assessed and recorded daily at about the same time of the day (every 24 h ± 2h) for a period of 10 days and, starting 24 ± 2 hours after start of the test period. Evaporations rates of the feeding solution was measured daily. |
| Evaporation control | The evaporation of feeding solution under the test conditions was measured daily to determine the exact amount of test chemical consumed during the test period. Five empty test cages without bumble bees were supplied with a pre-weighted feeder containing the untreated control feeding solution (No acetone controls were included because the dimethoate treatment groups did not contain acetone). These evaporation controls were placed in the test environment alongside the test cages. The actual food and test chemical intake of individual bumble bees can accurately be calculated by subtracting the average evaporation rate from the measured consumption rate. |

# Data evaluation

## Evaluation of Mortality and Behavioral Abnormalities

Results obtained from the bumble bees treated with the test chemical or the solvent control was compared to those obtained from the control fed with pure sugar solution. The percentage of mortality was calculated for each treatment group from the number of dead individuals in correlation to the number of introduced test organisms.

The number of bees showing behavioral abnormalities were counted and given as absolute number/day in the results summary.

The data of the test chemical and solvent control were not corrected for control mortality.

## Evaluation of Food Consumption

The weight of the feeders was recorded before and after feeding to the bees to calculate the exact food consumption per bee per day. The difference of the weight before and after feeding to the bees represents the food consumed by the bumble bees in one cage during one feeding interval (about 24 hours). The mean uptake of test chemical/bee is presented for each day as well as accumulated over the entire test period. The mean uptake of test chemical per bee per day over the whole testing period was calculated by summing up the daily uptake per bee and dividing by the relevant number of days.

## Data Analysis

Statistical analyses were performed with the statistical program R Studio v 4.3.0. The weight of the bees was analyzed for normality and equal variances via Shapiro-Wilk test and Levene's test, respectively. Only one of twelve datasets met these two assumptions and was analyzed via one-way ANOVA; all other datasets were analyzed via Kruskal-Wallis test.

The survival in the untreated control and solvent control groups were compared via Generalized Linear Mixed Model (maximum likelihood 'glmerMod', Family 'binomial', study number (laboratory) as random effect). A separate analysis was conducted for the laboratories that conducted the test with *B. terrestris* and for those that conducted the tests with *B. impatiens*. The dimethoate NOEC and NOEDD based on mortality were identified conducting Cochran-Armitage Trend tests, as recommended by OECD (OECD TG 54).

$LDD_{50}$ (expressed in µg/bee/day) and $LC_{50}$ (expressed in mg/kg) values with 95 % confidence intervals of the test chemical group were calculated by fitting a 3-parameter log-logistic function with the nominal concentration, daily dose, or dosage. Lab 7 dataset had an initial poor log-logistic function fit for the estimation of the $LC_{50}$, $LDD_{50}$ and $LDD_{50}$_dosage (normalized to weight), and the function slope was

fixed based on the average slope for the laboratories that conducted the test with *B. terrestris* (average $LC_{50}$ slope = -4.23; average $LDD_{50}$ slope = -5.03, average $LLD_{50}$_dosage = -5.16)

Definitions:

$LC_{50}$ (median Lethal Concentration) is a statistically calculated concentration of a test chemical that can cause death in 50 % of the test organisms at the end of the test period. It is expressed in, for example, mg or µg active ingredient or formulated product per kg food.

$LDD_{50}$ (median Lethal Dietary Dose) is a statistically calculated dietary dose of a test chemical that can cause death in 50 % of the test organisms at the end of the test period. It is expressed in, for example, µg or ng active ingredient or formulated product per bee per day.

NOEC (No Observed Effect Concentration) the highest tested concentration with no statistically significant effect on the test organisms, when compared to the control. It is expressed in, for example, mg or µg active ingredient or formulated product per kg food.

NOEDD (No Observed Effect Dietary Dose; some regulatory authorities interpret it as No Observed Effect Daily Dose) the highest tested dose per bee per day administered by chronic feeding exposure, with no statistically significant effect on the test organisms, when compared to the control. It is expressed in, for example, µg active ingredient or formulated product per bee per day.

# Validity criterion

| | |
|---|---|
| Control mortality | The validity threshold for mean mortality in the control groups at the end of the test was set to < 15%. This validity criterion was adopted from the validity criterion of the OECD TG 245. |

# Deviations to the protocol

No major deviations were reported by the participating labs.

# Results and discussion

## Analytical verification

The concentration of dimethoate in the feeding solution of the lowest and the highest treatment after preparation (sample 1) and if available at the end of the maximum storage period (sample 2) and additionally in the stock solution was analyzed. The results are summarized in Table 1 below. The recovery in the stock solutions was within a range of 89 to 123%. For the feeding solutions the recovery of dimethoate was in a range of 83 to 118%.

**Table 1 Summary of dimethoate concentrations in feeding and stock solutions.**

| | Lab1 | Lab2 | Lab3 | Lab4 | Lab5 | Lab6 | Lab7 | Lab9 | Lab10 | Lab11 | Lab12 | Lab13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Recovery from Target [%] | | | | | | | | | | | |
| Stock solution | 99 | 89 | 107 | 97 | 99 | 98 | 99 | 123 | 95 | 96 | 95 | 101 |
| Low concentration sample 1 | 108 | 93 | 105 | 100 | 100 | 106 | 100 | 110 | 83 | 101 | 88 | 105 |
| Low concentration sample 2 | 107 | 92 | 103 | 100 | 97 | 100 | 98 | NA* | 84 | 98 | 86 | 106 |
| High concentration sample 1 | 110 | 102 | 113 | 106 | 103 | 110 | 106 | 110 | 95 | 108 | 99 | 118 |
| High concentration sample 2 | 111 | 101 | 109 | 108 | 103 | 108 | 108 | NA | 89 | 105 | 99 | 117 |

* NA = Not Available

## Evaporation

Evaporation was measured in each laboratory in 5 additional cages not containing bees, only pre-weighed feeders. This evaporation figure is subtracted from the calculated food consumption to give the corrected food consumption accounting the loss by evaporation.

