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## Section 2 - Non-Apis bees

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### 2.1 Higher TIER bumble bees and solitary bees recommendations for a semi-field experimental design (ICPPR Non-Apis Working group)

**Silvio Knäbe<sup>1</sup>, Matthew J. Allan<sup>2</sup>, Annika Alscher<sup>3</sup>, Kristin Amsel<sup>4</sup>, Christian Classen<sup>3</sup>, Magdaléna Cornement<sup>5</sup>, Charlotte Elston<sup>6</sup>, Nina Exeler<sup>7</sup>, Lea Franke<sup>1</sup>, Malte Frommberger<sup>8</sup>, Hervé Giffard<sup>10</sup>, Juan Sorlí Guerola<sup>11</sup>, Sabine Hecht-Rost<sup>3</sup>, Bettina Hodapp<sup>5</sup>, Ines Hotopp<sup>9</sup>, Carole Jenkins<sup>12</sup>, Tobias Jütte<sup>8</sup>, Stefan Kimmel<sup>5</sup>, Olaf Klein<sup>1</sup>, Britta Kullmann<sup>9</sup>, Johannes Lückmann<sup>3</sup>, Markus Persigehl<sup>9</sup>, Ivo Roessink<sup>13</sup>, Christof Schneider<sup>14</sup>, Alexander Schnurr<sup>4</sup>, Verena Tänzler<sup>15</sup>, Jozef J.M. van der Steen<sup>16</sup>**

<sup>1</sup>Eurofins Agrosience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany, [SilvioKnaebe@eurofins.com](mailto:SilvioKnaebe@eurofins.com)

<sup>2</sup>Atlantic Pollination Ltd., 18 North Street, Glenrothes, KY7 5NA, United Kingdom

<sup>3</sup>Rifcon GmbH, Goldbeckstr. 13, 69493 Hirschberg, Germany

<sup>4</sup>BioChem agrar GmbH, Kupferstraße 6, 04827 Machern/OT Gerichshain, Germany

<sup>5</sup>Innovative Environmental Services (IES) Ltd, Benkenstrasse 260, 4108 Witterswil, Switzerland

<sup>6</sup>Syngenta Ltd, Jealott's Hill International Research Centre Bracknell, Berkshire, RG42 6EY, United Kingdom

<sup>7</sup>Bayer CropScience, 40789 Monheim, Germany

<sup>8</sup>Institute for Bee Protection, Julius Kühn-Institut Federal Research Centre for Cultivated Plants, Messeweg 11-12, 38104 Braunschweig, Germany

<sup>9</sup>tier3 solutions GmbH, Kolberger Strasse 61-63, 51381 Leverkusen, Germany

<sup>10</sup>Testapi, 464 Sarré, Gennes, 49350 Gennes-Val-de-Loire, France

<sup>11</sup>Trialcamp SLU, Avda. Antic Regne de Valencia 25, 46290 Alcasser, Spain

<sup>12</sup>Envigo, Woolley Road, Alconbury, Huntingdon, Cambridgeshire PE28 4HS, United Kingdom

<sup>13</sup>Wageningen Environmental Research, Droevendaalsesteeg 4, 6708 PB Wageningen, The Netherlands

<sup>14</sup>BASF, 67117 Limburgerhof, Germany

<sup>15</sup>IBACON GmbH, Arheilger Weg 17, 64380 Roßdorf, Germany

<sup>16</sup>Alveus AB Consultancy, Oisterwijk, The Netherlands

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#### Abstract

The publication of the proposed EFSA risk assessment guidance document of plant protection products for pollinators highlighted that there are no study designs for non-*Apis* pollinators available. Since no official guidelines exist for semi-field testing at present, protocols were proposed by the ICPPR non-*Apis* working group and two years of ring-testing were conducted in 2016 and 2017 to develop a general test set-up. The ringtest design was based on the draft EFSA guidance document, OEPP/EPPO Guideline No. 170 and results of discussions regarding testing solitary bees and bumble bees during the meetings of the ICPPR non-*Apis* workgroup.

