4.3.P Laboratory Acute Contact Toxicity Test with the Leafcutter Bee *Megachile rotundata*

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Abstract

So far little is known about the toxicity of Plant Protection Products (PPPs) to solitary bees other than *Osmia* spp. as well as the inter- and intra-species sensitivity differences of honey bees and solitary bees.

Megachile rotundata is a commercially bred solitary bee which is used worldwide mainly for the pollination of alfalfa. In general, bees can be exposed to PPPs directly by contact spray application (overspray) or indirectly via nectar and pollen. The leafcutter bees additionally can be exposed to (possibly) contaminated leaf pieces which are used for the building of brood cells. Therefore, contact toxicity might be of major importance within leafcutter bee species.

Acute contact toxicity tests with *M. rotundata* based on the existing honey bee testing guideline OECD No. 214 were carried out, to make a first step in the direction of the development of a standard test method and collect data for the comparison of inter- and intra-species contact toxicity sensitivity. The toxic reference substance dimethoate was used as test substance. LD50/24h values of *M. rotundata* were compared to values of *A.mellifera* generated in a similar period of time.

The low mortality observed in the control also after 96 hours, confirms the feasibility and reliability of the test method. The LD50/24h values of *M. rotundata* in all four tests were higher compared to those of *A. mellifera*. Accordingly, *M. rotundata* appeared to be slightly less sensitive to formulated dimethoate than *A. mellifera*.

Keywords: acute contact toxicity, *Megachile rotundata*, laboratory toxicity test

Introduction

The EFSA guidance document on the risk assessment of plant protection products on bees (EFSA 2013) requires testing of acute, chronic and larval toxicity of PPPs not only on honey bees, but eventually also on bumble bees and solitary bees. As a representative of solitary bees *Osmia* ssp. was chosen by the ICPPR ringtest group for the development of a suitable laboratory test method. Acute oral and contact toxicity ring tests have been conducted with *Osmia* ssp. and standard OECD test methods are on their way to be published. However, *Osmia* ssp. is only one out of hundreds of solitary bee species present in nature and the currently developed test methods do not consider the biology of all solitary bees. Therefore, to assess the risk of PPPs to solitary bees adequately, additional test methods and data on a number of solitary bee species are required.

Leaf cutting solitary bees like e.g. *M. rotundata* are building their brood cells with leaf pieces cut out of alfalfa leaves or leaves of other plant species. If these plants are contaminated with residues of PPPs, not only the adult bees but also the offspring (eggs and larvae) of leafcutter bees will be exposed to residues mainly via contact exposure.

M. rotundata was selected as a representative leafcutter bee species since it is spread in Europe, Northamerica and the northern part of Africa and because it is commercially bred and therefore, easily available.

The described test method was chosen to assess possible effects of PPPs on *M. rotundata* after contact exposure.

Materials and Methods

As test organism the leafcutter bee *Megachile rotundata* was used. Cocoons were obtained from Northstar Seed Ltd in Canada. Alltogether four tests were conducted over 2018 and 2019. For each test the cocoons were incubated at a temperature of 33°C and 50-70% relative humidity. The incubation phase took place in a hatching box (material: glass, dimensions: 30 x 30 x 40 cm) in the dark for a period of 3 to 4 weeks. Freshly hatched bees were fed with solid food Apifonda® (supplier:

Südzucker AG, Mannheim, Germany) which was daily re-moistured with a few drops of deionized water.

For the tests *M. rotundata* bees were collected out of the hatching box with tweezers under red light conditions. Bees were kept in cages made of stainless steel (base: 8 cm x 4 cm; height: 6 cm). The front side of the cages was equipped with a transparent glass to enable observation. The bottom of the cages consisted of perforated steel, which guaranteed sufficient air supply/circulation. The bottom and the side walls of the cages were lined with filter paper. Bees were kept in groups of 5 to 10 bees per cage. No fights among bees were observed.

During the test period the bees were fed with solid food offered in a small petri dish with a low rim.

The reference substance dimethoate (EC formulation: BAS 152 11 I; 400 g/L) served as test substance. The respective control groups were treated with deionized water. In one test two additional control groups were treated with pure acetone to test the suitability of acetone as a possible solvent.

In all tests the dose range of 0.06, 0.185, 0.56, 1.67, 5 µg dimethoate/bee was tested. An application volume of 1 µL per bee was used. The application solution was applied to the dorsal side of the thorax with a hand micro-applicator. In order to reduce the surface tension of the applied solution and to ensure that the drop of the test item solution spreads out immediately after application on the bees, all application solutions contained 0.1 % Triton X-100 as surfactant.