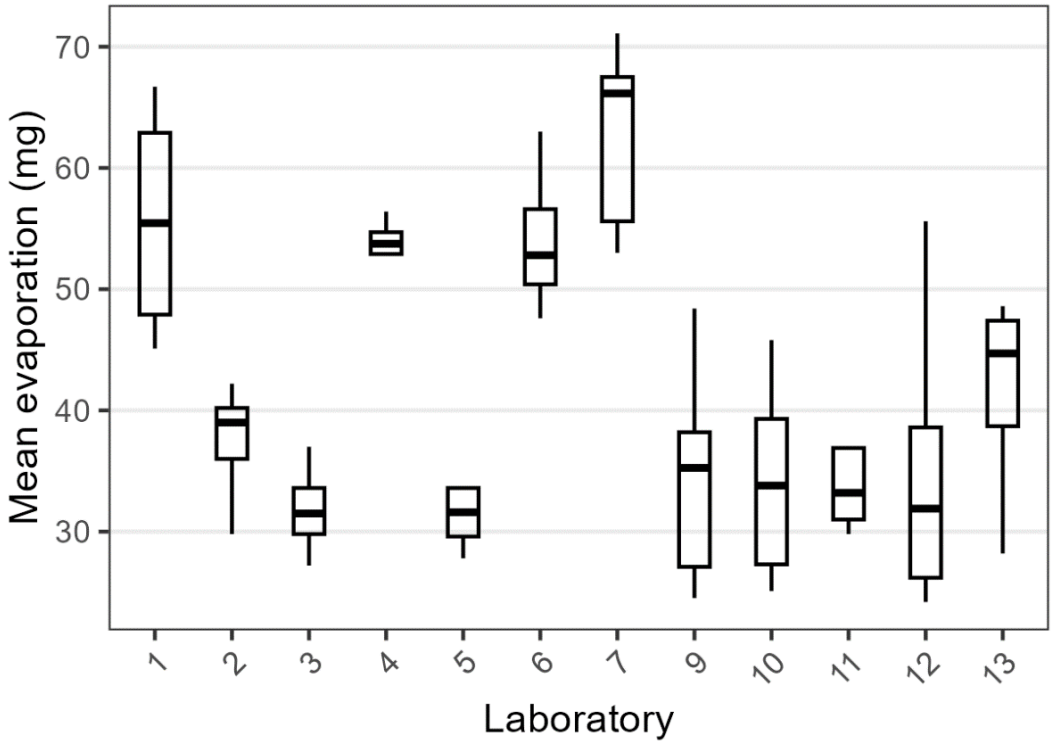



**Figure 1: Mean evaporation during 10 days in each laboratory**

## Mortality in the control and test chemical treatments

Ten days after the start of the exposure phase, control mortality of the bumble bees was on average 4.4% for *B. terrestris* and 0% for *B. impatiens*. In the acetone control group average mortality for *B. terrestris* was 4.1% and for *B. impatiens* 4.4%. TABLE 2 and TABLE 3 are presenting the cumulative mortality in the control group and the test chemical groups over the 10-day test period for *B. terrestris* and *B. impatiens*.

**Table 2 Cumulative mortality [%] in the control and test chemical treatment groups during the 10 days test period for *B. terrestris***

| Treatment | Lab1 | Lab2 | Lab3 | Lab4 | Lab5 | Lab6 | Lab7 | Lab 8*2 | Lab9 | Lab 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Control groups *1** | | | | | | | | | | |
| **C 0** | 0.0 | 0.0 | 0.0 | 13.3 | 0.0 | 10.0 | 3.3 | 33.3 | 6.7 | 6.7 |
| **AC 0** | 0.0 | 0.0 | 0.0 | 3.3 | 3.3 | 13.3 | 3.3 | 43.3 | 6.7 | 6.7 |
| **Test Chemical groups** | | | | | | | | | | |
| **T1 0.2** | 3.3 | 0.0 | 0.0 | 23.3 | 6.7 | 10.0 | 3.3 | 30.0 | 23.3 | 3.3 |
| **T2 0.4** | 20.0 | 36.7 | 6.7 | 73.3 | 23.3 | 33.3 | 73.3 | 33.3 | 56.7 | 40.0 |
| **T3 0.8** | 96.7 | 86.7 | 33.3 | 96.7 | 86.7 | 86.7 | 100.0 | 96.7 | 100.0 | 90.0 |
| **T4 1.6** | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| **T5 3.2** | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

*1C = untreated control group was fed with 50 % aqueous sucrose solution, AC = Acetone Control group fed with 50 % aqueous sucrose solution including 5% acetone

*2 Lab8 validity criterion not met, therefore excluded from further analysis

**Table 3 Cumulative mortality [%] in the control and test chemical treatment groups during the 10 days test period for *B. impatiens***

| Treatment | Lab11 | Lab12 | Lab13 |
|---|---|---|---|
| **Control groups*** | | | |
| **C 0** | 0.0 | 0.0 | 0.0 |
| **AC 0** | 10.0 | 3.3 | 0.0 |
| **Test Chemical groups** | | | |
| **T1 0.025** | 0.0 | 3.3 | 0.0 |
| **T2 0.05** | 0.0 | 3.3 | 3.3 |
| **T3 0.1** | 0.0 | 6.7 | 0.0 |
| **T4 0.2** | 6.7 | 46.7 | 23.3 |
| **T5 0.4** | 70.0 | 96.7 | 93.3 |
| **T6 0.8** | 100.0 | 100.0 | 100.0 |
| **T7 1.6** | 100.0 | 100.0 | 100.0 |

*C = untreated control group was fed with 50 % aqueous sucrose solution, AC = Acetone Control group fed with 50 % aqueous sucrose solution including 5% acetone

*Bombus terrestris*

A clear dose-response curve for the test chemical was observed in all 9 laboratories (1 laboratory excluded because validity criterion not met). At the lowest concentration level of 0.2 mg a.i./kg the mortality ranged between 0 % and 23.3 % at day 10.

In all laboratories, the mortality at the highest concentration level (T5) and the one below (T4) was 100 % after 10 days. The mean mortality levels over all labs were 8.1, 40.4, 86.3, 100, and 100 % following treatment with dimethoate concentrations of 0.2, 0.4, 0.8, 1.6 and 3.2 mg a.i./kg feeding solutions, respectively.

*Bombus impatiens*

A clear dose-response curve for the test chemical was observed in all 3 laboratories. At the lowest concentration level of 0.025 mg a.i./kg the mortality ranged between 0 % and 3.3 % at day 10.

In all laboratories the mortality at the highest concentration level (T7) and the one below (T6) was 100 % after 10 days. The mean mortality levels over all labs were 1.1, 2.2, 2.2, 25.6, 86.7, 100, and 100 % following treatment with dimethoate concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg a.i./kg feeding solutions, respectively.

Mortality levels for B. terrestris were constantly above 50% at test concentrations ≥ 0.6 mg a.i./kg feeding solution, for B. impatiens this was the case at test levels above 0.3 mg a.i./kg feeding solution. As to a standardized test method, this observation justifies the testing of only one concentration of the reference item which results in mortality levels above 50 % at the end of the test.