Ring-tests were conducted with two different test organisms, one representative of a social bumble bee species (*Bombus terrestris* L; Hymenoptera, Apidae) and one representative of a solitary bee species (*Osmia bicornis* L; Hymenoptera, Megachilidae). The species are common species in Europe, commercially available and widely used for pollination services. Several laboratories participated in the higher-tier ring tests. 15 semi-field tests were conducted with bumble bees and 16 semi-field tests were done with solitary bees in 2016 and 2017.

Two treatment groups were always included in the ringtests: an untreated control (water treated) and the treatment with dimethoate as a toxic reference item (optional other *i.e.* brood-affecting substances fenoxycarb or diflubenzuron). The toxic reference items were chosen based on their mode of action and long term experience in honey bee testing.

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A summary of the ringtest results will be given and the recommendations for the two semi-field test designs will be presented.

**Keywords:** Semi-field testing, non-*Apis* bees, bumble bees, solitary bees

### Introduction

All plant protection products have to be registered and approved under Regulation (EC) 1107/2009. The European Food Safety Authority (EFSA), published 2013 a new Draft Guidance Document on the risk assessment of plant protection products on bees (EFSA 2013 (hereafter called EFSA Bee GD)). Before the publication, only the European honey bee (Hymenoptera: Apidae - *Apis mellifera* L.), was used as a surrogate species to assess the risk of plant protection products to all insect pollinators. However, there was always controversy if this approach is protective enough to cover also other pollinator species (Heard et al. 2016). Non-*Apis* bees comprise a wide range of body sizes as well as biological and life history traits which may result in differences in sensitivity and exposure routes in comparison to honey bees. In the EFSA Bee GD it was advised to consider not only honey bees, but also bumble- and solitary bees in the plant protection product risk assessment. For solitary bees, EFSA recommends use of the closely related mason bee species *Osmia cornuta* (Latreille, 1805) and *Osmia bicornis* (Linnaeus, 1758, syn. *O. rufa* Linnaeus, 1758) (Hymenoptera: Megachilidae). But at the time of the publication of the EFSA Bee GD no suitable methods or guidelines were available to generate reliable data for the risk assessment of plant protection products (ppp) on non-*Apis* species, neither for lower-tier laboratory studies nor under more realistic conditions in higher-tier semi-field or field study situations. The lack of standardized test methods for non-*Apis* bees meant it was not possible to test the hypothesis that honey bees are a suitable surrogate organism that can be considered protective of non-*Apis* bees in the risk assessment.

To account for these data gaps and uncertainties in a regulatory context, standardized test systems were needed.

The International Commission for Plant-Pollinator Relationships (ICP-PR) established a non-*Apis* working group in 2014. It consists of experts from authorities, academia and industry and aims to develop and establish robust and reproducible test protocols to conduct standardized laboratory and semi-field tests with bumble bees and solitary bees.

First recommendations for higher tier tests with bumble bees were given in the late 1980's and 1990's by Tasei et al. (1987), Gretenkord & Drescher, (1993), Gretenkord (1997) and Sechser & Reber (1996). A comprehensive overview of ecotoxicological testing of bumble bees can also be found in Van der Steen (2001) and Tasei (2002). In the past years different test designs related to ecotoxicological field and semi-field testing were published just to name a few, by Wintermantel et al (2018), Arce et al. (2017), Scott-Dupree et al. (2017), Sterk et al. (2016), Sandrock & Candolfi (2015) and Thompson et al. (2013). Concerning higher tier studies with solitary nesting bee species reports of using *Osmia lignaria* and *O. bicornis*, *Megachile rotundata* (all Hymenoptera: Megachilidae) and *Nomia melanderi* (Hymenoptera: Halictidae) were available (Abbott et al. 2008; Alston et al. 2007; Artz, and Pitts-Singer 2015; Hodgson et al. 2011; Ladurner et al. 2008; Mayer et al. 1998; Peters et al. 2016; Ruddle et al. 2018; Rundlöf et al. 2015; Torchio, 1983). However, even though the number of studies is large, the variety of test designs and endpoints makes it difficult to compare the results. Based on preliminary work in 2014 and 2015 protocols were developed and 2016 and 2017 refined with ring testing.

