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This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank, Basel, Rotterdam and Stockholm Conventions and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

Foreword

This document describes the results of the validation effort for the method included in the new Test Guideline (TG) 254 describing the *Osmia* acute contact test. This TG 254 and its Validation Report (VR) are the result of a project included in the work plan of the Test Guidelines Programme in 2019, with Switzerland as the lead country.

A standardized guideline to determine the acute toxicity of pesticides for solitary bees (in this case mason bees, *Osmia* spp.) is of significant importance for evaluating the side effects of pesticides and other chemicals on pollinating insects, a requirement in various regulatory frameworks across the OECD. This protocol is designed to evaluate effects of a test chemical via contact exposure.

The WNT Expert Group provided comments on the initial draft new TG and its draft VR in the second half of 2023 and in 2024, with a subsequent Working Party of National Coordinators of the Test Guidelines Programme (WNT) review finalized in January 2025. The TG and VR were subsequently approved by the WNT at its 37th meeting in April 2025.

Following declassification in July 2025, the Validation Report is published under the responsibility of the Chemicals and Biotechnology Committee.

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Results of the International Ring Test Related to the Mason bee (*Osmia spp.)* Acute Contact Toxicity Test

Compiled by ICPPR Non-Apis group

Authors: Bettina Wenzel, Ivo Roessink

Introduction and Rationale for the Proposed Method

There have been reports these last years of declines in some pollinator species in several regions of the world. Potential factors associated with these declines are hypothesized to include habitat destruction, pests, certain agricultural practices, bee management practices, pathogens, climate change, nutrition and pesticides.

In 2009, the OECD conducted a Survey on Pollinator Testing, Research, Mitigation and Information Management and published a report in 2010 [1]. This survey was conducted to gauge efforts to determine the extent to which pesticides are influencing losses of pollinators. Up until recently, the potential for adverse effects on bees has been evaluated using honey bees (*Apis mellifera*) as a surrogate for both Apis and non-Apis bees. In the Survey on Pollinator Testing, the ranking of testing requirements based on the analysis of need and feasibility showed the need for laboratory testing on non-Apis bee species.

A new test, aiming to determine the acute contact toxicity of pesticides on Mason bees (i.e., *Osmia* spp.) is of significant importance for evaluating the side effects of pesticides and other chemicals on solitary bees. Solitary bees differ from honey bees in their life history strategies and determination of acute contact toxicity in solitary bees may be required, e.g., when exposure of these bees to a given chemical is likely.

This test is based on the well-established acute contact toxicity test for honey bees [2] as well as the newer contact test for bumble bees [3], but is specifically designed to test Mason bees.

The evaluation of the side effects of pesticides, biocides and other chemicals on solitary bees is required according to the European guidance document for bees [4] and can be used to fulfil the data requirements for bees set in Regulation (EC) 1107/2009 for the placement of both active substances as well as plant protection products on the market (data requirements are described in Commission Regulation (EU) No 283/2013 8.3.1.1 and Commission Regulation (EU) no 284/2013 10.3.1.1) [5]. The U.S./Canada harmonized guidance on assessing risks to bees [6] also identifies the need to evaluate risks to native species, e.g., solitary and social non-Apis bees, since the focus of Apis and non-Apis bee testing has been on species which are of commercial value (i.e., provide pollination services). The generation of toxicity data for solitary bees, specifically Mason bees, in addition to honey bees and bumble bees, is also a matter of interest for other regulatory authorities and authorities, such as the Japanese MAFF [7], the Swiss authorities [8], and Brazilian authorities [9].

This need has been recognized by the ICP-PR (International Commission for Plant-Pollinator Relationships). The organizations' working group "Non-Apis bees" has organized several preliminary as well as larger scale international ring tests on the methodology of the acute contact test for *Osmia spp*.. Based on the results of these ring tests, a protocol proposal to determine the acute contact toxicity in Mason bees was made. This validation report describes the results of three ring tests performed in the years 2014 through 2016.

Pollinators such as mason bees (*Osmia spp*.) may be exposed to residues of plant protection products or chemicals either by foraging on treated crops, by contact with contaminated soils from treated fields, and potentially from foraging on non-target plants exposed to spray drift.

To address this potential risk, an acute contact study can be conducted in the laboratory by exposing adult female Mason bees to a single dose of the respective chemical substance at varying concentrations. The method aims at the determination of the LD_{50} (median lethal dose) and should be seen as a lower tier test in the context of an overall risk assessment scheme for pollinators (step-wise programs for evaluating the risks of chemicals to pollinators, based on sequential progression from laboratory toxicity tests to semi-field and field experiments [4]).

Information on the Ring Test Group

In total, 14 laboratories from 8 countries participated in the ring tests of 2014 to 2016. Participants represented a wide range of stakeholder groups, including universities, contract laboratories (CRO's) and the crop protection industry. The ring test was organized by the ICP-PR (International Commission for Plant-Pollinator Relationships) working group "non-Apis bees". The tests were conducted under non-GLP conditions; however, many of the participating labs were GLP (Good Laboratory Practice) certified. Participating Laboratories are listed in

Table 1. Laboratories Participating in Testing below.

Table 1. Laboratories Participating in Testing

Laboratory	Responsible person(s)	Participated in testing		
		2014	2015	2016
CRA-API, University of Bologna, Italy	Piotr Medrzycki in consultation with Jordi Bosch, CREAF, Barçelona	X	X	
BASF SE, Germany	Nicole Hanewald (co-organizer ring test) Christoph Schneider	X	X	X
Bayer CropScience AG, Germany	Anja Quambusch Nina Exeler	X	X	X
University of Belgrade, Serbia	Ljubiša Stanisavljević	X	X	
BioChem Agrar GmbH, Germany	Markus Barth	X	X	X
Eurofins Ecotox GmbH, Germany	Annette Kling Lea Franke Silvio Knäbe	X	X	X
University of Ghent, Belgium	Guy Smagghe		X	X
ibacon GmbH, Germany	Stephan Schmitzer Verena Tänzler	X	X	X
Innovative Environmental Services (IES) Ltd, Switzerland	Bettina Wenzel Stefan Kimmel		X	X
Knoell GmbH, Germany	In consultation with the University of Landau, Germany	X	X	
Syntech Research, France	Eric Ethier	X	X	
Testapi SARL, France	Hervé Giffard		X	X
Trialcamp, Spain	Eugenia Soler		X	X
Wageningen Environmental Research, Netherlands	Ivo Roessink (organizer ring test)	X	X	X

In order to avoid any conflict of interest associated with disclosure of the names of laboratories that participated in this ring test, the names were anonymised by numbering the labs in a random order.

Ring Test Schedule

Once a rough design of the test had been created, the following activities were performed in order to ring test the method/protocol within the ICPPR group:

Table 2. Meetings held

Activity	Dates
Meeting to set up protocols	• Brussels, SETAC Special Science Symposium, October 2013 • Niefern (DE) workshop, March 2014
Experimental phase	• April-June 2014 • April-September 2015 • April-August 2016
Meetings of the ring test group for discussion of results and fine tuning of the Protocol	• Limburgerhof (DE), February 2015 • Braunschweig (DE), February 2016 • Wageningen (NL), February 2017

Substances Used for Validation of the Proposed Test Method

The test chemical used in the ring test comprised Dimethoate (CAS Number 60-51-6). This chemical was selected for the following reasons:

1. It has well-documented effects on other bee species, such as honey bees and bumble bees
2. It is the standard reference item used in acute honey bee studies
3. It is relatively easy and inexpensive to analyze at the concentrations used

Each participant purchased the chemical test chemical either as formulated product (Perfekthion 400 EC, Roxion 400 EC, Rogor plus or Dimetogal) or as technical substance/analytical standard from a local supplier. The following formulations containing Dimethoate were used by ring test participants:

Table 3. Used Dimethoate products, solvents and wetting agents per participant per year

Laboratory	Dimethoate formulation used in ring test		
	2014	2015	2016
CRA-API, University of Bologna, Italy	Analytical standard; Wetting agent: Tween 80	Test not included in validation report	Did not participate
BASF SE, Germany	Perfekthion 400 EC; Wetting agent: Tween 80	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)
Bayer CropScience AG, Germany	Test not included in validation report	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)
University of Belgrade, Serbia	Test not included in validation report	Dimetogal 400g/L; Solvent: acetone, no solvent, formulated product	Did not participate
BioChem Agrar GmbH, Germany	Perfekthion 400EC; Wetting agent: Tween 80	Perfekthion 400 EC; Solvent: acetone	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)
Eurofins Ecotox GmbH, Germany	Perfekthion 400 EC;	a) Perfekthion 400 EC;	Perfekthion 400 EC;

	Wetting agent: Tween 80	Wetting agent: Triton X-100 b) Perfekthion 400 EC; Solvent: acetone	Wetting agent: Triton X-100 (0.1%)
University of Ghent, Belgium	Did not participate	Test not included in validation report	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)
ibacon GmbH, Germany	Did not participate	Perfekthion 400 EC; Solvent: acetone	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)
Innovative Environmental Services (IES) Ltd, Switzerland	Did not participate	Roxion 400 EC; Solvent: Acetone	Roxion 400 EC; Wetting agent: Triton X-100 (0.1%)
Knoell GmbH, Germany	Perfekthion 400 EC; Wetting agent: Tween 80	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)	Did not participate
Syntech Research,	Test not included in validation report	Perfekthion 400 EC; Solvent: Acetone	Did not participate
Testapi SARL, France	Did not participate	Analytical standard; Solvent: Acetone	Rogor plus; Wetting agent: Triton X-100 (0.1%)
Trialcamp, Spain	Did not participate	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)	Test not included in validation report
Wageningen Environmental Research, Netherlands	Perfekthion 400 EC: Wetting agent: Tween 80	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)	Perfekthion 400 EC; Wetting agent: Triton X-100 (0.1%)

Test Method Protocol

The method followed in the ring-test is described in detail in the protocol in Appendix I. This method, with all subsequent changes made, has been submitted to the OECD as a draft OECD Test Guideline. Changes in methodology that resulted from experience gained during the ring testing phase are presented in Section 6.1.

This test is a laboratory test designed to assess the acute contact toxicity of pesticides and other chemicals to adult female Mason bees (*Osmia spp*.). The test is based on OECD 214 [2] and OECD 246 [3]. The method aims at the determination of the LD_{50} following a single exposure to a test chemical via dorsal application.

Results

Results from a total of 36 tests performed with Dimethoate/Dimethoate-Formulations in the three summers of 2014 to 2016 by 14 laboratories were taken into account. All considered tests have been given the same trial ID throughout the report for easier understanding. Although further trials were done in the scope of the ring test, tests using males, tests done using single housing, tests done with food other than sugar water, tests using CO_2 to anesthetize the bees and tests where mortality was above 50% in all treatments including the controls were not included here and results are not shown. This was done in order to include only tests that are as close to the guideline proposal as possible.