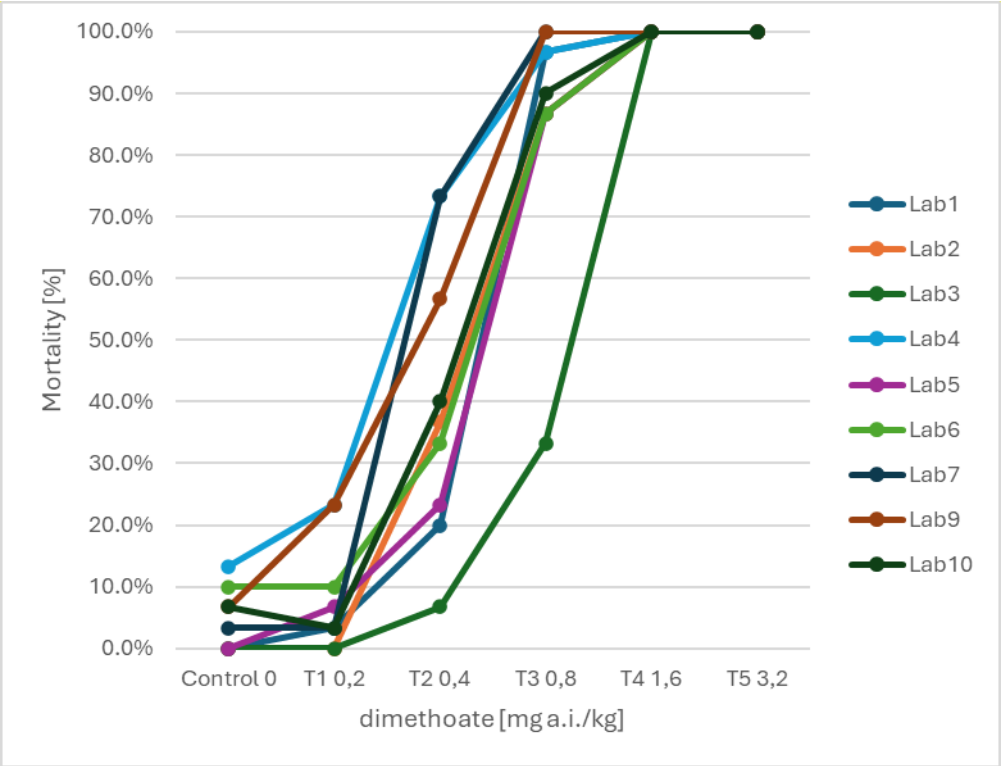



**Figure 2** **Cumulative mortality in the reference item treatment group after10 days for *B. terrestris***

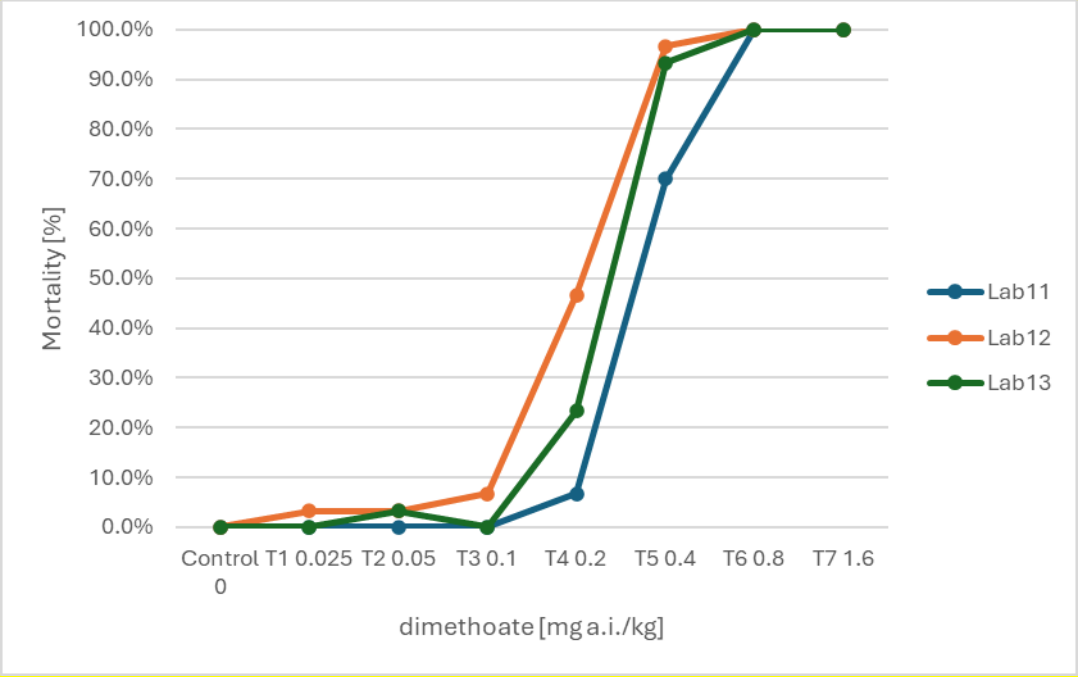



**Figure 3 Cumulative mortality in the reference item treatment group after 10 days for *B. impatiens***

*Solvent control*

As a potential solvent for use with non-water-soluble test chemicals, acetone in a concentration of 5% in the feeding solution, was added as an additional control group in the ring test.

The food uptake in the solvent control group was slightly higher than the food consumption in the untreated control group for all participating labs (APPENDIX B). *B. terrestris* bees in the solvent control group consumed on average 29 mg sucrose solution/bee/d more than the bees that consumed untreated sucrose solution (t value = -4.013, $p \leq 0.0001$). Similarly, B. impatiens bees in the solvent control group consumed on average 40 mg sucrose solution/bee/d more than the bees that consumed untreated sucrose solution (t value = -3.832, p = 0.00018).

The mortality levels in the solvent control group ranged from 0.0 % to 13.3 %. In 12 out of 13 labs the mortality level was below the validity criteria of 15%. There were no statistical differences in survival among the water control and solvent control with B. terrestris (z value = -0.216, p value = 0.829) and with B. impatiens (z value = 0, p value = 1).

**Table 4 Food uptake (corrected for evaporation) and cumulative mortality [%] in the Control and Solvent control group during the 10 days test period**

| Bumble bee species | *Bombus terrestris* | | | | | | | | | *Bombus impatiens* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| treatment group | Lab1 | Lab2 | Lab3 | Lab4 | Lab5 | Lab6 | Lab7 | Lab9 | Lab10 | Lab11 | Lab12 | Lab13 |
| Mean daily feeding solution consumption per BB over the course of the study [mg/bee/d] | | | | | | | | | | | | |
| Control | 185.2 | 121.2 | 268.9 | 157.2 | 194.4 | 123.9 | 210.5 | 142.6 | 130.0 | 220.4 | 256.3 | 196.1 |
| Solvent Control | 183.5 | 130.6 | 271.5 | 201.8 | 215.2 | 163.2 | 259.2 | 230.2 | 139.2 | 245.9 | 300.6 | 245.2 |
| Control mortality over the course of the study [%] | | | | | | | | | | | | |
| Control | 0.0 % | 0.0 % | 0.0 % | 3.3 % | 0.0 % | 10.0 % | 3.3 % | 6.7 % | 6.7 % | 0.0 % | 0.0 % | 0.0 % |
| Solvent Control | 0.0 % | 0.0 % | 0.0 % | 13.3 % | 3.3 % | 13.3 % | 3.3 % | 6.7 % | 6.7 % | 10.0 % | 3.3 % | 0.0 % |

## $LC_{50}$, $LDD_{50}$, NOEC, and NOEDD

The results for each of the 10-day tests with bumble bees are presented in TABLE 5. The $LC_{50}$/$LDD_{50}$ also include their corresponding lower and upper confidence interval (CI). The NOEC/NOEDD values were also identified for each dimethoate dose-response test.

**Table 5 $LDD_{50}$, $LC_{50}$, NOEDD, and NOEC values of dimethoate**

| Participating Laboratory | $LDD_{50}$ [µg/BB/day] | lower CIL | upper CI | $LC_{50}$ [mg a.i./kg] | lower CI | upper CI | NOEDD [µg/BB/day] | NOEC [mg a.i./kg] |
|---|---|---|---|---|---|---|---|---|
| *Bombus terrestris* | | | | | | | | |
| Lab1 | 0.123 | 0.107 | 0.140 | 0.493 | 0.429 | 0.557 | 0.045 | 0.2 |
| Lab2 | 0.072 | 0.063 | 0.081 | 0.479 | 0.410 | 0.548 | 0.039 | 0.2 |
| Lab3 | 0.231 | 0.206 | 0.256 | 0.800 | 0.694 | 0.905 | 0.132 | 0.4 |
| Lab4 | 0.052 | 0.044 | 0.060 | 0.329 | 0.256 | 0.402 | 0.037 | 0.2 |
| Lab5 | 0.110 | 0.097 | 0.123 | 0.516 | 0.405 | 0.626 | 0.062 | 0.2 |
| Lab6 | 0.048 | 0.042 | 0.054 | 0.516 | 0.419 | 0.612 | 0.027 | 0.2 |
| Lab7 | 0.084 | 0.073 | 0.095 | 0.335 | 0.285 | 0.385 | 0.045 | 0.2 |
| Lab9 | 0.087 | 0.054 | 0.121 | 0.644 | 0.322 | 0.966 | 0.039 | 0.2 |
| Lab10 | 0.076 | 0.064 | 0.088 | 0.465 | 0.392 | 0.538 | 0.041 | 0.2 |
| **Mean *B.t.*** | **0.098** | **0.083** | **0.113** | **0.509** | **0.401** | **0.615** | **0.052** | **0.22** |
| **SD** | **0.055** | **0.051** | **0.061** | **0.145** | **0.126** | **0.200** | **0.031** | **0.07** |
| *Bombus impatiens* | | | | | | | | |
| Lab11 | 0.043 | 0.039 | 0.047 | 0.337 | 0.293 | 0.380 | 0.021 | 0.1 |
| Lab12 | 0.027 | 0.025 | 0.030 | 0.205 | 0.175 | 0.235 | 0.019 | 0.1 |
| Lab13 | 0.041 | 0.037 | 0.044 | 0.258 | 0.221 | 0.295 | 0.021 | 0.1 |
| **Mean *B.i.*** | **0.037** | **0.034** | **0.040** | **0.267** | **0.230** | **0.303** | **0.020** | **0.1** |
| **SD** | **0.009** | **0.008** | **0.009** | **0.066** | **0.059** | **0.073** | **0.001** | **---** |