### METHODS

#### Solitary bees

Ring-test studies with solitary bees were conducted in 2016 and 2017 by 9 laboratories from Germany, Switzerland and France, which performed a total of 21 studies.

As test organism the red mason bee, *O. bicornis* was selected. Additional studies with a second species, *O. cornuta* were also performed, to test if the study design would also be feasible with other

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*Osmia* species. Both species were chosen because they are polylectic species native to Europe (Peters, 1977) and cocoons can be ordered from commercial suppliers. Their natural activity begins between March and April (*O. cornuta*) or April and June (*O. bicornis*).

The life cycle of the mason bee starts each year in spring. The bees start to emerge from cocoons, in which they overwintered as imagines. Males are emerging a few days before the females (proterandry). After mating several times, the females start to build nests in pre-existing cavities using moist soil as nesting material. Each female builds up to 30 brood cells consisting of a provision of pollen mixed with nectar and a single egg (Scheuchl and Willner 2016). Only the females take care of the brood, meaning that reproductive success mainly depends on the vitality of the females.

A number of different assessments were performed to investigate lethal and sublethal effects on adult *O. bicornis* and *O. cornuta* and their brood:

Nest occupation (nesting activity): was assessed by counting the number of females occupying the cavities inside the nesting units after the end of bee flight or very early in the morning before bee flight. In this way the establishment of females before the application was monitored. After application the nest occupation was assessed in regular intervals as an indirect measure of mortality until the end of the exposure phase in the tunnels.

Flight activity: was noted shortly before the application to ensure a sufficient exposure of adult bees and directly after the application to assess sublethal effects. To assess flight activity the number of females entering the nesting cavities in a defined time interval was counted.

Cell production/reproductive performance (fecundity): was assessed by counting the number of cells built in the nesting cavities after application. This was done either by counting, photo documentation and/or marking on a transparent sheet. A cell was defined if an egg was placed on a food provision (mass of pollen and nectar) and a mud wall to seal was visible. Cells completely built or cells under construction containing pollen provisions, also with egg and/or mud wall before the application were excluded from further analysis, as developing larvae were not exposed to residues in the food provisions.

The total number of produced cells in the test item treatment was compared to the control to determine, whether the test item had an impact on the offspring population size ("cell production per nesting unit"). The reproductive performance (fecundity) of female bees was calculated as "cell production per nesting female".

Cocoon production: the development of eggs was monitored until cocoon formation and the number of cocoons was counted in autumn. Additionally, the immature mortality was calculated: immature mortality = % of dead eggs and larvae (calculated as difference of cocoon and cell production in % of total cell production per nesting unit).

Offspring production: in the following spring, after the hibernation period, the emergence success of male and female bees from overwintered cocoons was assessed. For this purpose, cocoons were incubated at 22±2°C and the number of emerged bees was determined. All emerged bees were weighed, and the sex was determined to assess potential effects on offspring weight and the sex ratio.

#### Bumble Bees

Ring-test studies with bumble bees were conducted in 2016 and 2017 by 9 laboratories from Germany, Spain, Switzerland and the United Kingdom which performed a total of 16 semi-field studies.

As a test organism the buffed tailed bumble bee, *Bombus terrestris* (Linnaeus, 1758; Hymenoptera, Apidae) was used. The species was chosen because it is polylectic species native to Europe and colonies can be ordered from commercial suppliers.

The life cycle of the buffed tailed bumble bee starts each year in spring. The queens start to build nests preferably in pre-existing soil cavities. First the foundress queen is foraging alone. After the first workers are emerged from the first brood cells they start for forage and look after the brood.

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Now the eusocial phase is running, and the queen stays in the hive. After the hive reaches maximum development males (drones) and females (queens) are produced. Queens are mating and feed until overwintering in the soil individually.

For the studies young queen right bumble bee colonies were obtained from commercial bumble bee breeders. Colonies were kept in containers, which were equipped with a nutrition system (*i.e.*, a sugar solution tank). The nutrition system was closed off or taken out so that the bees could not access it during the exposure period.