Of the total amount of tests, 27 were performed using *Osmia bicornis* while nine tests were performed using *Osmia cornuta*. Performing the tests with the different Mason bee species had the goal to validate the test for the two commercially available *Osmia* species in Europe. As there was no access to other *Osmia* species for testing, the representativeness for other *Osmia* species is yet to be determined.

Experimental Design

The detailed protocol provided to each participant in 2016 is described in Annex I. Major changes that took place over the ring test years are described below.

In the 2014 protocol, it was left up to the experimenter to decide whether the bees should be kept in group or individual housing. As most labs had the feedback that control mortality seemed to be lower in group housing, all further tests were done in group housing of either 5 or 10 bees per cage. All tests performed using single housing were excluded, only tests performed with group housing are included in the validation.

Although female bees were recommended from the 2014 protocol on, a few labs tested males as well to see if it is feasible. Only tests performed on females were considered here, although the protocol seems to be just as applicable to male bees. The tests were performed with female bees that had not been mated, as this was recommended by experts in 2014 and consequently got documented in the ring test protocol. However, independent observations by two participating labs indicated that no difference occurs between testing non-mated or mated females. In addition, it is practically rather difficult to ensure females hatch in isolation and consequently are not mated. This is because as soon as one male hatches amongst the female cocoon batch, mating occurs. Therefore, no specific recommendation is given in the proposed test guideline on using mated or unmated bees for the test.

The test requires the test item to be dissolved or dispersed in either water, acetone, or some other solvent. If the test item is dissolved in acetone or another solvent, it can be applied directly on the bees' hairy back without forming a droplet and draining off. If the test item is dissolved in water, the addition of a wetting agent is required to be able to apply the solution to the bees. In 2014, all labs tested Tween 80. It was not used in 2015, as Tween 80 at a concentration of 0.5% did not sufficiently reduce the surface tension and therefore caused an improper exposure of the bees during the ring test In 2015, the labs tested either acetone or Triton X-100, and in 2016 all labs tested Triton X-100. Consequently, this protocol was validated for the use of pure acetone (or percentage of acetone) in amounts of up to 2 µl per bee and of solutions containing the wetting agents Triton X-100 (Dow Chemical Company) at concentrations up to 0.1% or Tween 80 (Croda International PLC) at concentrations up to 0.5%, which is not recommended.

An important factor for the handling of the bees is how they are anaesthetized. In the 2014 protocol, it was left open if the bees should be chilled or anaesthetized using CO_2. As in the first year, using CO_2 caused mortality, it was decided from 2015 onwards to exclusively chill the bees before handling. Chilling of bees is defined by putting them into a lab fridge at about 4°C for at least 15 minutes, which puts them into a state where they will not move, thus facilitating the pre-experimental weighing and dosing process. All tests shown here were done with chilling the bees.

In order to facilitate the feeding of the bees during the test a lot of different feeder types, including cups placed on the bottom of the cage, devices hanging from the side or top of the cage, and even devices with natural flower petals were used by the different labs. The goal was to provide food during the test period and as a result the type of feeder was not specified in the draft guideline, thus tests with all feeder types are included here.

Two types of food, namely a 50% sugar solution (Water/sugar in a 50:50 ratio w/v) and sucrose paste (Apifonda®, Südzucker AG, Germany) were provided during the ring test. Sugar solution is identical to the food specified in the honeybee and bumblebee test guidelines and consequently recommended in the protocol, but tests done using sucrose paste were not included in this validation.

Another factor that was not specified in the ring test protocol was the light intensity that the bees needed. As a consequence, the tests were done with the same light regime but with different light intensities. Light regime was 16:8 for most tests performed, although three labs used a 12:12 or a different light regime.

As for the other physical parameters, all tests were done at a constant temperature of 22±2°C and relative humidity of 60±20%. Although there were some deviations from this, this does not seem to affect the studies, e.g., as demonstrated by average performance of controls.

All bees were acquired from commercial suppliers. All labs obtained their bees from either Kommilch (DE), DSP Dr Schubert Pflanzenzucht (DE), WAB-Mauerbienzucht (DE), bijbijen.nl (NL), or Getico, D. Teper (PL). Bees became available from November onwards and were tested by the labs before July the following year. It should be noted that both *Osmia bicornis* and *Osmia cornuta* have a strong seasonal cycle and testing late in the year will seriously diminish their survival rate and performance in the test.

For the evaluation, all tests were evaluated using a Probit analysis using linear maximum likelihood regression. Other methods can be used, as appropriate.

A full list of the 36 tests that were considered for the validation of the method, with experimental details, is specified in Table 4. .

Table 4. Summary of the ring test trials considered in the validation

Trial ID	Lab no.	Year	Species	Month performed	Solvent/Wetting agent	Concentration of solvent/wetting agent (%)	Bees/test unit	Volume applied (µL)	Average weight of bees in the trial (mg)	Light regime	Analytical verification
1	1	2014	*O. cornuta*	April	Tween 80	0.50	10	2	131.0	16:8	101.1 ±9.2[a]
2	2	2014	*O. cornuta*	April	Tween 80	0.50	5	1	n.a.	16:8	Approx. 110%[b]
3	3	2014	*O. bicornis*	June	Tween 80	0.50	10	1	94.5	12:12	Approx. 100%[b]
4	6	2014	*O. bicornis*	July	Tween 80	0.50	10	1	90.1	12:12	Approx. 100%[b]
5	7	2014	*O. bicornis*	May	Tween 80	0.50	10	1	102.5	Not controlled	99.6±2.6[b]
6	8	2014	*O. bicornis*	May	Tween 80	0.50	10	1	93.7	16:8	103.4 ±6.6[a]
7	8	2014	*O. bicornis*	May	Tween 80	0.50	10	1	96.2	16:8	103.4 ±6.6[a]
8	8	2014	*O. bicornis*	June	Tween 80	0.50	10	1	99.1	16:8	103.4 ±6.6[a]
9	1	2015	*O. bicornis*	June	Triton X-100	0.10	10	2	86.8	16:8	96.2±7.6[a]
10	3	2015	*O. bicornis*	May	Triton X-100	0.10	10	2	87.4	16:8	Approx. 100%[b]
11	4	2015	*O. bicornis*	June	Triton X-100	0.10	10	2	98.3	16:8	Approx. 100%[b]
12	5	2015	*O. cornuta*	April	Acetone	100	10	2	150.1	16:8	n.a.
13	5	2015	*O. cornuta*	April	No solvent, formulated product	0.10	10	2	145.7	16:8	n.a.
14	6	2015	*O. cornuta*	June	Acetone	100	10	2	102.7	16:8	88.8±2.8[b]
15	7	2015	*O. bicornis*	April	Acetone	100	10	2	102.4	16:8	100.0±3.6[b]
16	7	2015	*O. bicornis*	April	Triton X-100	0.10	10	2	103.3	16:8	100.0±3.6[b]

17	12	2015	*O. bicornis*	June	Acetone	100	10	1	91.0	16:8	104.3±2.9[b]
18	13	2015	*O. bicornis*	Sept.	Acetone	100	10	1	97.7	16:8	108.1±6.7[b]
19	8	2015	*O. bicornis*	May	Triton X-100	0.10	10	2	90.0	16:8	n.a.
20	9	2015	*O. bicornis*	June	Acetone	100	10	1	98.3	16:8	n.a.
21	10	2015	*O. bicornis*	April	Acetone	100	10	2	82.0	16:8	n.a.
22	14	2015	*O. bicornis*	July	Triton X-100	0.10	10	2	89.7	16:8	n.a.
23	1	2016	*O. cornuta*	April	Triton X-100	0.10	10	2	126.9	16:8	99.1±9.0[a]
24	1	2016	*O. bicornis*	May	Triton X-100	0.10	10	2	92.6	16:8	99.1±9.0[a]
25	3	2016	*O. bicornis*	May	Triton X-100	0.10	10	2	83.1	16:8	Approx. 100%[b]
26	3	2016	*O. bicornis*	May	Triton X-100	0.10	10	2	90.1	16:8	Approx. 100%[b]
27	4	2016	*O. bicornis*	June	Triton X-100	0.10	5	2	92.1	16:8	Approx. 100%[b]
28	4	2016	*O. cornuta*	Aug.	Triton X-100	0.10	5	2	136.5	16:8	Approx. 100%[b]
29	6	2016	*O. bicornis*	July	Triton X-100	0.10	10	2	103.1	16:8	n.a.
30	7	2016	*O. cornuta*	June	Triton X-100	0.10	10	2	89.5	16:8	n.a.
31	7	2016	*O. cornuta*	June	Triton X-100	0.10	10	2	101.0	16:8	n.a.
32	11	2016	*O. bicornis*	June	Triton X-100	0.10	10	2	86.8	16:8	127.5±76.7[a]
33	12	2016	*O. bicornis*	June	Triton X-100	0.10	10	2	100.0	16:8	n.a.
34	13	2016	*O. bicornis*	Aug.	Triton X-100	0.10	10	2	73.8	16:8	Approx. 100%[b]
35	10	2016	*O. bicornis*	April	Triton X-100	0.10	10	2	89.1	16:8	99.8±8.8[a]
36	10	2016	*O. bicornis*	May	Triton X-100	0.10	10	2	94.0	16:8	99.8±8.8[a]