The $LDD_{50}$ of dimethoate after 10 days was similar among the labs for *B. terrestris* and ranged from 0.048 to 0.231 µg a.i./BB/day over the participating labs. The $LDD_{50}$ values estimated for *B. impatiens* ranged between 0.027 and 0.043 µg a.i./BB/day. The resulting mean $LC_{50}$ value for *B. terrestris* was 0.509 ± 0.145 mg a.i./kg, for *B. impatiens* was 0.267 ± 0.066 mg a.i./kg diet.

Confidence limits were calculated for all data sets. NOEDD values were determined for all studies. Mean NOEDD for dimethoate was 0.052 ± 0.031 for *B. terrestris* and 0.020 ± 0.001 µg a.i./BB/day for *B. impatiens.*

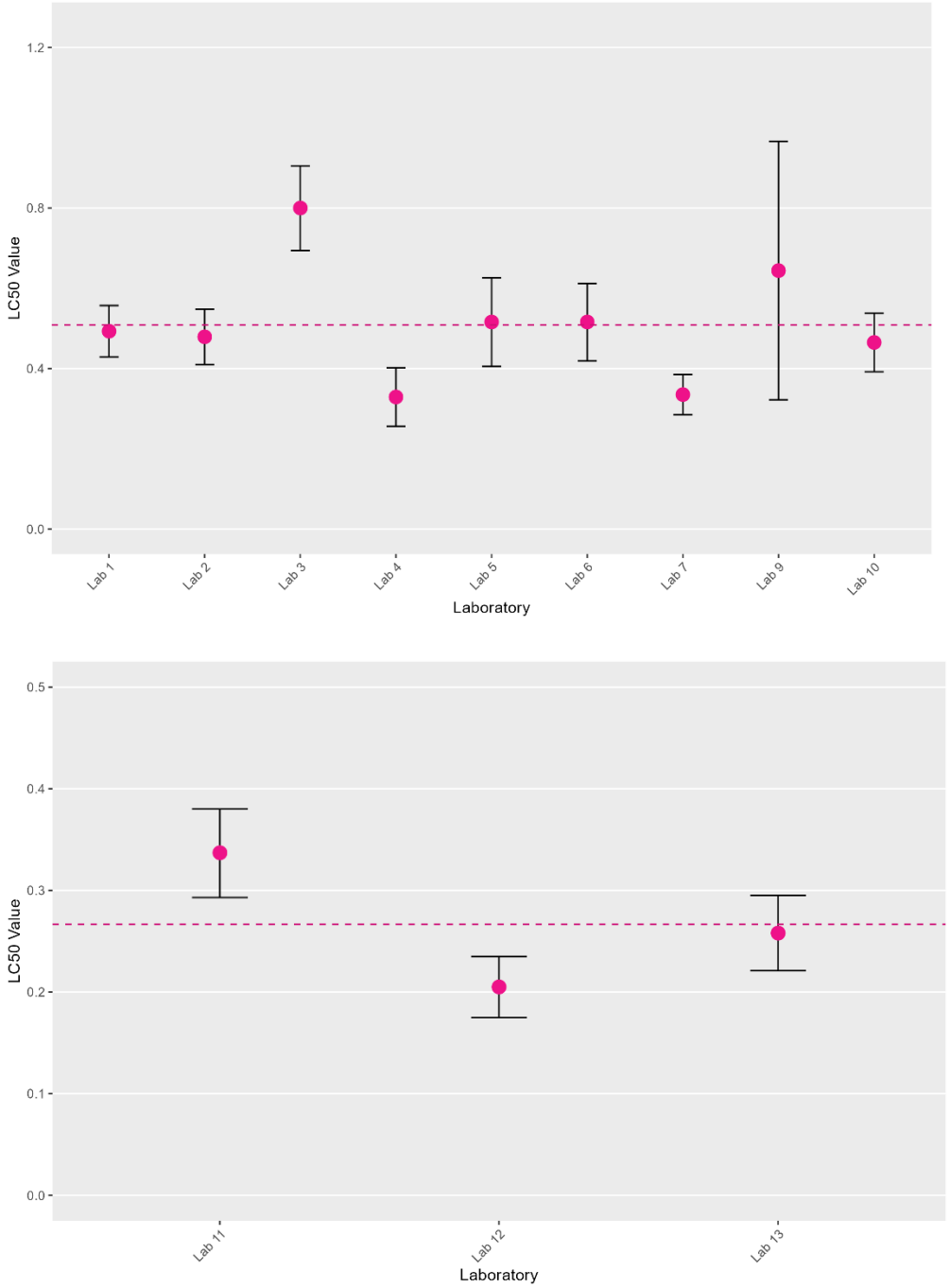



**Figure 4 Dimethoate $LC_{50}$ values in 10-day tests with bumble bees. Top graph corresponds to laboratories that conducted the test with *B. terrestris* and the bottom graph corresponds to the laboratories that conducted the test with *B. impatiens*. Mean $LC_{50}$ values are visible in both figures as a dashed line. Mean $LC_{50}$ value for *B. terrestris* = 0.509 mg dimethoate/kg diet; mean $LC_{50}$ value for *B. impatiens* = 0.267 mg dimethoate/kg diet**

