Improved protocols for testing agrochemicals in bees

Deliverable D3.2

February 2021

Author(s)

Piotr Medrzycki[1], Sara Hellström[2*], Edward Straw[3*], Alberto Linguadoca[3*], Margret Jürison[4*], Cedric Alaux[5*], Lena Barascou[5], Mark Brown[3], Cecilia Costa[1], Pilar De la Rúa[6], Joachim Rodrigues de Miranda[7], Gennaro di Prisco[1], Eva Forsgren[7] ,Reet Karise[4], Yves Le-Conte[5], Marika Mänd[4], Vicente Martínez-López[6], Peter Neumann[8], Piero Onorati[6], Robert Paxton[2], Deborah Sene[5], Verena Strobl[8], Orlando Yanez[8]*

** These authors have contributed equally to the work*

Author affiliations

[1]CREA-AA, [2]MLU, [3]RHUL, [4]EMU, [5]INRA, [6]UM, [7]SLU and [8]UBERN

PoshBee

Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

Prepared under contract from the European Commission

Grant agreement No. 773921
EU Horizon 2020 Research and Innovation action

Project acronym:	**PoshBee**
Project full title:	**Pan-european assessment, monitoring, and mitigation of stressors on the health of bees**
Start of the project:	June 2018
Duration:	60 months
Project coordinator:	Professor Mark Brown Royal Holloway, University of London www.poshbee.eu
Deliverable title:	Improved protocols for testing agrochemicals in bees
Deliverable n°:	D3.2
Nature of the deliverable:	Demonstrator
Dissemination level:	Public
WP responsible:	WP3
Lead beneficiary:	EMU
Citation:	Medrzyck, P., Hellström, S., Straw,E., Linguadoca, A., Jürison, M., Alaux, C. et al. (2021). *Improved protocols for testing agrochemicals in bees.* Deliverable D3.2. EU Horizon 2020 PoshBee Project, Grant agreement No. 773921 .
Due date of deliverable:	Month n° 27
Actual submission date:	Month n° 33

Deliverable status:

Version	Status	Date	Author(s)
1.0	Final	February 2021	Various WP3 members, c/o Marika Mänd EMU

Table of Contents

Summary

Within the PoshBee Project, we have worked out precise protocols for testing the toxicological endpoints and metabolisation rate of agrochemicals on and in bees. Three agrochemical classes (insecticides, fungicides and herbicides) were tested on three bee groups (honey bees, bumble bees and solitary mason bees). The specific protocols, different for each combination of the pesticide class and bee group, were designed based on the existing toxicity testing methods and modified according to the specificities of the different bee groups, their castes and the different exotoxicological features of the compounds. Sulfoxaflor, Azoxystrobin, and Glyphosate were used respectively as examples of the three pesticide classes. *Apis mellifera*, *Bombus terrestris* and *Osmia bicornis* were chosen as the examples of the three bee groups.

The protocols for honey bees and bumble bees were designed based on the official existing guidelines edited by OECD. Nevertheless, given that the OECD guidelines provide recommendations to fit with most agrochemicals, many of the parameters are defined with low precision in order to be flexible and adapt to different categories of molecules. While OECD has no guidelines for testing solitary bees, we used information from the unpublished ring test protocols for *Osmia bicornis* by the ICPPR non-*Apis* working group.

Introduction

Modern agriculture has become dependent on intense use of agrochemicals with a consequent risk of negative impact on the environment. For a long time synthetic fertilizers and pesticides have assured high yields (Goklany 1998), however, we now understand better the problems related to the use of agrochemicals: pest resistance to pesticides (Sparks and Lorsbach 2017), pesticide residues in human and animal food (Malalgoda, Simsek 2021) and declines of biodiversity (Raven, Wagner, 2021). Pollinator decline is caused by multiple interacting factors, among which pesticides play an important role (Powney et al., 2019). Still, for various reasons, there are uncertainties around the extent to which pesticides contribute to bee decline. This is important for the policy makers and organizations, such as EFSA and US-EPA, that are working to enable sustainable agriculture. The EU HORIZON 2020 project PoshBee aims to contribute to the understanding of pesticide effects on bees.