The tests were carried out with females (one test) and males (3 tests) with 3 to 5 replicates per dose level (Tab 1).

Tab. 1 Test design details

Test No.	Date	Sex of bees	No. of treatment groups	No. of replicates/ treatment group	Bees per replicates	Total number of bees/ treatment group
1	20.07.18	female bees (♀)	5	3	5	15
2	19.07.18	male bees (♂)	5	4	10	40
3	29.08.19	male bees (♂)	5	5	10	50
4	13.09.19	male bees (♂)	5	4	10	40

In 2018 two tests were carried out: one with females and one with males of *M. rotundata*. As only a small number of females had hatched in the following year, two more tests were carried out only with male bees in 2019.

Assessments on mortality were made 24, 48, 72 and 96 hours after application.

For all tests, the calculated endpoint was the LD50 at all assessment intervals. They were determined by means of Weibull and Probit analysis using linear maximum likelihood regression as well as Trimmed Spearman Karber with the statistical program ToxRat Professional 3.3.0.

As the LD_{50} value for dimethoate after 24 hours is the validity criterion for honey bee acute contact studies, the LD_{50} values after 24 hours were taken for the comparison of the sensitivity between *M. rotundata* and *A. mellifera*. Data of *A. mellifera* were generated at the same lab during the same time frame.

Results

Results are presented in Tab. 2 to Tab. 5.

Tab. 2 Results of the acute contact toxicity test with *M. rotundata*; test 1; female bees; 2018

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control)	0.0	6.7	6.7	20.0
0.06	0.0	13.3	13.3	20.0
0.19	6.7	33.3	33.3	40.0
0.56	86.7	86.7	86.7	86.7
1.67	86.7	93.3	93.3	93.3
5.00	100	100	100	100

Control: deionized water containing 0.1 % Triton X-100

Tab. 3 Results of the acute contact toxicity test with *M. rotundata*; test 2; male bees; 2018

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control)	0.0	2.5	7.5	20.0
0.06	2.5	17.5	37.5	40.0
0.19	20.0	22.5	32.5	37.5
0.56	62.5	65.0	75.0	75.0
1.67	97.5	100	100	100
5.00	100	100	100	100

Control: deionized water containing 0.1 % Triton X-100

Tab. 4 Results of the acute contact toxicity test with *M. rotundata*; test 3; male bees; 2019

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control)	0.0	0.0	0.0	0.0
0.06	0.0	0.0	0.0	0.0
0.19	8.0	8.0	8.0	16.0
0.56	40.0	46.0	60.0	70.0
1.67	74.0	82.0	84.0	88.0
5.00	100	100	100	100

Control: deionized water containing 0.1 % Triton X-100

Tab. 5 Results of the acute contact toxicity test with *M. rotundata*; test 4; male bees; 2019

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control 1)	2.5	2.5	2.5	2.5
0.00 (Control 2)	0.0	2.5	5.0	5.0
0.00 (Control 3)	0.0	2.5	5.0	7.5
0.06	0.0	5.0	15.0	20.0
0.19	15.0	30.0	30.0	30.0
0.56	67.5	77.5	77.5	85.0
1.67	90.0	97.5	100	100
5.00	100	100	100	100

Control 1: deionized water containing 0.1 % Triton X-100; Control 2 and 3: pure acetone

The control mortality ranged from 0.0 % to a maximum of 20 % after 96 hours (Fig. 1). The control mortality in all tests remained below 10 % up to the 72 hour assessment. There was no increased mortality in the acetone control groups compared to the control groups treated with deionized water containing 0.1 % Triton X-100. Therefore, control mortality after 96 hours confirmed feasibility and reliability of the test method.

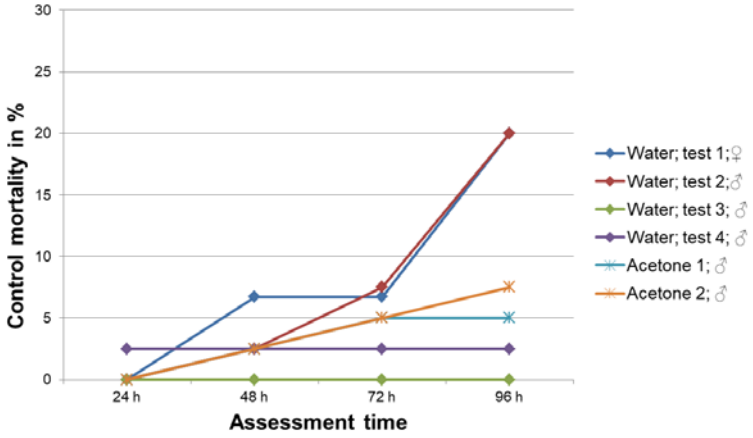

Fig. 1 Mortality of *M. rotundata* in water and acetone controls in 2018 and 2019

In all studies a clear dose response correlation was observed and LD_{50} values could be calculated for all assessment intervals in all tests (Tab. 6).