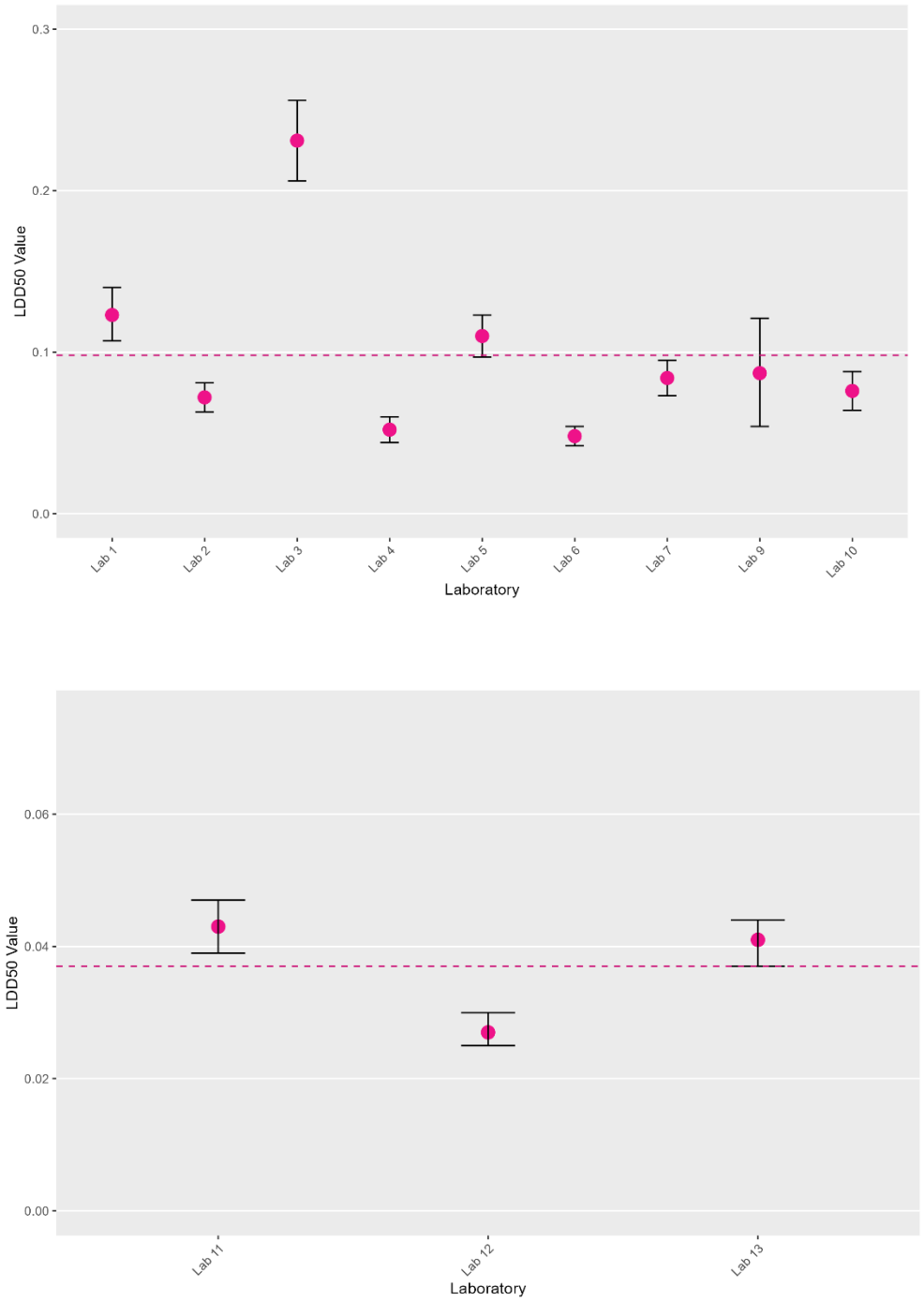



**Figure 5 Dimethoate $LDD_{50}$ values in 10-day tests with bumble bees. Top graph corresponds to laboratories that conducted the test with *B. terrestris* and the bottom graph corresponds to the laboratories that conducted the test with *B. impatiens*. Mean $LDD_{50}$ values are visible in both figures as a dashed line. Mean $LDD_{50}$**

**value for *B. terrestris* = 0.098 µg dimethoate bee/d; mean $LDD_{50}$ value for *B. impatiens* = 0.037 µg dimethoate/bee/d**

## Behavioral Abnormalities

Related to the effects caused by the dimethoate treatment, behavioral abnormalities occurred mainly at the higher concentration/dose levels. Most of these bees were categorized as affected, apathetic or moribund.

## Replication

A bootstrapping analysis of the datasets indicated that the number of replicates could be lowered from 30 to 20 bumble bees per treatment group (detailed information is given in APPENDIX C). Additionally, one lab performed a test with only 20 replicates and confirmed the results of the bootstrap analysis as well as the results from the ring test with 30 replicates. The calculated LC and LDD values are in the same range as the endpoints calculated with a data set conducted with 30 replicates.

# Conclusions

An international ring test was conducted to develop a robust test method to address chronic effects of oral exposure on bumble bees by the ICPPR (International Commission on Plant-Pollinator Relations) Non-*Apis* working group. Thirteen laboratories from 6 countries participated in the ring test, conducting these tests with either *Bombus terrestris* (10 labs) or *Bombus impatiens* (3 labs).

A 10-day feeding test for bumble bees was developed and validated using dimethoate as the reference substance. The test developed was based on the honey bee 10-day chronic feeding OECD test guideline 245 (TG 245) and the bumble bee acute oral toxicity OECD TG 247. The aim of the ring test was to demonstrate that, under standardized conditions, this method can be used to collect reproducible and robust data on the toxicity of PPP`s in a chronic 10 day feeding test with bumble bees. Required and relevant endpoints for a risk assessment like the $LC_{50}$, $LDD_{50}$, NOEC, and NOEDD could be estimated with the given protocol for all the participating laboratories.

A predefined validity criterion of not more than 15% mortality in the untreated control group was met by 12out of 13 participating laboratories. The same validity criterion is applied to the solvent control when a solvent is needed in the system. Included in the ring test protocol, the participating laboratories tested acetone at 5% concentration, which is a commonly used solvent in bee tests and at levels currently deemed safe for honey bees (OECD TG 245). There was no statistical difference in survival between the untreated control and the solvent control. However, there were statistical differences among the untreated control and solvent control for consumption. Thus, the importance of including a solvent control group when a solvent is needed to get a test material into solution has been demonstrated.

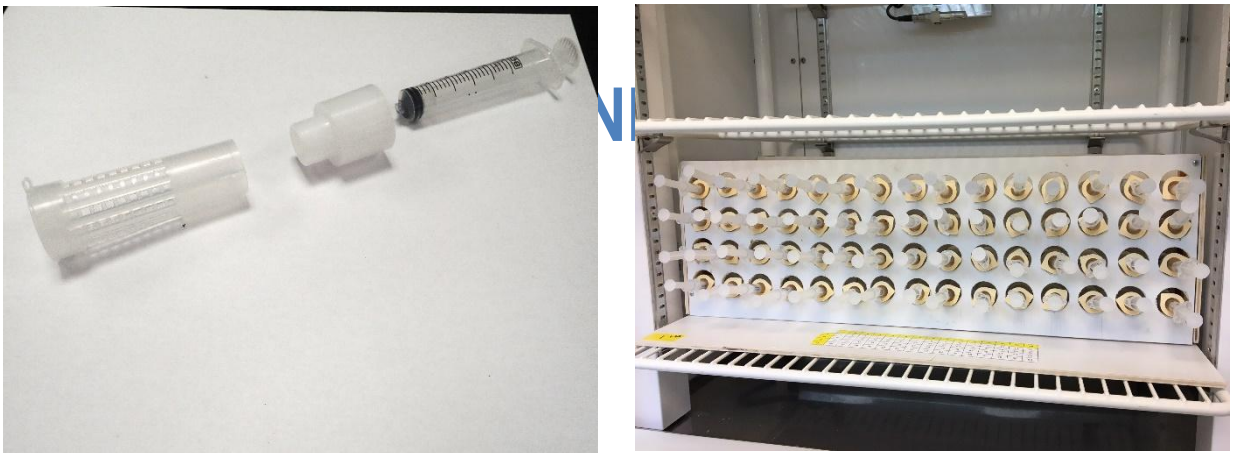
The described test method proved to be suitable to assess the chronic effects of PPPs or other chemicals on bumble bee workers in the laboratory. Adult worker bumble bees can be collected under red light from colonies obtained from a preferred supplier. The test chemical dimethoate either as a formulated product or the technical substance, is suitable to demonstrate the sensitivity of the test system in a chronic oral feeding test.

# References

OECD (2006), *Current Approaches in the Statistical Analysis of Ecotoxicity Data: A guidance to application (annexes to this publication exist as a separate document)*, OECD Series on Testing and Assessment, No. 54, OECD Publishing, Paris, https://doi.org/10.1787/9789264085275-en.