[a] Analysed by Wageningen Environmental Research

[b] Analysed by laboratory performing the actual test

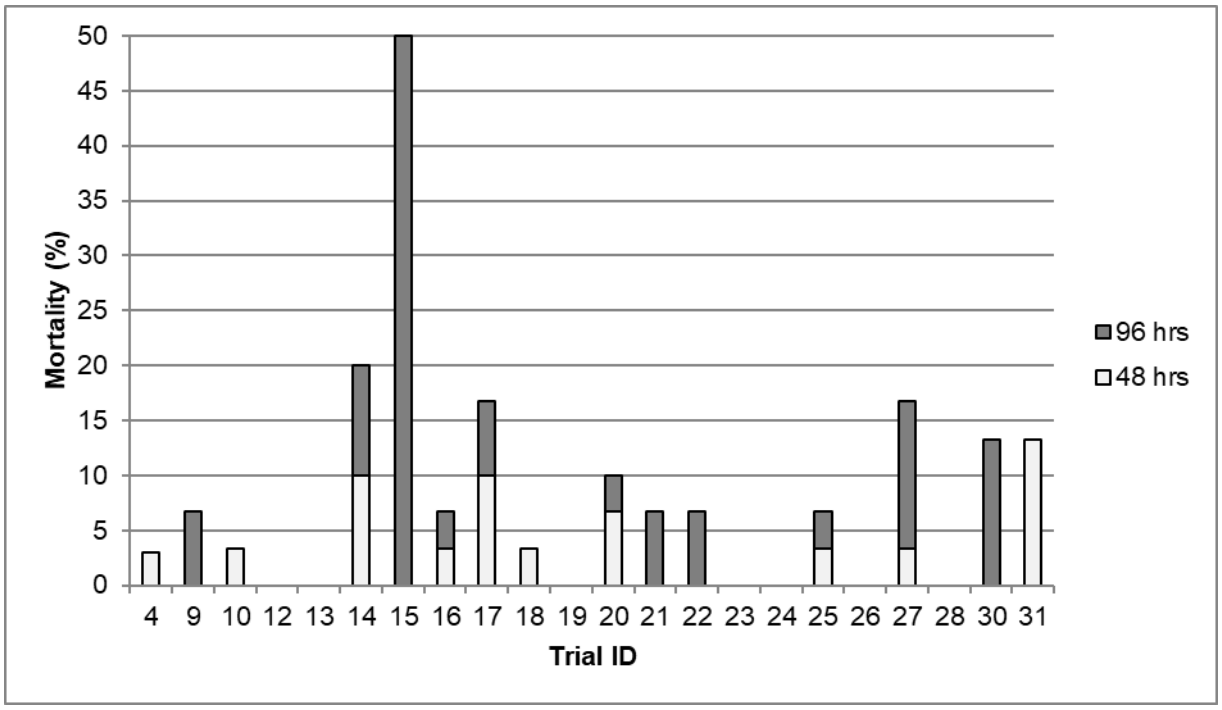
n.a. = not available, as no analytical verification was performed or no weight measurements were performed

Control mortality (Validity of the Tests)

According to the acceptance criteria, control mortality should not exceed 15% in order for the test to be valid. In most of the tests showing high control mortality, all or most of the bees exposed to the dimethoate formulations were dead as well. It should be noted that mortality was especially high in tests of first-time participants and that test performance increased with increasing experience of the labs. A summary of all control mortality values can be seen in Table 5.

Of the 36 tests from 14 participating labs considered for this validation report, 22 had a water control in addition to the solvent control. Mortality values for the water control are shown in Table 1. After 48 hours, none of the tests exceeded 15% mortality in the control. After 96 hours, four of the labs (18%) exceeded the 15% control mortality. Of these four labs, one had 50% mortality, the other three labs showed mortalities below 20 %.

Figure 1. Mortality in water controls

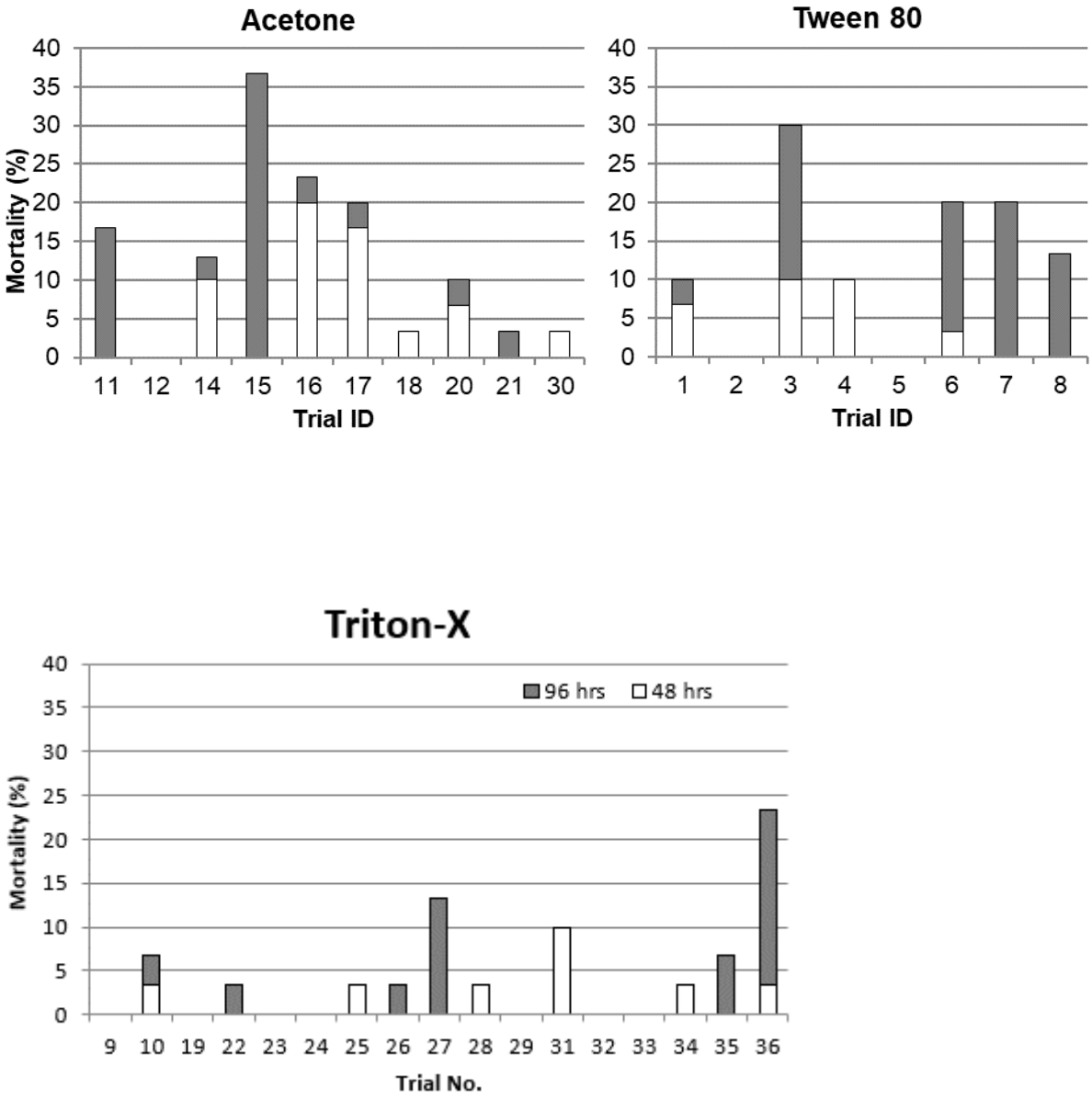


Of the 36 tests considered, ten used acetone as the solvent control, 17 used Triton X-100 and eight used Tween 80, the latter two were used in the test to reduce the surface tension of the application solution and for the applied solution to better stay on the hairy bees back and not drain off.

As can be seen in Figure 2, only two tests (both with acetone as a solvent) had control mortalities above 15% after 48 hours (5.6% of all tests).

At 96 hours, eight tests including tests with all three solvents had mortalities above 15% (resulting in 22.2% of all tests being invalid). Again, all solvents were represented in the high mortality values.

Figure 2. Mortality of solvent controls



Note: Some trials were performed using Triton X-100 but were run with an acetone solvent control instead of a Triton X-100 control. In cases where a 48-hour mortality is shown but no 96-hour value, the mortality did not increase after 48 hours and the 96-hour value is the same as the 48-hour value.

LD_{50} values

Analysis of the test item in the dosing solutions used was performed by either the participating laboratory itself or by sending the samples to Wageningen Environmental Research. Analysis revealed that most participating laboratories indeed achieved the intended dose per bee. An exception are the results from Laboratory 11, where all measured values, including the control, were a bit higher than they should be. Consequently, the results from this specific lab should be interpreted with caution but were included here as they are well in line with all the other results.

For all tests that were considered, the LD_{50} value was calculated for both 48 and 96 hours with a Probit analysis using linear maximum likelihood regression (Table 6. LD50 **values of individual tests**

).

Overall, nine tests were performed using *O. cornuta*; the other 27 tests were done using *O. bicornis*. Although *O. cornuta* females were on average heavier, there was very little difference in the LD_{50} values of *O. bicornis* and *O. cornuta*, with the 48hr-LD_{50} values being 1.49 ±1.19 µg a.i./bee for all tests with *O. bicornis* and 1.66 ± 0.81 µg a.i./bee for all tests with *O. cornuta*. Figure 3 shows the relationship between the LD_{50} values and the LD_{50} values normalized to the weight of the bees.