In Tab. 6 and in Fig. 2 it can be seen that the LD_{50}/24h values of *M. rotundata* were consistent between test 1, 2 and 4, whereas the LD_{50}/24h of test 3 was slightly higher (but still in the same range). According to the classification provided by EFSA (2019) the determination of these values could be done with good precision. Fig. 2 shows that the LD_{50}/24h values of *M. rotundata* were higher in all four tests compared to the LD_{50}/24h values of *A.mellifera* generated at the same lab during the same time frame.

Tab. 6 LD50 values for dimethoate after 24, 48, 72 and 96 hours in acute contact toxicity tests with *M. rotundata*

Test	**LD_{50} (95 % confidence limits) µg dimethoate/bee**			
	24h	**48h**	**72h**	**96h**
1	0.40 (0.29 to 0.55)[a]	0.32 (0.18 to 0.49)[b]	0.32 (0.18 to 0.49)[b]	0.39 (0.23 to 0.58)[b]
2	0.45 (0.35 to 0.57)[b]	0.36 (0.27 to 0.46) [b]	0.24 (0.01 to 0.59) [b]	0.30 (0.05 to 0.66) [b]
3	0.75 (0.61 to 0.94) [c]	0.65 (0.53 to 0.80) [c]	0.56 (0.45 to 0.68) [c]	0.44 (0.35 to 0.54) [c]
4	0.46 (0.37 to 0.58) [c]	0.30 (0.24 to 0.38) [c]	0.25 (0.20 to 0.32) [c]	0.22 (0.09 to 0.47) [c]

[a]Trimmed Spearman Karber
[b]Weibull analysis using linear maximum likelihood regression
[c]Probit analysis using linear maximum likelihood regression

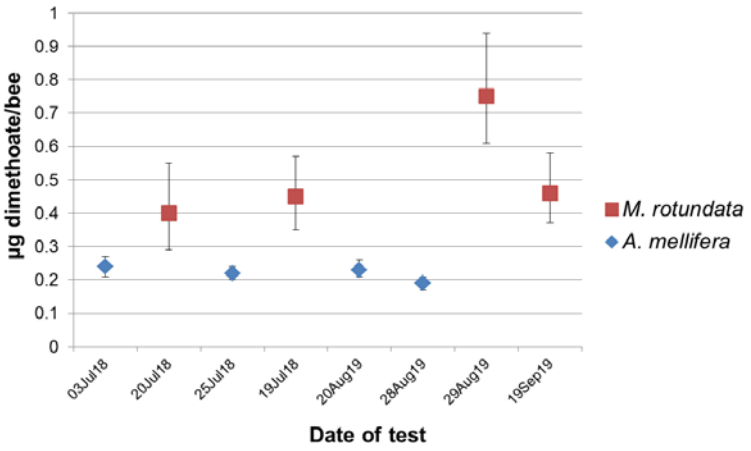

Fig. 2 LD_{50}/24h values (error bars indicate 95% confidence levels) of *M. rotundata* and *A. mellifera* determined at about the same time period 2018 and 2019

Conclusions

The mortality did not exceed 20% in all control treatments (water or acetone) with *M. rotundata* after 72 hours. The low mortality observed in the control also after 96 hours, confirms the feasibility and reliability of the test method.

The LD_{50}/24h values for formulated dimethoate in both bee species were reproducible (*A. mellifera*: 0.19 – 0.24 µg dimethoate/bee; *M. rotundata*: 0.40 – 0.75 µg dimethoate/bee) and could be determined with good precision according to the classification provided by EFSA (2019).

The LD_{50}/24h values of *M. rotundata* in all for tests were higher compared to those of *A. mellifera*. Accordingly, *M. rotundata* appeared to be slightly less sensitive to formulated dimethoate than *A. mellifera*.

Pure acetone was tolerated by *M. rotundata* and did not cause higher mortality compared to water treatment. Hence, acetone is a solvent which can be used in acute contact toxicity tests with *M. rotundata*.

References

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4.4.P Recent experiences with bumblebee (*Bombus terrestris*) semi-field tunnel testing following ICPPR Non-Apis 2016 and 2017 workshop recommendations to investigate the insecticide chlorantraniliprole

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