OECD (2017), *Test No. 247: Bumblebee, Acute Oral Toxicity Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264284128-en.

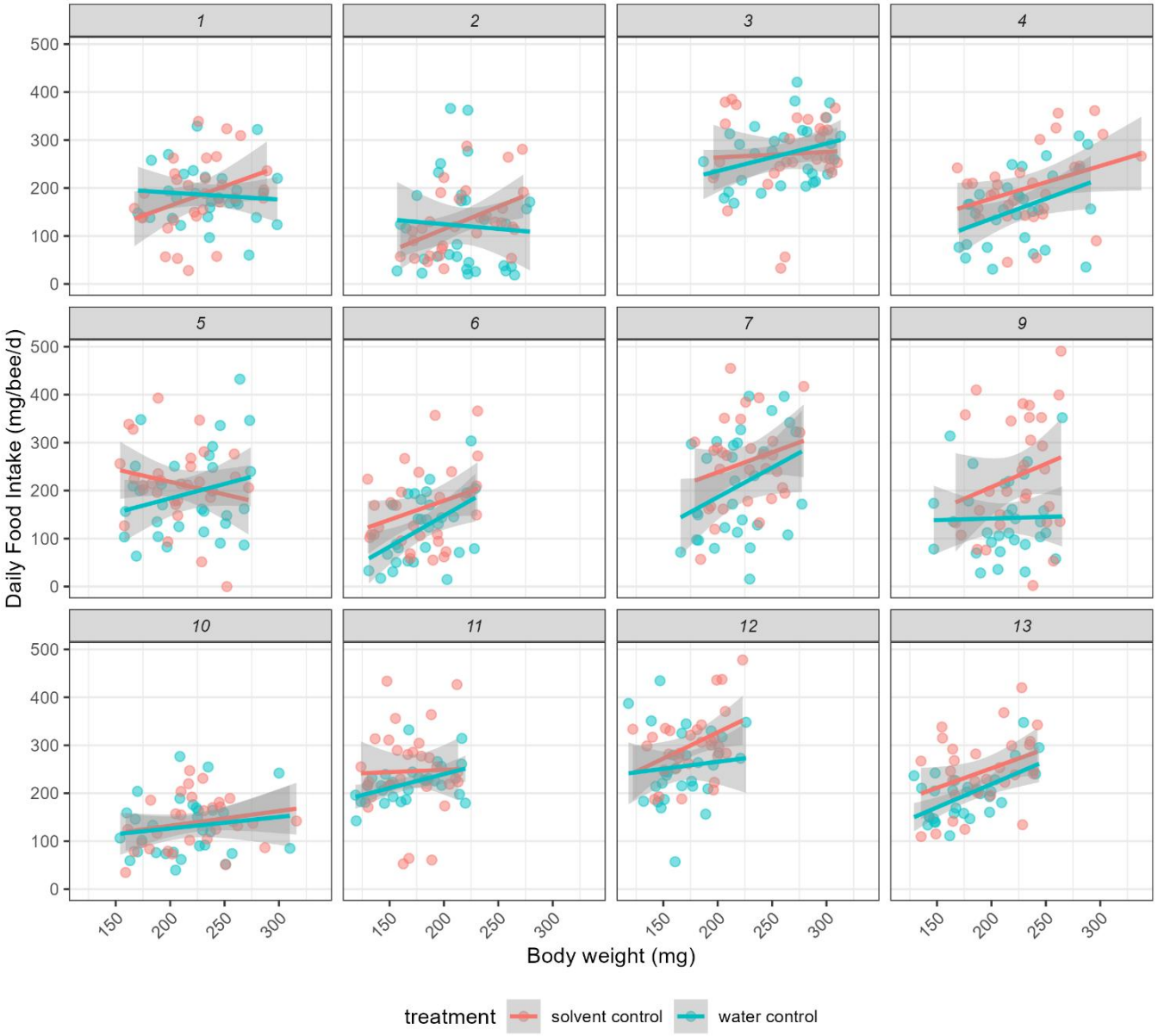
OECD (2017), *Test No. 245: Honey Bee (Apis Mellifera L.), Chronic Oral Toxicity Test (10-Day Feeding)*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264284081-en.



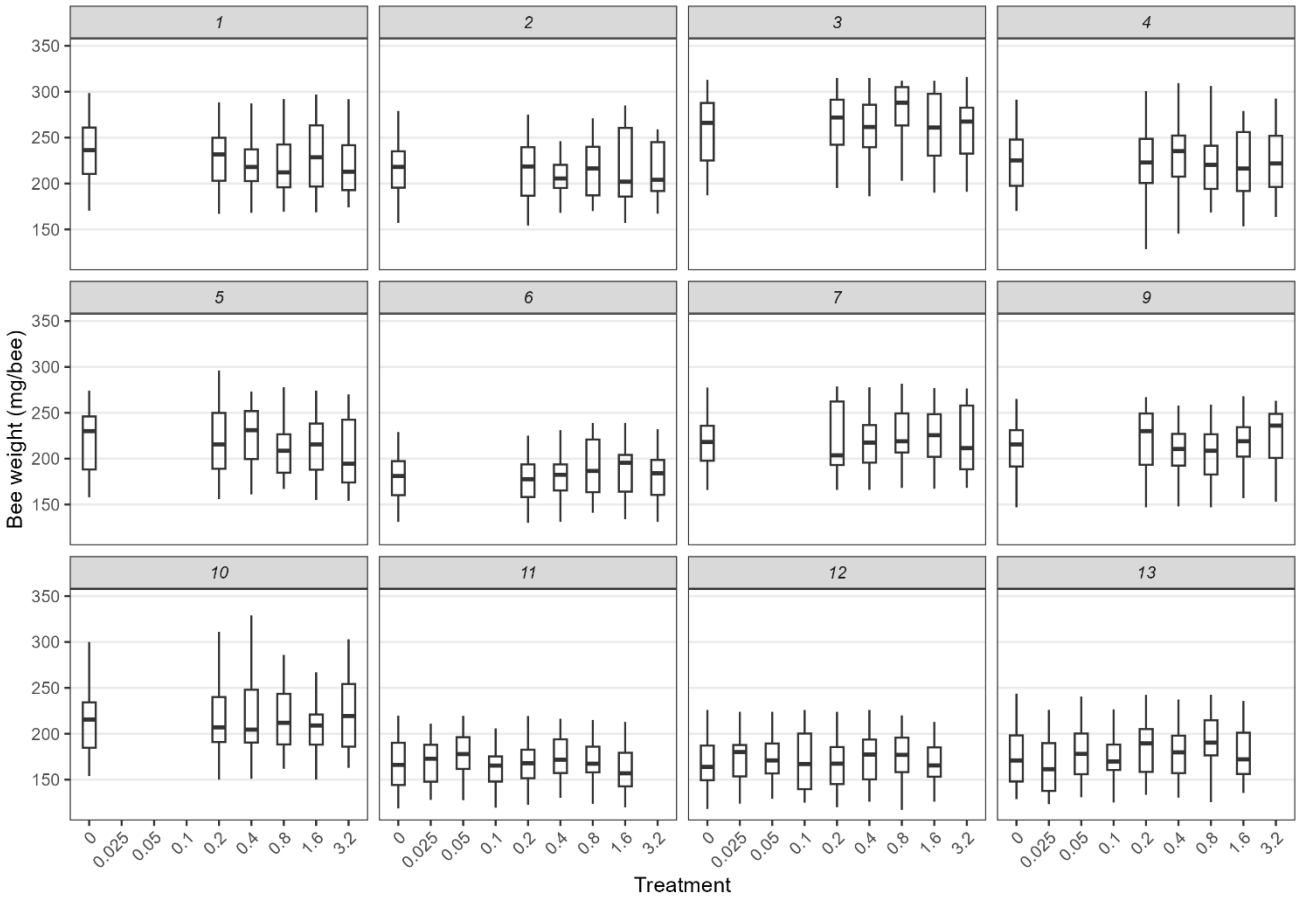
Individual nicot (a.k.a. hair roller) cage (left) and several cages randomly arranged in a customized rack inside a incubator. Picture credits: Smithers Viscient (left) and Bayer CropScience LP (right) (USA).