Figure 3. LD_{50} values after 48 hours

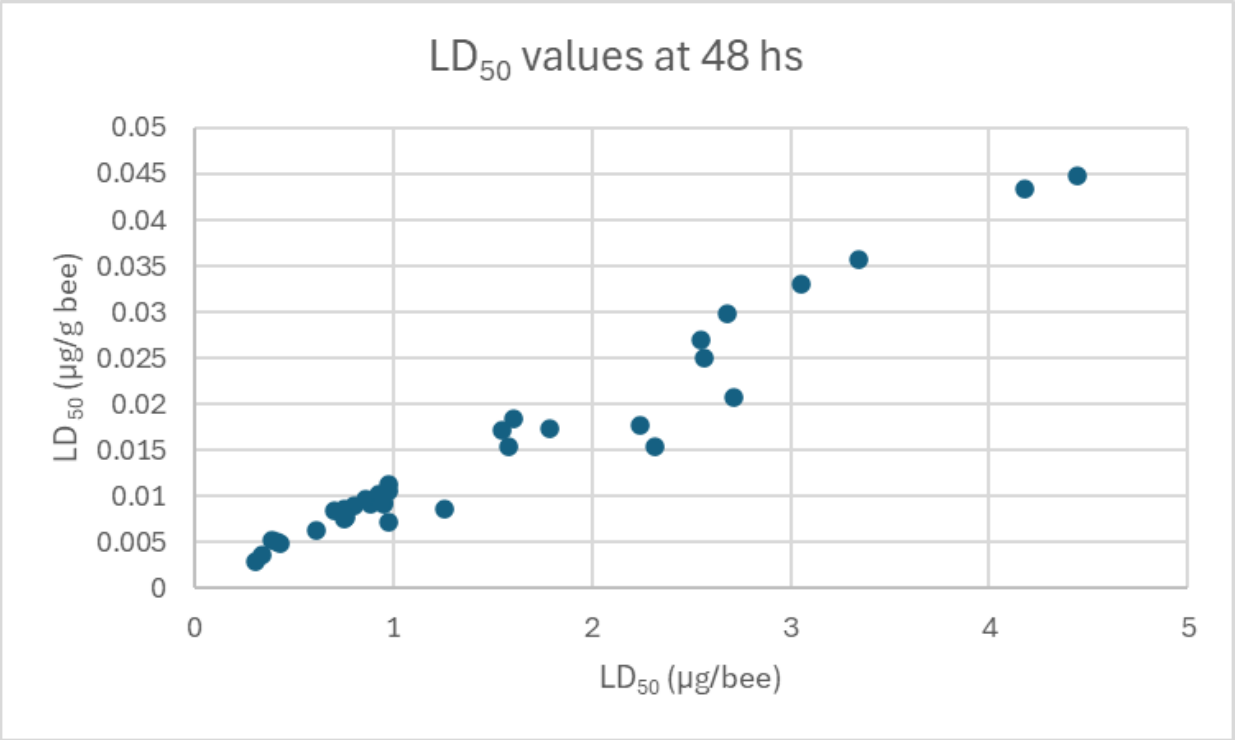
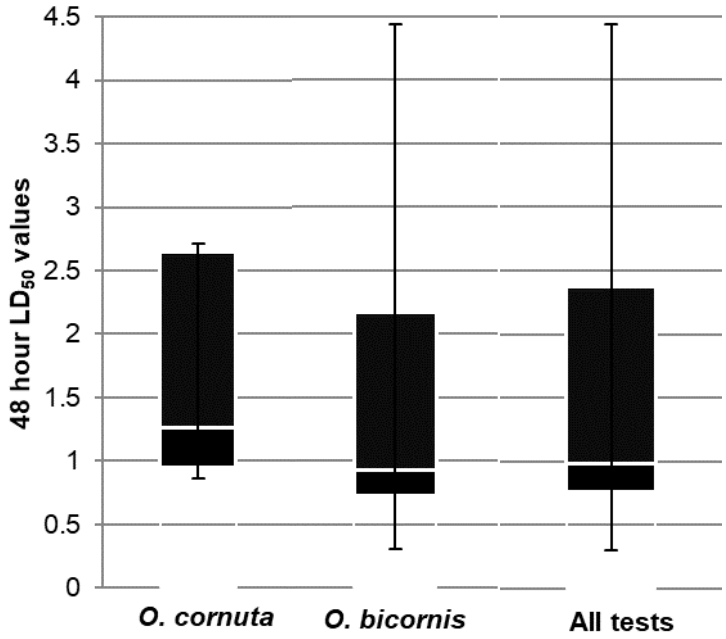


Table 4 shows a box-and-whiskers plot summarizing all LD_{50} values determined from the 36 tests. There, the lowest value obtained was 0.305 µg a.i./bee and the highest 4.44 µg a.i./bee. The average value over all tests was 1.53 ± 1.10 µg a.i./bee, but about half of the tests had an LD_{50} value below 1 µg a.i./bee (Median: 0.977 µg a.i./bee).

Figure 4. Box-and-Whiskers Plot of LD_{50} values (in µg/bee) after 48 hours



Tests with Tween 80 showed that the addition of Tween 80 did not result in a proper contact of the dimethoate with the test subject. Note that this was observed in both bumblebees as well as Mason bees. In addition, blue spots of droplets containing dimethoate were observed on the bottom of the testing cages. This indicates that using Tween 80 results in an inadequate exposure of the bees, as can also be seen by the higher LD_{50} values (Table 5). This is noted in Annex II of the Guideline proposal.

The use of both acetone and Triton X-100 resulted in a good flow of the droplet over the dorsal body surface of the bee and no overspill to the bottom of the cage was observed. Thus, it can be assumed that the bees got exposed to the actually dosed substance. It should be noted, however, that some labs using acetone reported abnormal behaviour, i.e. tremors and increased activity, in the bees after dosing.

Figure 5. 48 hour-LD_{50} values (in µg/bee) testing dimethoate with acetone, Triton X-100 or Tween 80.

One Lab in 2015 used formulated product instead of Triton X-100 as solvent. Product was dissolved in water.

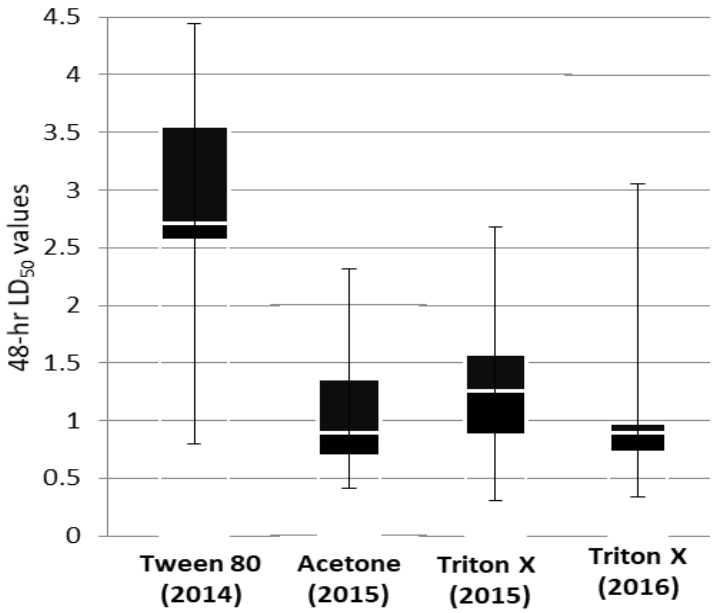


Figure 6. LD_{50} values of individual tests done in 2014.

See Table 4. for details about the individual tests. That year, all tests were done using Tween 80. Test 1 and 2 used *O. cornuta*, tests 3 through 8 used *O. bicornis*.

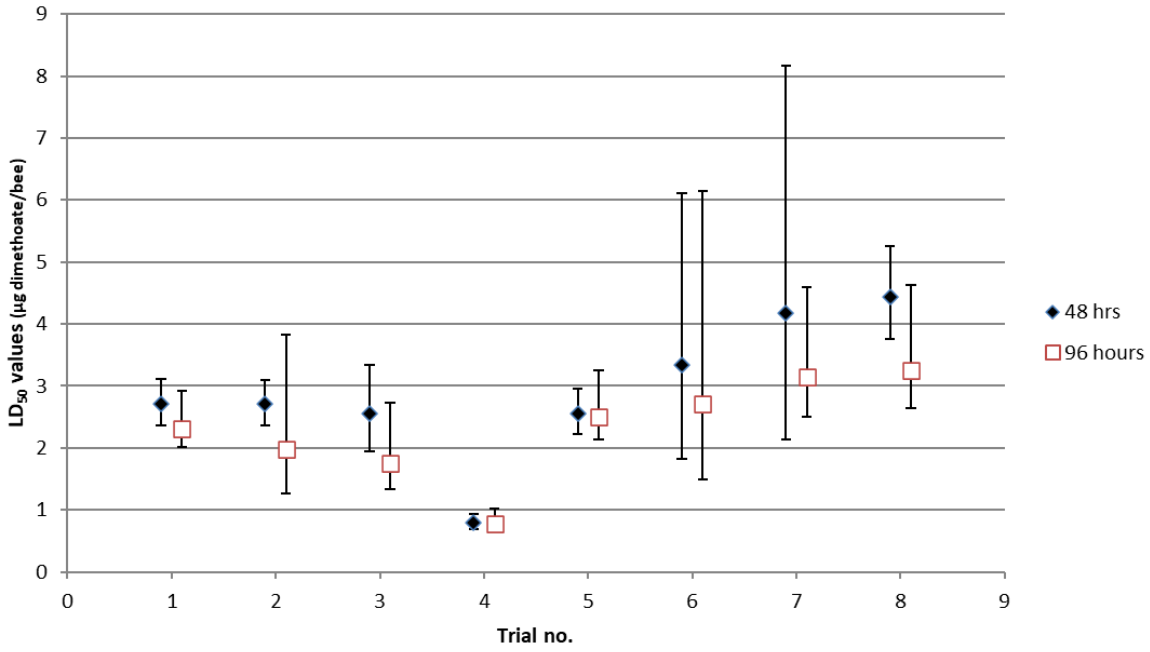


Figure 7. LD_{50} values of individual tests done in 2015.

See Table 4. for details about the individual tests. That year, tests were done using either acetone or Triton X-100. In test 13 no solvent, but a formulated product was used. Tests 12-14 used *O. cornuta*, the remaining tests used *O. bicornis*.