The following assessments were performed: Flight activity: the number of foragers entering and exiting the hive entrance per time interval (10 minutes) was counted during the exposure phase; assessments were conducted at the day of application once just before application to guarantee a sufficient exposure and just after application (minimum 1 hour after application) and at 1, 2 and 4 days after application to assess sub-lethal effects. Mortality: dead adult bees and dead larvae inside the hive box were counted and removed once before application and then two times per week. Weight development of colony: once before application and then two times per week the colonies were weighed. Queen production: the number of queen larvae, pupae and emerged young queens were counted and the weight of individual young queens assessed.

After deep-freezing a final brood assessment was performed and the following brood stages and observations were documented:

- Number of young queens
- Weight of young queens (individually)
- Number of egg cells
- Number of worker/drone larvae and pupae
- Number of queen larvae and pupae
- Number of workers
- Number of drones

**Results**

Based on the experiments the following recommendations are given for solitary bees in Table 1 with timelines in Figure 3.

**Tab. 1** Recommendations for a semi-field test with mason bees.

<b>Test species</b>	<b><i>Osmia</i> spp.</b>
<b>Crop</b>	<i>Brassica napus</i> , early <i>Phacelia tanacetifolia</i> (other crops are possible, <i>e.g.</i> fruit orchards)
<b>Reference item</b>	Dimethoate (75 g a.i./ha) (possible IGR: Diflubenzuron (216 g a.i./ha))
<b>Experimental unit</b>	Nesting unit with MDF trays (min. 1.5 cavities per female)
<b>Size of tunnel</b>	approx. 1 m <sup>2</sup> per female
<b>No. of replicates</b>	4
<b>Sex ratio (females:males)</b>	1 : 1.5
<b>Exposure period</b>	BBCH 59-60 (first flowers open) to BBCH 69
<b>Post-exposure period</b>	9 to 11 months
<b>Assessments (A) and endpoints (E)</b>	Nest occupation (A), flight activity (A), cell production (A), cocoon production (E), offspring production (E) (emergence success, sex ratio, weight)

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Based on the results the following recommendations for a test design with bumble bees are given in Table 2 with timelines in Figure 4.

**Tab. 2** Recommendations for a semi-field test with bumble bees

<b>Test species</b>	<b><i>Bombus</i> spp.</b>
<b>Crop</b>	<i>Phacelia tanacetifolia</i> , <i>Brassica napus</i> (other crops are possible, e.g. potato, tomato, ...)
<b>Reference item</b>	Dimethoate (800 g a.i./ha) (possible IGR: Diflubenzuron (216 g a.i./ha))
<b>Size of tunnel</b>	Minimum 30 m <sup>2</sup> crop size, better 60 m <sup>2</sup> (maximum 1 worker per m <sup>2</sup> at set-up of colonies in the tunnels; minimum should be at least 15 workers per colony)
<b>No. of replicates</b>	6
<b>Exposure period</b>	2 weeks (depending on crop)
<b>Post-exposure period</b>	approx. 4 weeks
<b>Assessments (A) and endpoints (E)</b>	Flight activity (A), mortality in hive (A), colony weight (A), queen production (E)

**Conclusions**

The recommended test design was based on experiences from different labs before starting ring-testing in 2016 and includes all available information from literature. Overall, the ring-test protocols were feasible for the majority of labs and the results improved in the second year (2017) in the labs with increasing experience. It was shown, that semi-field studies with bumble bees and solitary bees in purple tansy (*P. tanacetifolia*) or winter oil seed rape (*B. napus*) are feasible. However, success of a study strongly depends on the experience of the experimenter, on the crop quality (provision of nectar and pollen), the quality of the starting colonies/cocoons and the weather conditions. It could be observed that the availability of food (nectar and pollen) and thus the quality of the crop during the exposure phase in the tunnels is an important factor influencing the outcome of the study. If the conditions during the exposure phase are not favourable, reproduction can be very low and results are not reliable.

At the time being, dimethoate can be used as a toxic reference item, but further experience is needed on the use of the IGRs (e.g., diflubenzuron).

Reproduction of the following generation is an appropriate endpoint and can be used for both solitary bees and bumble bees.

Further research and experience are necessary to get a better understanding of what triggers and influences queen production within such a semi field set-up.

More detailed publications will be prepared by the working group and published within 2020.

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