# APPENDIX B

Mean daily food intake (corrected for evaporation) and bumble bee body weight in the two control groups, water control and solvent control, across the participating laboratories. Laboratories 1-10 conducted their tests with *Bombus terrestris*, while Laboratories 11-13 conducted their tests with *Bombus impatiens*.

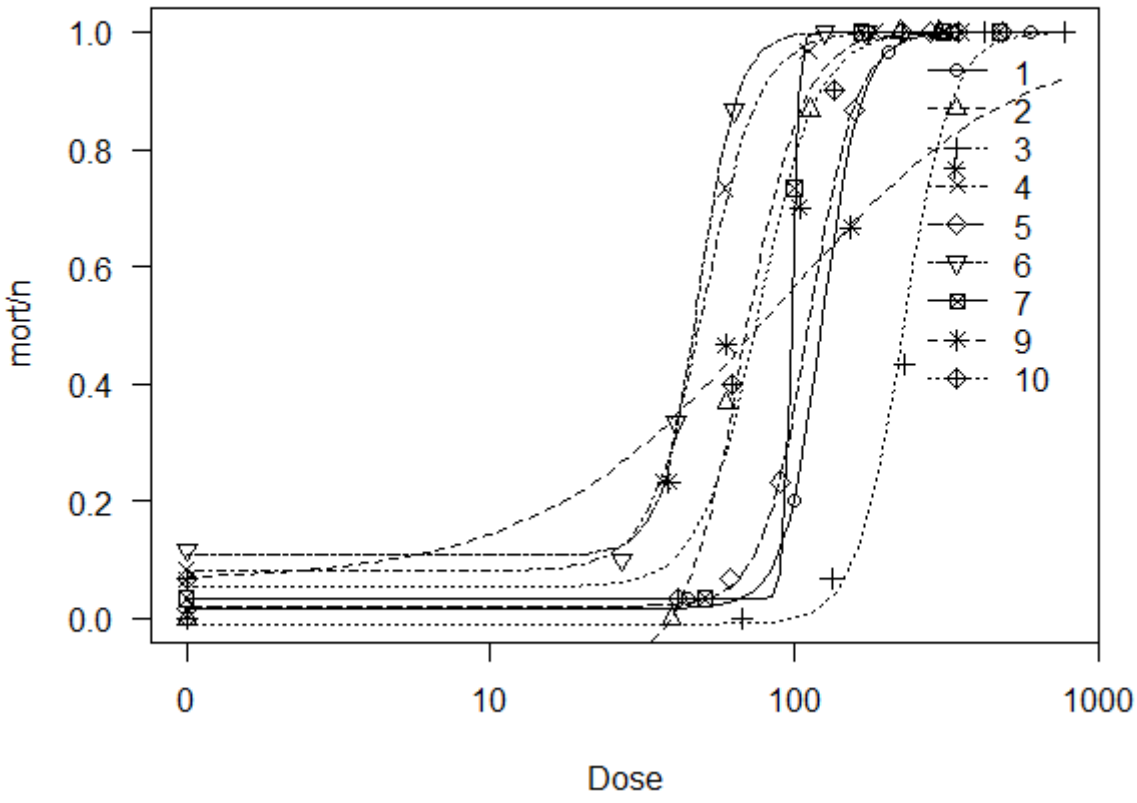


Average bumble bee body weight (mg/bee) per treatment group. Laboratories 1-10 conducted their tests with *Bombus terrestris*, while Laboratories 11-13 conducted their tests with *Bombus impatiens*.

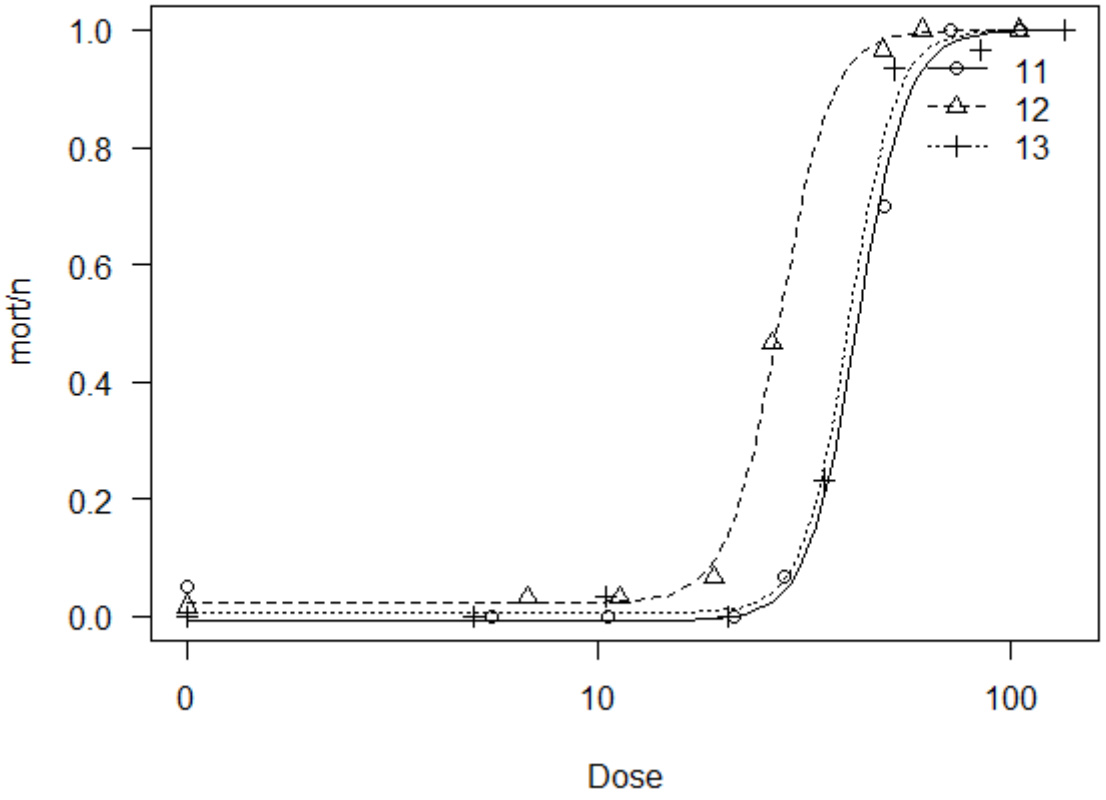


**Survival curves**

Dose-response curves for each laboratory conducting the test with *Bombus terrestris* (Laboratories 1-10; dataset generated by Laboratory 8 did not meet validity criterion).



Dose-response curves for each laboratory conducting the test with *Bombus impatiens* (Laboratories 11-13.



# APPENDIX C

**Replication - Number of bees necessary for the detection of a certain level of effect**

**Introduction**

Raw data were provided from the bumble bee chronic ring test and consisted of 13 studies total conducted with two species. Each study contained five treatment levels of dimethoate along with a negative and solvent control. As no solvent was utilized in any of the dimethoate treatments, solvent control data were excluded from the bootstrap exercises described in this appendix. Each treatment group was replicated 30 times for each study. One study was omitted from the analyses due to high control mortality.