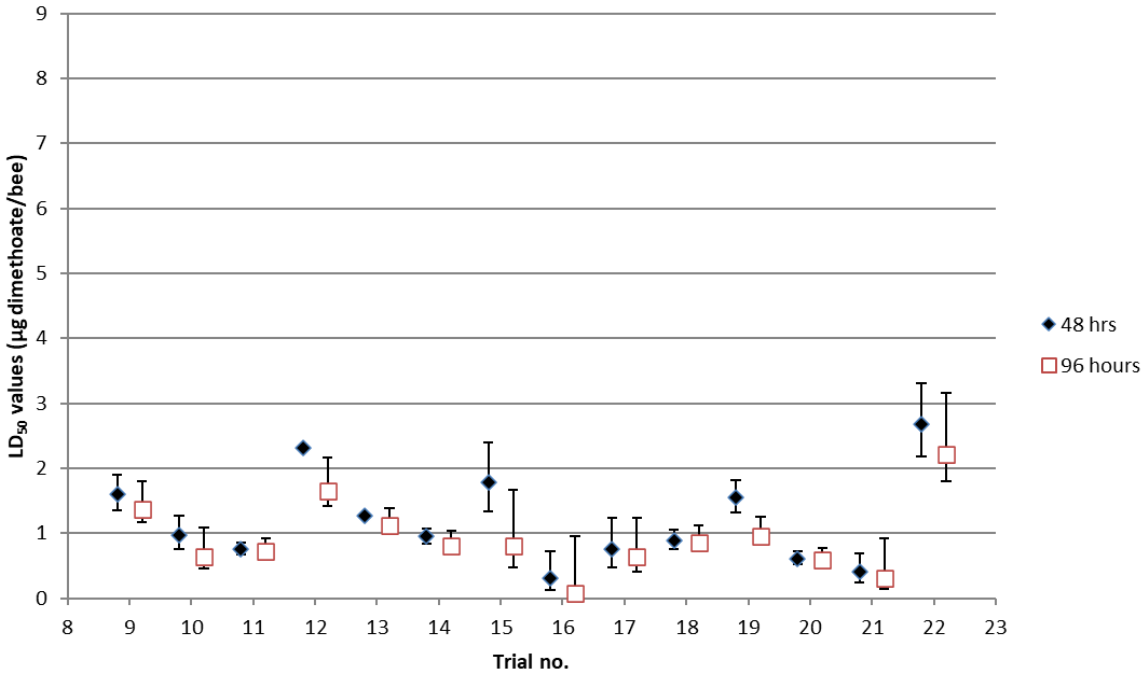
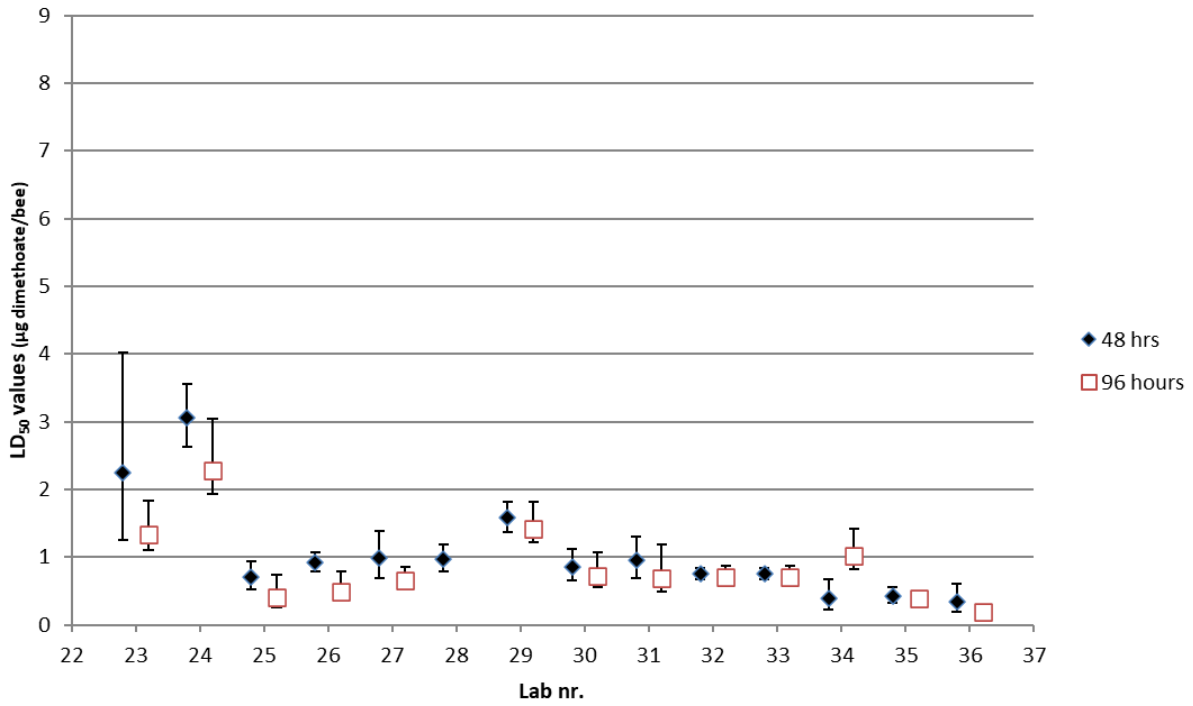


Figure 8. LD_{50} values of individual tests done in 2016.

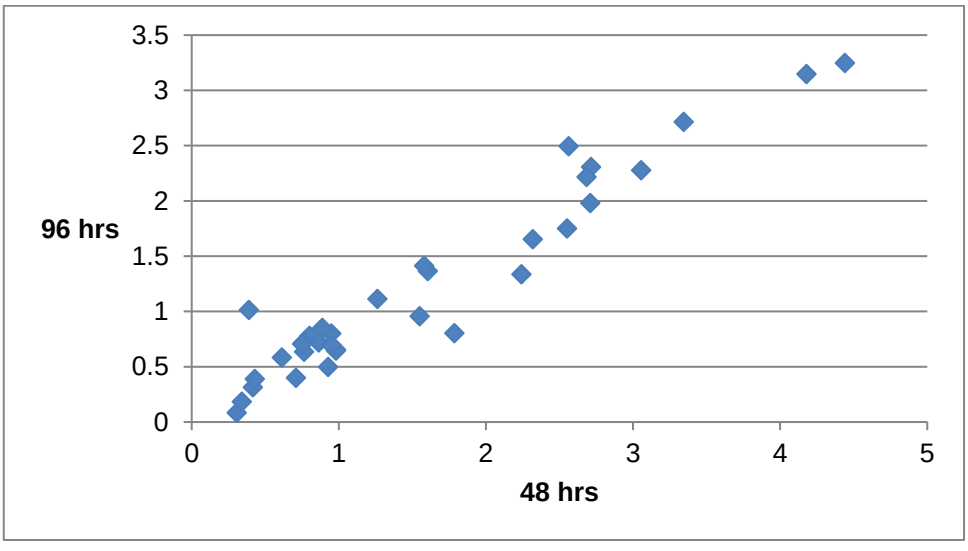
See Table 4. for details about the individual tests. That year, tests were done using Triton X-100. Tests 23, 28, 30 and 31 used *O. cornuta*, the remaining tests used *O. bicornis*.



All ring tests were performed over a period of 96 hours to test the feasibility of the test over this time period. However, the protocol requires a test period of 48 hours which is prolonged only if there is a mortality of more than 10% in the preceding 24 hours.

Figure 9 shows the LD_{50} values after 48 and 96 hours. It can be seen that the effect of Dimethoate was visible after 48 hours. After 96 hours, values were generally somewhat lower.

Figure 9. LD_{50} values (in µg dimethoate/bee) compared after 48 and 96 hours



Test Method Performance (Accuracy)

Accuracy has been defined by ICCVAM as determinations of concordance, sensitivity, specificity, positive and negative predictivity, and false positive and negative rates. Comparisons with a reference method were not made since in ecotoxicological testing there is generally no accepted reference value. The closest to a reference value in ecotoxicology is where a weight-of-evidence approach is taken.

Ecotoxicity test values derived from testing methods with related species may cluster together to give a mean toxicity value. This approach may give a rough indication of method accuracy. In this case, there are standardized tests that are very similar to the test described here with the honey bee and the bumble bee as a test species. The 24-hour LD_{50} value for dimethoate stated in the honeybee (*A. mellifera*) guideline for acute contact toxicity testing [2] is 0.1 to 0.3 µg dimethoate/bee. For bumble bees (*B. terrestris)* [3], a 48-hour LD_{50} value of 10 µg dimethoate/bee is considered as standard. The average LD_{50} value of 1.5 µg dimethoate/bee reached in these tests would then lie between these two values. That means that the honey bee, even though it has a similar (but not so broad) weight range, is more sensitive to dimethoate than the solitary bee. The bumble bee is much less sensitive, but it also has a much higher body mass.

Test Method Reliability (Repeatability/Reproducibility)

Quality control criteria were derived and expressed in terms of the deviation from the “consensus” mean LD_{50} (the mean of the LD_{50} values obtained by each laboratory). Estimates of inter-laboratory variance were calculated. When used in conjunction with reference toxicant data, these criteria may be used by laboratories to assess the accuracy with which they perform tests. They may also be used by regulatory authorities when assessing the quality of data to be used in hazard identification/assessment and in risk assessment of chemicals, so that excessive bias and variability can be identified.

The 48-hour EC_{50} values obtained from most of the dimethoate tests show a good level of agreement within the years. In 2014, the LD_{50} value was generally higher than in 2015 and 2016. Of the eight tests done in 2014, seven tests (88% of tests done) had values between 2 and 3.5 µg a.i./bee. Of the 28 tests done in 2015 and 2016, 19 tests (68%) had values between 0.5 and 2.0 µg a.i./bee. Four of the tests (14%) had values between 2.0 and 3.5 µg a.i./bee.

The values found in the year 2014 were higher than in the following two years. This is probably due to the fact that Tween 80 did not guarantee a proper exposure of the bees. Additionally, this may be due to the method overall becoming more accurate over time due to better handling and stronger compliance with the protocol as well as improvements within the protocol.

Overall, for the guideline, the ranges suggested serve as “targets” for judging accuracy of *Osmia* tests with dimethoate. From all 36 tests performed, the tests of 2014 should not be considered for setting this range, as the exposure was not guaranteed using Tween 80. Considering all tests done in 2015 and 2016 (using Triton X-100 and acetone), the 48-hour LD_{50} values ranged from 0.31 µg a.i./bee to 3.06 µg a.i./bee with an average of 1.14 ± 0.72 µg a.i./bee and a median of 0.94 µg a.i./bee. Looking at the tests done only in 2016, when the test protocol was closest to the proposed OECD test guideline, there was an average of 1.07 ± 0.75 µg a.i./bee and a median of 0.90 µg a.i./bee. Based on this, a value of 2.0 µg a.i./bee would be appropriate as the target 48-hour value to reach a mortality greater than 50% using dimethoate for *Osmia bicornis* as well as *Osmia cornuta*.

Discussion

Comparison with similar tests

The proposed test method is consistent with existing OECD pollinator guidelines on acute toxicity (i.e., OECD 213, 214, 246 and 247) ([9], [2], [3],[11]) and helps to address gaps in the current guidelines for pollinator testing with respect to testing solitary non-*Apis* bees. Tests on solitary bees using Mason bees may be requested by regulatory authorities when there is a concern that the honey bee is not a reasonable surrogate or when solitary bees are the more appropriate test species (*e.g*. for pollination of certain crops or specific applications during a special seasonal approach). This is also an important and indispensable approach for the protection of wild populations of pollinating insects.

As this method has a similar scope as the other pollinator guidelines on acute toxicity OECD 213, 214, 246 and 247, the costs will be in a similar range to these guidelines. This means that this test will be in a similar cost range as these tests, which are widely used and accepted to be cost effective.

The scope of this validation report includes 36 tests done over 3 years. In contrast, for most validations, much fewer tests done in only one year are considered. Additionally, the ring test included analytical verification of the test solutions used, many of them even verified by the same lab. This helped in ruling out control contamination and verifying the actual doses used, which leads to more robust data for setting of the validity criteria for the test.

Conclusions about the tested protocol

The results of these ring tests showed, that the described test method is suitable to assess the effects of plant protection products or other chemicals on solitary bees in the laboratory. As shown above, the test is robust and transferable and allows for standardisation. No highly specialised equipment was needed and the tests could be performed across eight countries without difficulties. Experience in laboratory-based toxicity tests of bees was a benefit in the beginning of the ring test, however, as the guideline became clearer on details of how to perform the test, even labs without previous experience of testing solitary/Mason bees were able to follow the protocol and to complete the test.

The test method was refined throughout the test. All tests considered here were done with females only. Although during the ring test successful trials were performed with males and results seemed to not be very different (data not shown), the protection goal is aimed towards females.