Agrochemical companies, alongside non-governmental organizations and scientists, are working to find chemicals that could be effective against target pests (Damalas, Eleftherohorinos, 2011) and at the same time selective for non-target organisms (Nishimoto, 2019). For many years the western honey bee *Apis mellifera* L. has been the traditional model organism used for the assessments of pesticide effects on pollinators. More recently, the large earth bumble bee *Bombus terrestris* L has also been included in the obligatory selectivity studies. Still, there are many other bee species providing pollination services (other bumble bees, solitary bees) that need to be considered in ecotoxicological research. In order to guarantee comparable results of toxicological studies, OECD standardized test guidelines for honey bees and bumble bees are normally used. However, the protocols are designed only for workers and larvae, excluding queens and males, who are responsible for reproduction, and whose exposure to chemicals is much different compared to workers. In addition, the exposure and susceptibility can vary also between species or even subspecies.

The pesticide industry is in constant development and new compounds are designed at a regular basis. In parallel with the development and improvement of analytical methods and scientific knowledge, new evidence about environmental risks emerge. Neonicotinoids, as the most commonly sold insecticides, are already partially banned in the European Union due to their impacts on bees (Goulson, 2013). PoshBee aims to test new chemicals on three different bee groups: honey bees

(comprising its four subspecies), bumble bees and solitary bees, including all the existing castes of these insects and honey bee larvae.

To determine which chemicals the PoshBee studies would focus on, an evidence-gathering exercise took place (August 2018) to produce evidence summaries for a range of suggested chemicals. Members of PoshBee WP3 assessed a range of chemicals. The ideal candidate chemicals are those with the greatest likelihood of being used in the near future against a broad spectrum of pests and with probable high exposure risk to bees. We selected one chemical from each pesticide class. The insecticide Sulfoxaflor may replace the banned neonicotinoids, its registration to European countries is broadening and, based on previous studies, it has been suggested as having low risk to bees (but see Siviter et al. 2018). The fungicide Azoxystrobin is one of the most widely used fungicides. Its mode of action is inhibition of the respiration system, which can also affect insects, and its usage coincides with sulfoxaflor and hence might threaten bees with mixed exposure. The herbicide Glyphosate is the most commonly sold pesticide of all, while the impact on non-target organisms is still unclear and controversial. We adapted OECD guidelines to these particular chemicals and target species or castes of bees.

The PoshBee Work Package 3 (WP3) developed improved protocols for controlled laboratory approaches to identify lethal and chronic effects of chemicals on bee health, as well as their toxicokinetics in bee bodies. The detailed aims were divided into three tasks. Task 3.1 aimed to determine the dose-response relationships of Sulfoxaflor, Azoxystrobin and Glyphosate in three model bee species, including four honey bee subspecies. According to the presumed pesticide toxicity, its physical-chemical properties, the test bee species and the exposure route (contact or oral), pure active ingredients or commercial formulations were used. In the case of the latter, the formulations were based on one active ingredient only. For the determination of the toxicological endpoints the OECD protocols were used as a baseline, but important adaptations were necessary, principally when applied to queens and males. Range finding tests were carried out in order to approximately individuate the range of doses to be tested. In the case of too low toxicity (e.g. glyphosate), the limit test was used. Task 3.2 aimed to investigate the toxicokinetics of these agrochemicals in the selected model bee species, following their exposure to sub-lethal doses. The protocols for carrying out experiments for toxicokinetic were developed. Task 3.3 aims to evaluate toxicodynamics of chronic and sub-lethal exposure of agrochemicals in laboratory on bumble bees and solitary bees as well as honey bees.

References

Damalas, C. A., Eleftherohorinos, I. G. (2011). Pesticide exposure, safety issues, and risk assessment indicators. International Journal of Environmental Research and Public Health, 8(5), 1402–9.

Goklany, I. M. (1998). Saving habitat and conserving biodiversity on a crowded planet. BioScience 48, 941–953.

Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. Journal of Applied Ecology, 50, 977–987.

Malalgoda, M., Simsek, S. (2021). Pesticide residue in grain-based food: Effects on health, grain quality, and chemical properties of biomacromolecules. Cereal Chemistry, 98(1), 8–16.

Nishimoto R. (2019). Global trends in the crop protection industry. Journal of Pesticide Science, 44(3), 141–147.

Powney, G. D., Carvell, C., Edwards, M., Morris, R. K., Roy, H. E., Woodcock, B. A., Isaac, N. J. B. (2019). Widespread losses of pollinating insects in Britain. Nature Communications, 10, 1–6.