The impact of replication with this study design was investigated using Monte Carlo bootstrapping techniques. This involved either resampling from the empirical data from the study results or sampling from binomial distributions to create simulated data sets with varying levels of replication. For each bootstrapped data set, a 3-parameter log-logistic function was fit to the data as described below:

$$M = c + \frac{1 - c}{1 + e^{-b(\log(x) - \log(e))}}$$

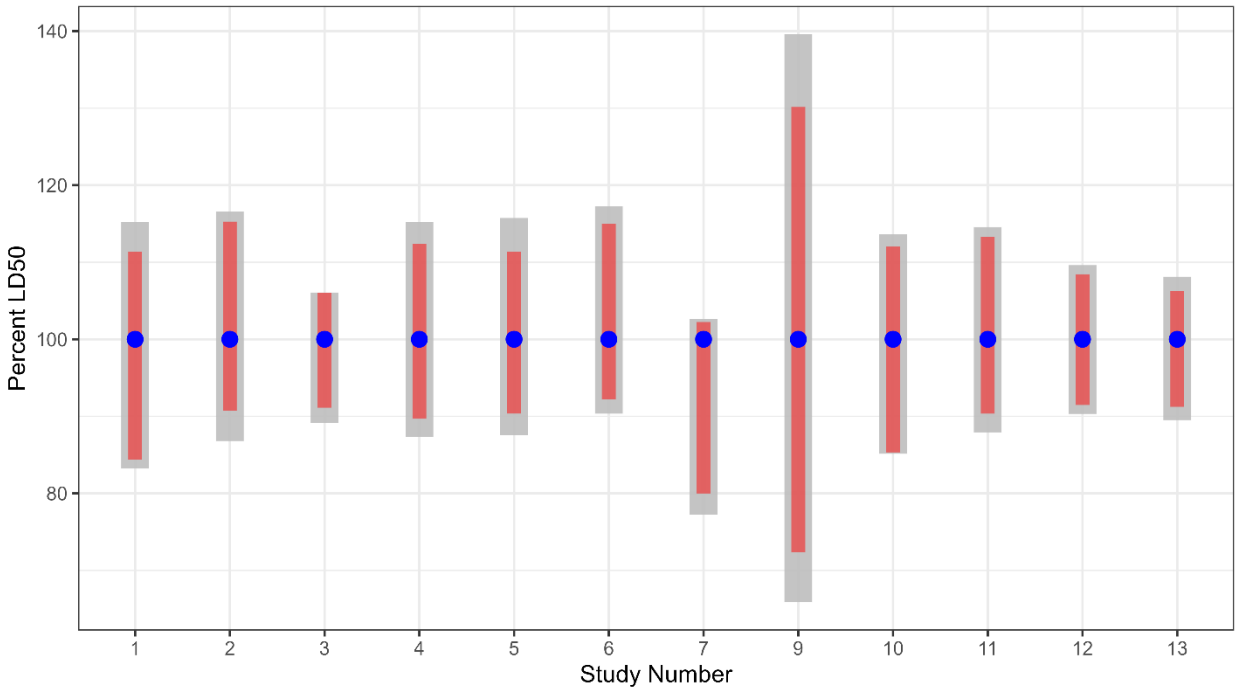
Where M is the proportion of mortality observed at treatment level x, c is the control mortality, e is a location parameter equal to the $LD_{50}$ and b is a scale parameter. All analyses were conducted using R statistical software (version 4.4.0) with the `drc` package (version 3.0-1). The `drm` function was used to fit `LL.3u` model to each bootstrapped data set. In a few, very rare instances (<1%), this resulted in an error using the default starting algorithms. In these cases, the simulated data sets were not used in creating the $LD_{50}$ distributions.

These bootstrapping analyses inform on the value of different replication levels in this study design. As the endpoint from these studies is derived from regression analyses and not hypothesis testing, a power analysis is not applicable. There is no determination of a minimum detectable difference. Rather the bootstrapping results give output with respect to likely sizes of confidence limits (empirical bootstrap) with varying levels of replication, or overall accuracy when the true $LD_{50}$ is known (parametric bootstrap).

**Empirical Bootstrap**

The first half of the analyses was conducted by resampling the data from the 12 valid bumblebee chronic studies. For each treatment of a study, the empirical, measured values were used as sampling populations. For each simulated study, these populations were sampled with replacement with either an n of 20 or 30 individual bees. For each study and replication value, a N of 10000 bootstraps were conducted from which the $LD_{50}$ value was determined. Therefore, for each study two distributions of $LD_{50}$ values were generated, one for a replication level (n) of 20 and one of 30. From these distributions, the 5th and 95th percentiles were calculated. For the replication level of n = 30, these values are equivalent to 90% confidence intervals based on an empirical bootstrap.

The overall $LD_{50}$ value for each study using the original data was also calculated for each study. For presentation of the results, the 5th and 95th percentiles from the bootstrapped $LD_{50}$ distributions are expressed as the percentage of the $LD_{50}$.




**The empirical bootstrapped $LD_{50}$ values show similar results for either replication level of 20 or 30 bees per treatment.** The calculated $LD_{50}$ value from each study is presented as a blue circle. The $5^{th}$ to $95^{th}$ percentile ranges are presented as filled bars for each n = 20 (grey) or n = 30 (red). The higher replication always results in a narrower distribution; however, this difference is not large.

### Parametric Bootstrap

One shortcoming of the empirical bootstrap analysis of the dimethoate data is its relevance for other compounds. In particular, the scale (slope) of the response curve can have an important impact on the width of the $LD_{50}$ distributions. Additional bootstrapping analyses were conducted to describe the impact of scale, control mortality and replication.

For these simulations, it was assumed that the underlying response followed a log-logistic curve with defined location and scale parameters. For all random generation of responses, the location parameter was set at a value of 1. The doses were then set at five levels with a 2X dilution factor starting at a value of 0.25. This resulted in a treatment series of 0.25, 0.5, 1, 2 and 4 in addition to a control. The proportion of mortality expected for each treatment group was then calculated for a given slope. For example, if the slope and control mortality evaluated were b = 4 and c = 10% respectively, then expected mortality at each treatment level would be calculated as:

| Treatment | Calculation | Mortality (%) |
|---|---|---|
| Control | $c$ | 10.0 |
| 0.25 | $0.1 + \frac{1 - 0.1}{1 + e^{-4(\log(0.25) - \log(1))}}$ | 10.4 |
| 0.5 | $0.1 + \frac{1 - 0.1}{1 + e^{-4(\log(0.5) - \log(1))}}$ | 15.3 |
| 1 | $0.1 + \frac{1 - 0.1}{1 + e^{-4(\log(1) - \log(1))}}$ | 55.0 |
| 2 | $0.1 + \frac{1 - 0.1}{1 + e^{-4(\log(2) - \log(1))}}$ | 94.7 |
| 4 | $0.1 + \frac{1 - 0.1}{1 + e^{-4(\log(4) - \log(1))}}$ | 99.6 |

These mortality values were then used to randomly draw from binomial distributions to determine the number of mortalities for each treatment level in a simulated study based on different replication structures.

Each combination of four different slopes (2, 4, 6 and 8), four different control mortality levels (0, 5, 10 and 15%) and five different replication levels (5, 10, 15, 20, 25 and 30) were bootstrapped a total of N = 10000 times. As in the first exercise the 5$^{th}$ and 95$^{th}$ percentiles of the calculated $LD_{50}$ values were determined and used to compare the results. For these simulations, the true $LD_{50}$ is known as this was set at 1 and percentiles are expressed as a percentage of this true $LD_{50}$.