Unmated (tests performed in 2014) as well as very likely mated bees were used. As it is practically very difficult to assure bees are unmated and explicit mating times cause stress on the bees, the mating status was excluded from the guideline, as it also did not seem to make a difference and just causes a practical obstacle.

A validity criterion of ≤ 15% for the untreated control mortality was set and met by all laboratories for 48 hours. This demonstrated the feasibility and robustness of the test. For 96 hours, control mortality increased above 15% for four labs in the untreated water control and for eight labs in the solvent control. 15% was chosen as the amount of control one can have over the test material is rather limited compared to for instance honeybees and bumblebees. In the guidelines of these latter tests, a lower value of 10% is set [2] [3]. However, this would not be practically feasible for the solitary bee testing system, as control mortality in this genus is varying considerably. A higher value of 15% is shown to be feasible. This value is still within range of other NTA test guidelines and lower than the validity criterion of the Chironomus test, using lab cultured material, where 30% is still accepted [12].

Prolonging the test will always increase the risk of increasing the control mortality. However, prolonging the test to 96 hours in case of ongoing mortality is a standard procedure in the acute pollinator tests. When the test is prolonged, increased attention needs to be paid to have proper access to food, as bees not

properly feeding is probably one of the main factors leading to increased mortality when the test is prolonged.

Regarding the reference item treatment, the observations of this ring test justify the testing of only one concentration (2.0 µg dimethoate/bee) of the reference item which results in a mortality of ≥ 50% following exposure at the end of the test period. Both validity criteria can be used for a standardized test guideline.

The ring test indicated that solvents might cause a problem as solitary bees seem to be sensitive to them. A section in Annex II is dedicated in the guideline to choosing the correct wetting agent. However, the guideline will require a solvent control to be run in addition to the non-treated water control. This will show if the bees are particularly sensitive to the solvent used. Additionally, it is stated that high application volumes should only be used in absolute exceptions.

Specifically, no limitations are set on the types of feeders or cages used. This is as many different setups were tried out by different labs to actually get the bees to feed. This was one of the biggest challenges in the beginning of the test, and also explains why the guideline is set up for group housing, as survival was much better with group housing. However, as labs gain increased experience and the oral guideline is being developed, this has steadily become less of a problem.

Animal Welfare Considerations (Refinement, Reduction, and Replacement)

These considerations are not relevant to this method and ring-testing.

Practical Considerations (Evaluation of strengths and limitations of the test method)

The main strength of the test is that it helps to address gaps in the current guidelines for pollinator testing and helps with the generation of toxicity data for solitary bees, which is a matter of interest for regulatory authorities. The protocol was tested on two different species and was applicable to both species, generating very similar results for both.

Limitations of the test method might be:

- uncertainties related to the extent to which Mason bees and data generated regarding their sensitivity to chemicals are representative of other solitary non-Apis bees.
- the selectivity to the group of species, as the methodology was developed for Mason bees and results on acute contact toxicity may not be transferable to other solitary living bee species due to differences in sensitivity and breeding behaviour.
- Limited testing season due to limited flight period of the bees.

Further on limitations of this test might be its specific applicability. Acute contact toxicity testing in Mason bees may only be necessary for certain applications (certain season of the year, or to a specific crop).

References

[1] OECD (2010), OECD Survey of Pollinator Testing, Research, Mitigation and Information Management: Survey Results , Series on Pesticides No. 52, ENV/JM/MONO(2010)24.

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

[3] OECD (2017), *Test No. 246: Bumblebee, Acute Contact Toxicity Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264284104-en.

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

[5] EC, 2009. Regulation (EC) No 1107/2009 of the European parliament and of the council concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal L 309.1: 24.11.2009.

[6] Guidance for Assessing Pesticide Risks to Bees. Office of Pesticide Programs, United States Environmental Protection Agency, Washington, D.C. 20460; Health Canada Pest Management Regulatory Agency, Ottawa, ON, Canada; California Department of Pesticide Regulation, Sacramento, CA. June 23, 2014.

[7] The notification No.30-Shouan-6278, issued on March 29, 2019 by Director-General, Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries https://www.acis.famic.go.jp/eng/shinsei/6278_2nd_e.pdf.

[8] Schweizerische Eidgenossenschaft (2014). Nationaler Massnahmenplan für die Gesundheit der Bienen Bericht des Bundesrates. file:///C:/Users/U80833062/Downloads/Nationaler_Massnahmenplan_f%C3%BCr_die_Gesundheit_der_Bienen_d_final-2.pdf.

[9] Cham, M, K. de O.; Rebelo, R. M.; Olivieira, R. de P.; Ferro, A. A; Viana-Silva, F. E. de C.; Borges, L. de O.; Saretto, C. O. S. D.; Tonelli, C. A. M.; Macedo, T.C. Manual de avaliação de risco ambiental de agrotóxicos para abelhas. Brasília: Ibama/Diqua, 2020. 114 p https://www.gov.br/ibama/pt-br/assuntos/quimicos-e-biologicos/agrotoxicos/arquivos/2020-12-10-Mamual_ARA_Abelhas_2ed-Ibama.pdf .

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

[11] 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.

[12] OECD (2023), *Test No. 218: Sediment-Water Chironomid Toxicity Using Spiked Sediment*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264070264-en.

[13] Johansen, C.A., et al., Pesticides and Bees. Environmental Entomology, 1983. 12(5): p. 1513-1518.

[14] Steen, J.J.M.v.d. The effect of the size of the bumble bee (Bombus terrestris L.) on the susceptibility to the pesticide dimethoate 40%. Proceedings 7th International Symposium of the ICPBR Bee Protection Group co-organised by INRA and ACTA. 1999:213-216. INRA Editions RD 10-78026 Versailles Cedex, France. ISBN 2-7380-0966-2. 2001.

[15] Abbott, W.S., A method for computing the effectiveness of an insecticide. Jour. Econ. Entomol., 1925. 18: p. 265-267.

Definitions

Bee	Is used to describe adult female Osmia bees in this guideline
Dose	The amount of test chemical applied. Dose is expressed as mass of test chemical per test animal (µg/bee)
LD_{50}	(Median Lethal Dose) A statistically-derived single dose of a chemical that can cause death in 50% of animals when administered by dermal uptake. The LD_{50} value is given in g of test chemical per bee. For pesticides, the test chemical may either be an active ingredient (a.i.) or a formulated product containing one or more than one active ingredient
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods

Annex I. Ring test protocol

INTRODUCTION

This test guideline is a laboratory test method, designed to assess the acute contact toxicity of pesticides and other chemicals to adult solitary bees. It is based principally on the OECD guidelines for the testing of chemicals 214 [1] and Methods to determine the acute oral and contact LD_{50} of pesticides for bumble bees (*Bombus terrestris* L.) [2] and results of the discussions regarding ring testing solitary bees during the meeting of the ICPPR non-Apis testing working group, March 6th, 2014 in Niefern, Germany, February 19th, 2015 in Limburgerhof, Germany and February 29th, 2016 in Braunschweig, Germany.

INITIAL CONSIDERATIONS

In the assessment and evaluation of toxic characteristics of substances, determination of acute contact toxicity in solitary bees may be required, e.g., when exposure of these bees to a given chemical is likely. The acute contact toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals. The results of this test should be used to define the need for further evaluation. In particular, this method can be used in step-wise programmes for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments [1]. Pesticides can be tested as either active ingredients (a.i.) or as formulated products.

The effect of pesticides on solitary bees depends on the body size of the test subject. As solitary bee workers between different species, within one 'colony' of one species and between 'colonies' can have significantly different sizes and related weights, they have a different surface to volume ratio. This affects the susceptibility of these individuals to plant protection products. Smaller bees have a greater surface to volume ratio and have less weight [3] [4]. For practical reasons not the surface to volume ratio of solitary bees is assessed but instead the bees are weighed. In this way the LD_{50} can be calculated as µg PPP bee^{-1} and µg PPP gram bee^{-1} which will make the evaluation of the LD_{50} for non-apis bees more consistent.

To avoid great variation in susceptibility in one test, solitary bees of an average size / weight should be selected and tested.

The method is tested on *Osmia* sp. and may be adjusted for other solitary bees.

Definitions used are given in the Annex.

PRINCIPLE OF THE TEST

Adult female solitary bees are exposed to a range of doses of the test chemical dissolved in appropriate carrier, by direct application to the dorsal side of thorax (droplets). The test duration is at max 96 h. If the mortality rate is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e., <10%, it is appropriate to extend the duration of the test to a maximum of 96 h. Mortality is recorded daily

and compared with control values. The results are analysed in order to calculate the LD_{50} at 24, 48h, 72h, and 96h (see Annex for definitions).

Note that for this ring test the full test duration of 96h is required.

VALIDITY OF THE TEST

For a test to be valid the following conditions apply:

- The average mortality for the total number of controls should not exceed 10% at the end of the test.
- The LD_{50} of the toxic standard Dimethoate 40% meets the specified range. As solitary bees differ more in size / weight than honeybees, a larger variation in LD_{50} values can be observed. For *Osmia* the LD_{50} of Dimethoate approximates 1.5 µg a.i./bee. For other solitary bee species, the LD_{50} may be significantly different.

Note that depending on the results of the ring test the control mortality criterion might be changed to 15 to 20% in accordance to other non-target arthropod testing.

DESCRIPTION OF THE METHOD

Collection of bees

Newly emerged female bees (preferably of modal size) are selected for the test. Cocoons containing females are generally of larger size than those containing males. Do not manipulate the cocoons in order to facilitate hatching and/or sexing of the bees (i.e. do not open or cut prior to hatching). When hatching proper sized cocoons in a flight cage, any males still present will emerge earlier than the females and should be removed from the cage. Emerged females should be non-mated and meconium-free and are to be stored in the refrigerator at 5 ºC until enough bees have been collected to populate the test. Note that this can take up to 4 days since 30 bees per treatment group are required.

Number of bees per treatment group

Thirty (30) non-mated meconium-free solitary bee females.

Number of doses

Per test the bees are treated with 5 doses of the test chemical: two between the presumed LD_{100} and LD_{50}, one at the presumed LD50 and two between the presumed LD_{50} and LD_0, a negative control (in case a solvent is used) and at least three concentrations of the positive control.

Note that in the current ring test, dimethoate (positive control substance when testing other chemicals) is tested so no positive control is required in the current ring test.

Number of replicates

An acute contact LD_{50} consists of three [5] replicates in parallel to be executed as 3 x 10 bees from the same geographic pool/supplier. However, good results have also been obtained by participants using 6x5 bees. Both designs are considered adequate. At all times the origin, normal flight period in the year and wintering conditions of the cocoons of the bees used in the test should be specified in the raw data.

Note that in the current test only group housing i.e. 3x10 or 6x5 bees per replicate will be tested.

Test cages

Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g., stainless steel, wire mesh, plastic, disposable plastic cages, et cetera. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space and feeding opportunity (i.e. all individuals should have access to the sugar solution). This can be arranged by using bigger cages with multiple feeders or using less bees per cage, but increasing the number of cages per treatment level. In principle, however, groups of 10 bees per cage are tested. Provide cage enrichment like a piece of gauze and/or (filter)paper, since *Osmia* bees like to play around/have hiding places. Food should be available *ad libitum* and feeders should be placed on the ground of the test cage (*Figure 10*).

Figure 10. Some examples of test cages with feeders positioned on the ground.

Note that feeders positioned on the ground appear to work better than suspended feeders. Hence in the ring test, feeders need to be positioned on the ground. Feeders containing a reservoir with some kind of wick or cotton from which the Osmia feed give good results, but good results are also obtained with feeders equipped with a flower petal. Participants are asked to fine-tune their choice feeders with the coordinator before testing so that a balanced ring test using both feeder types can be performed.

Preparation of bees

The collected bees are anaesthetized by chilling by putting them for at least 30 minutes at 4-5 °C or using an ice bath. Cold storage can be prolonged but the amount/duration of anaesthetic used and times of exposure should be minimised. Note that using CO_2 for anaesthesia can result in mortality for *Osmia* species and should therefore be avoided. All bees are weighed before application of the test chemical to determine the average weight, standard deviation and min-max weight of animals used in the test. Moribund bees should be rejected and replaced by healthy bees before starting the test.

Preparation of doses

All test item doses will be dissolved in water. Add Triton X-100 (0.1%) as surfactant or any other low toxic surfactant which equally distributes the droplet on the bee body.

Note that in this ring test Triton X-100 should be used as a surfactant. When testing formulated products, the test chemical is dissolved in water.

Housing and keeping of the solitary bees

The bees are kept under light: dark conditions (16:8h) in a climate room at a temperature of 22 ± 2°C and a relative humidity of 60 ±10%. During the test the bees have access to sucrose solution 50% (w/w) ad libitum.

Per test cage ten bees will be housed (3x10). If using the 6x5 option, five bees per test cage will be used.

Handling and feeding conditions

Handling procedures, including treatment and observations may be conducted under (day)light.

Test item ring test

The ring test will be performed using Dimethoate 40% (e.g. Dimethoate 400 EC).

Test concentrations ring test

The proposed test-range for the ring test is: control, 0.5, 1.0, 2.0, 4.0, 8.0 µg active ingredient / bee

Note that this range has been slightly adapted, compared to the range used in 2015.

Administration of doses

Anaesthetized bees are individually treated by topical application. The bees are randomly assigned to the different test chemical doses and controls. A volume of 2 µL of solution containing the test chemical at the suitable dose should be applied with a micro-applicator to the dorsal side of the thorax of each bee between the neck and wing base. After application, the bees are allocated to test cages in groups of 10 bees and supplied with sucrose solutions 50% ad libitum.

Residue analyses test chemical

At minimum, the stock solution, the lowest, and the highest test concentrations are analysed for Dimethoate levels. Till analysis, the solution of the test chemical is stored in the freezer (-18 ºC).

PROCEDURE

Test and control groups

The number of doses and replicates tested should meet the statistical requirements for determination of LD_{50} with 95% confidence limits. Normally, five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for $LD_{50,}$ are required for the test. However, the number of doses has to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate doses (not applicable for the current dimethoate ring test).

Note that participants are asked to send in their raw data in the distributed format so that all data can be processed in an uniform manner.

A minimum of three replicate test groups, each of 10 bees, should be dosed with each test concentration (not applicable for the current dimethoate ring test).

A minimum of three replicate cages, each containing 10 bees, should be used with each test dose. Note that when 6 replicates are used, each can contain 5 bees.

EXPOSURE

Test conditions

The bees should be held under light: dark conditions (16:8h) in a climate room at a temperature of 22 ± 2°C and a relative humidity of 60 ±10%. During the test the bees have access to sucrose solution 50% (w/w) ad libitum.

Duration

The duration of the test is 96 h.

Observations

Mortality is recorded at 4 h after dosing and thereafter at 24h, 48 h, 72 h, and 96 h. All abnormal behavioural effects observed during the testing period should be recorded.

DATA AND REPORTING

Data

Data should be summarised in tabular form, showing for each treatment group, as well as control and toxic standard groups, the number of bees used, mortality at each observation time and number of bees with adverse behaviour. Analyse the mortality data by appropriate statistical methods (e.g., probit analysis, moving-average, binomial probability) [5] [6]. Plot dose-response curves at each recommended

observation time (i.e., 24h, 48h, 72h, and 96h) and calculate the slopes of the curves and the median lethal doses (LD_{50}) with 95% confidence limits. Corrections for control mortality could be made using Abbott´s correction [7] or Scheider Orelli [8]. LD_{50} should be expressed in µg of test chemical per bee and µg of test chemical per gram bee.

Note that participants are asked to send in their raw data in the distributed format so that all data can be processed in an uniform manner.

Test report

The test report should include the following information:

Test chemical:

- physical nature and relevant physical-chemical properties (e.g. stability in water, vapour pressure);
- chemical identification data, including structural formula, purity (i.e. for pesticides, the
- identity and concentration of active ingredient (s)).

Test bees:

- scientific name, race, approximate age (in weeks), collection method, date of collection;
- all relevant information on colonies used for collection of test bees, including health, any adult disease, any pre-treatment, etc.

Test conditions:

- temperature and relative humidity of experimental room;
- housing conditions including type, size and material of cages;
- methods of administration of test chemical, e.g. carrier solvent used, volume of test solution applied, anaesthetics used;
- test design, e.g. number and test doses used, number of controls; for each test dose and control, number of replicate cages and number of bees per cage;
- date of test.

Results:

- results of preliminary range-finding study if performed;
- raw data: mortality at each concentration tested at each observation time;
- graph of the dose-response curves at the end of the test;
- LD_{50} values, with 95% confidence limits, at each recommended observation time, for test chemical and toxic standard;
- statistical procedures used for determining LD_{50};
- mortality in controls;
- other biological effects observed and any abnormal responses of the bees;
- any deviation from the Test Guideline procedures and any other relevant information.

Note that participants are asked to send in their raw data in the distributed format so that all data can be processed in an uniform manner.

LITERATURE

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

2. Steen. J.J.M. van der, C. Gretenkord, and H. Schaefer. Methods to determine the acute oral and contact LD50 of pesticides for bumble bees (Bombus terrestris L.) in Proceedings ICPBR 6th Symposium on the Hazard of Pesticides to Bees 1996. Braunschweig, Germany.

3. Johansen, C.A., et al., Pesticides and Bees. Environmental Entomology, 1983. 12(5): p. 1513-1518.

4. Steen, J.J.M.v.d. The effect of the size of the solitary bee (Bombus terrestris L.) on the susceptibility to the pesticide dimethoate 40% in Proceedings 7th International Symposium of the ICPBR Bee Protection Group co-organised by INRA and ACTA 1999:213-216. INRA Editions RD 10-78026 Versailles Cedex, France. ISBN 2-7380-0966-2. 2001.

5. Finney, D.J., Probit Analysis. 3rd ed., Cambridge, London and New York. 1971, Cambridge, London and New York. 333.

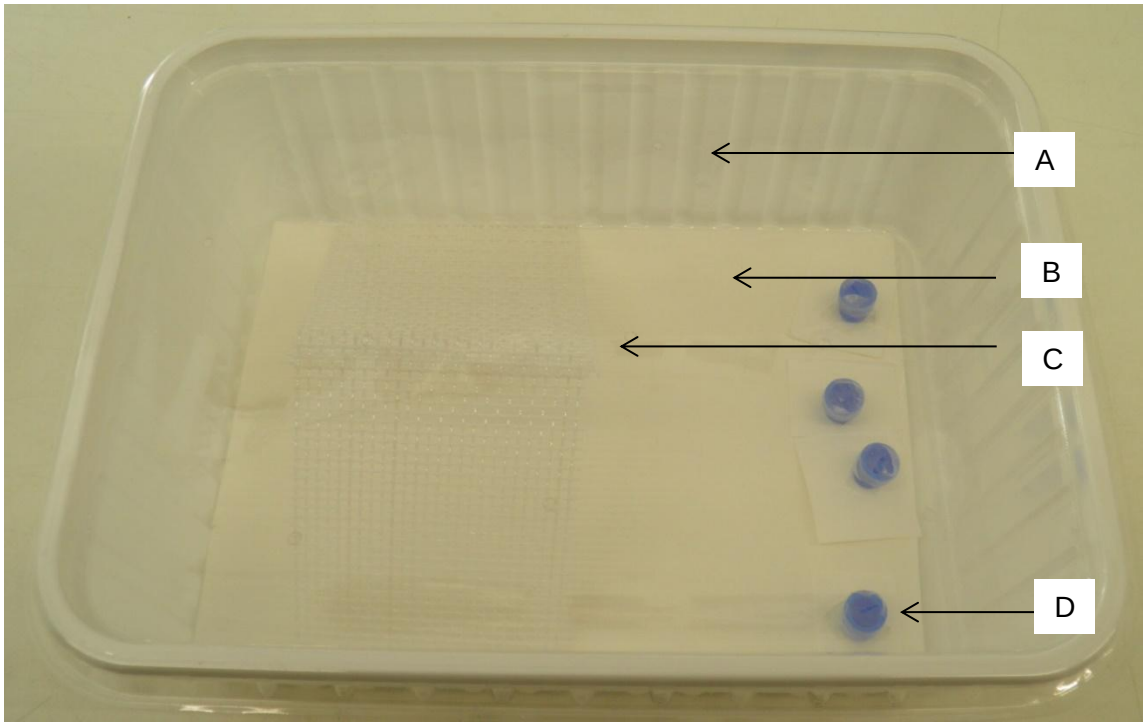
6. Litchfield, J.T. and F. Wilcoxon, A simplified method of evaluating dose-effect experiments. Journal of Pharmacology and Experimental Therapeutics, 1949. 96(2): p. 99-113.

7. Abbott, W.S., A method for computing the effectiveness of an insecticide. Jour. Econ. Entomol., 1925. 18: p. 265-267.

8. Schneider - Orelli, O., Entomologisches Praktikum : Einfuehrung in die land- und forstwirtschaftliche Insektenkunde. 1947, Aarau: Sauerlaender.

Annex II. Examples of test units

Figure 11. Examples of test units for the contact tests



A: 1 mm diameter holes to allow aeration of the test unit

B: Filter paper

C: Structures as cage enrichment

D: Feeding vial consists of a small cap covered by a septum. The septum has a hole in the middle just big enough for the bees' proboscis to enter it. The cap and septum are of blue, yellow or purple colour. More than one feeding vial may be used per box.



Annex III. Complimentary data

Table 5. Control Mortalities

Trail no.	Lab no.	Performed in		Solvent		Mortality (%)			
						48 hours		96 hours	
		Year	Month	Type	Concentration	Control	Solvent control	Control	Solvent control
1	1	2014	April	Tween 80	0.50%	n.a.	6.7	n.a.	10
2	2	2014	April	Tween 80	0.50%	n.a.	0.0	n.a.	0.0
3	3	2014	June	Tween 80	0.50%	n.a.	10	n.a.	30
4	6	2014	July	Tween 80	0.50%	3.0	10.0	3.0	10
5	7	2014	May	Tween 80	0.50%	n.a.	0.0	n.a.	0.0
6	8	2014	May	Tween 80	0.50%	n.a.	3.3	n.a.	20
7	8	2014	May	Tween 80	0.50%	n.a.	0.0	n.a.	20
8	8	2014	June	Tween 80	0.50%	n.a.	0.0	n.a.	13
9	1	2015	June	Triton-X	0.10%	0.0	0.0	6.7	0.0
10	3	2015	May	Triton-X	0.10%	3.3	3.3	3.3	6.7
11	4	2015	June	Triton-X	0.10%	3.3	0.0	23	17
12	5	2015	April	Acetone	100%	0.0	0.0	0.0	0.0
13	5	2015	April	Triton-X	0.10%	0.0	0.0	0.0	0.0
14	6	2015	June	Acetone	100%	10	10	20	13
15	7	2015	April	Acetone	100%	0.0	0.0	50	37
16	7	2015	April	Triton-X	0.10%	3.3	20	6.7	23
17	12	2015	May	Triton-X	0.10%	10	17	17	20
18	13	2015	June	Acetone	100%	3.3	3.3	3.3	3.3
19	8	2015	June	Acetone	100%	0.0	0.0	0.0	0.0
20	9	2015	September	Acetone	100%	6.7	6.7	10	10
21	10	2015	April	Acetone	100%	0.0	0.0	6.7	3.3
22	14	2015	July	Triton-X	0.10%	0.0	0.0	6.7	3.3
23	1	2016	April	Triton-X	0.10%	0.0	0.0	0.0	0.0
24	1	2016	May	Triton-X	0.10%	0.0	0.0	0.0	0.0
25	3	2016	May	Triton-X	0.10%	3.3	3.3	6.7	3.3
26	3	2016	May	Triton-X	0.10%	0.0	0.0	0.0	3.3
27	4	2016	June	Triton-X	0.10%	3.3	0.0	17	13
28	4	2016	August	Triton-X	0.10%	0.0	3.3	n.a.	n.a.
29	6	2016	July	Triton-X	0.10%	n.a.	0.0	n.a.	0.0
30	7	2016	June	Triton-X	0.10%	0.0	3.3	13	10
31	7	2016	June	Triton-X	0.10%	13	10	13	10
32	11	2016	June	Triton-X	0.10%	n.a.	0.0	n.a.	0.0
33	12	2016	June	Triton-X	0.10%	n.a.	0.0	n.a.	0.0
34	13	2016	August	Triton-X	0.10%	n.a.	3.3	n.a.	3.3
35	10	2016	April	Triton-X	0.10%	n.a.	0.0	n.a.	6.7
36	10	2016	May	Triton-X	0.10%	n.a.	3.3	n.a.	23

For further details for the respective tests, see Table 4. .

Table 6. LD_{50} values of individual tests

Trail no.	48hours										96 hours									
	Smallest dose with sublethal effects	LD_{10}			LD_{20}			LD_{50}			Smallest dose with sublethal effects	LD_{10}			LD_{20}			LD_{50}		
		Value	Lower limit	Upper limit	Value	Lower limit	Upper limit	Value	Lower limit	Upper limit		Value	Lower limit	Upper limit	Value	Lower limit	Upper limit	Value	Lower limit	Upper limit
1		1.69	1.37	2.08	1.99	1.68	2.35	2.71	2.37	3.11		1.45	1.18	1.78	1.70	1.43	2.01	2.31	2.02	2.64
2	1.25	1.70	1.38	2.09	1.99	1.68	2.36	2.71	2.37	3.10		1.13	0.56	2.25	1.37	0.78	2.42	1.98	1.26	3.11
3	5.00	0.56	0.33	0.94	0.94	0.63	1.40	2.55	1.95	3.34	2.50	0.39	0.22	0.70	0.66	0.42	1.02	1.75	1.33	2.30
4		0.49	0.37	0.63	0.58	0.47	0.71	0.80	0.69	0.93		0.46	0.35	0.61	0.55	0.44	0.69	0.78	0.67	0.91
5		1.55	1.20	2.01	1.84	1.50	2.27	2.56	2.22	2.96		1.47	1.11	1.96	1.76	1.41	2.21	2.50	2.14	2.91
6	1.25	1.47	0.58	3.74	1.95	0.91	4.16	3.34	1.83	6.11	1.25	1.16	0.46	2.96	1.56	0.73	3.32	2.72	1.50	4.93
7		1.43	0.51	3.98	2.06	0.92	4.63	4.18	2.14	8.16		0.94	0.64	1.37	1.42	1.06	1.91	3.15	2.51	3.96
8		2.17	1.67	2.81	2.77	2.25	3.42	4.44	3.75	5.25		1.12	0.80	1.57	1.61	1.24	2.11	3.25	2.64	4.01
9		0.75	0.57	1.00	0.98	0.78	1.22	1.61	1.35	1.91		0.71	0.55	0.92	0.89	0.72	1.09	1.37	1.16	1.60
10	1.25	0.32	0.19	0.54	0.47	0.30	0.71	0.98	0.76	1.27		0.22	0.11	0.45	0.32	0.18	0.56	0.65	0.46	0.90
11		0.54	0.49	0.64	0.61	0.53	0.70	0.76	0.68	0.86		0.46	0.37	0.56	0.53	0.45	0.63	0.72	0.63	0.82
12		1.98			2.09			2.32				0.89	0.71	1.13	0.10	0.91	1.34	1.66	1.42	1.93
13		1.09			1.14			1.26				0.76	0.62	0.92	0.86	0.74	1.01	1.11	0.99	1.26
14	0.63	0.63	0.52	0.76	0.72	0.62	0.85	0.95	0.84	1.08	1.25	0.49	0.37	0.63	0.58	0.47	0.71	0.80	0.69	0.93
15		0.35	0.18	0.67	0.61	0.37	1.00	1.79	1.33	2.40		0.10	0.03	0.35	0.20	0.08	0.55	0.81	0.48	1.35
16	*	0.06	0.01	0.32	0.10	0.03	0.42	0.31	0.13	0.72	0.63	0.01			0.02			0.09	0.01	0.88
17	1.25	0.34	0.15	0.76	0.45	0.24	0.86	0.76	0.47	1.24		0.30	0.14	0.65	0.39	0.21	0.72	0.64	0.40	1.00
18		0.47	0.35	0.64	0.59	0.46	0.75	0.89	0.75	1.05		0.47	0.35	0.64	0.58	0.46	0.73	0.85	0.73	1.00
19	2.50	0.79	0.61	1.02	1.00	0.81	1.23	1.55	1.32	1.82		0.53	0.41	0.70	0.65	0.53	0.81	0.96	0.82	1.12
20		0.30	0.23	0.40	0.38	0.30	0.48	0.61	0.52	0.73		0.30	0.23	0.40	0.38	0.30	0.47	0.59	0.50	0.69
21	*	0.17	0.06	0.50	0.23	0.10	0.56	0.42	0.25	0.70	0.63	0.13	0.03	0.64	0.17	0.04	0.67	0.32	0.14	0.74
22	0.63	0.93	0.66	1.31	1.34	1.02	1.75	2.68	2.18	3.30	0.63	0.75	0.52	1.08	1.09	0.82	1.45	2.22	1.80	2.74
23		0.97	0.39	2.42	1.30	0.62	2.72	2.24	1.25	4.03		0.56	0.41	0.76	0.75	0.58	0.97	1.34	1.11	1.61
24		1.66	1.31	2.10	2.05	1.69	2.48	3.06	2.62	3.56		1.12	0.87	1.44	1.43	1.16	1.76	2.28	1.93	2.69
25		0.22	0.12	0.40	0.32	0.20	0.53	0.71	0.53	0.95		0.15	0.06	0.35	0.21	0.10	0.42	0.40	0.26	0.61
26	0.50	0.52	0.41	0.66	0.63	0.52	0.77	0.93	0.80	1.08	1.00	0.21	0.12	0.39	0.29	0.18	0.46	0.50	0.38	0.66
27	1.00	0.57	0.33	0.98	0.69	0.44	1.07	0.98	0.70	1.38		0.38	0.29	0.50	0.46	0.37	0.57	0.65	0.56	0.76
28		0.38	0.26	0.56	0.53	0.39	0.72	0.97	0.79	1.20										
29	1.00	0.92	0.74	1.15	1.11	0.93	1.33	1.58	1.37	1.82		0.83	0.67	1.03	1.00	0.83	1.19	1.41	1.23	1.63
30	0.50	0.26	0.15	0.45	0.39	0.26	0.60	0.86	0.66	1.12	2.00	0.26	0.16	0.43	0.37	0.25	0.55	0.72	0.56	0.92
31		0.20	0.10	0.41	0.34	0.20	0.60	0.95	0.69	1.31	2.00	0.16	0.08	0.35	0.27	0.15	0.49	0.70	0.49	0.99
32		0.57	0.48	0.66	0.62	0.54	0.72	0.75	0.67	0.84		0.56	0.48	0.64	0.60	0.53	0.69	0.71	0.63	0.79
33		0.57	0.48	0.66	0.62	0.54	0.72	0.75	0.67	0.84		0.56	0.48	0.64	0.60	0.53	0.69	0.71	0.63	0.79
34	0.50	0.09	0.03	0.28	0.15	0.06	0.37	0.39	0.22	0.67	0.50	0.40	0.28	0.58	0.55	0.41	0.74	1.02	0.83	1.24
35	0.50	0.24	0.13	0.43	0.29	0.18	0.47	0.43	0.33	0.56		0.32			0.34			0.39		
36	0.50	0.13	0.05	0.32	0.18	0.09	0.38	0.34	0.19	0.61		0.05	0.03	0.08	0.07	0.05	0.11	0.19	0.14	0.24

*Sublethal effects were reported in the control or solvent control

For further details for the respective tests, see Table 4.