Raven, P. H., Wagner, D. L. (2021). Agricultural intensification and climate change are rapidly decreasing insect biodiversity. Proceedings of the National Academy of Sciences of the United Stated of America, 118, 2, Article Number: e2002548117

Siviter, H., Brown, M. J. F., Leadbeater, E. (2018) Sulfoxaflor exposure reduces bumblebee reproductive success. Nature 561, 109-112

Sparks, T. C., Lorsbach, B. (2017). Perspectives on the agrochemical industry and agrochemical discovery. Pest Management Science, 73, 672–677.

1. Task 3.1 Protocols: assessment of acute oral and contact LD_{50} on bees

1.1. Honey bees

Piotr Medrzycki, Gennaro Di Prisco, Cecilia Costa
Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA-AA), Bologna, Italy

For the experiments in the framework of task 3.1, the specific protocols were designed based on the official existing guidelines edited by OECD. Nevertheless, given that the OECD guidelines provide recommendations to fit with most agrochemicals, many of the parameters are defined with low precision in order to be flexible and adapt to different categories of molecules. Within the context of the PoshBee project, we have developed precise protocols that are based on the OECD guidelines, but have been adapted specifically to each of the considered agrochemicals: Sulfoxaflor, Azoxystrobin and Glyphosate.

The toxicological endpoints (LD_{50}) were assessed separately for four *Apis mellifera* subspecies: *mellifera, iberica, ligustica*, and *carnica*.

1.1.1. Assessment of acute oral LD_{50} on adult honey bees

The method is based on the OECD TG 213 (OECD, 1998a), which should be read in conjunction with the adapted protocol below. The following details are fixed:

- Flying bees (foragers) are obtained using either one of the two methods described in Williams et al. (2013), par. 4.3.3.2 or 4.3.3.3 as appropriate (Collecting flying adult workers of an undefined age for laboratory experiments using a container, or, Collecting flying adult workers of an undefined age for laboratory experiments using an entrance trap, respectively).
- Bees are anaesthetised by a CO2:air mixture (3:2 to 2:1 v/v) for a sufficient time interval, which should never exceed 1h.
- The bees are then randomly allocated to test cages.
- Each replicate (cage) contains 20 bees that originated from the same colony.
- In the exposure phase, the bees receive a feeder containing 200 microliters of test feeding solution (=10µL per bee). Caps of 2 mL Eppendorf tubes are used for this purpose.
- Once the test feed is finished (not later than 4 hours), a feeder with pure sucrose solution is provided *ad libitum*. A 5mL syringe with its tip cut away is used for this purpose.
- Three replicates are carried out with bees provided by three different colonies.
- The test temperature is 25°C ±1°C. A data logger is placed close to the test cages and the real temperature is recorded at least twice per hour.

Observations:

- The bee mortality in each cage is assessed at 24 and 48h after the exposure.

Validity criteria: the test is valid when:

- The bees have consumed all the test feed within 4h from the beginning of the exposure.
- The water and solvent (where foreseen) control mortality does not exceed 10% within 48h.
- The mortality in the toxic standard treatment is >50% at 24h.

Sulfoxaflor

- The agrochemical is tested in pure (active substance) form. No commercial formulation is used.
- The assessment of LD_{50} foresees seven increasing doses with a 2-fold difference where at least two doses are higher and two lower than the presumed LD_{50} which should be between 0.040 and 0.146µg/bee.
- Moreover, two negative controls (one with water and one with acetone) and one reference chemical (Dimethoate) are also prepared. Thus 10 cages per replicate are needed.
- The stock solution S7 is prepared by dissolving Sulfoxaflor in acetone at a rate of 6mg/mL.
- The solution S6 is prepared by diluting the S7 in acetone 1:1.
- The solution S5 is prepared by diluting the S6 in acetone 1:1.
- The solution S4 is prepared by diluting the S5 in acetone 1:1.
- The solution S3 is prepared by diluting the S4 in acetone 1:1.
- The solution S2 is prepared by diluting the S3 in acetone 1:1.
- The solution S1 is prepared by diluting the S2 in acetone 1:1.
- The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 5mg/mL.

The bees are fed with sucrose solution in distilled water (500g/L). The test feeding solutions (T1-T7, TD) are prepared by adding the relative stock solution to the feed at a rate of 1% (v/v). For solvent control 1% of acetone is added to the syrup. Water control is pure feed.

Azoxystrobin

The low solubility in water (less than 7mg/L) and low toxicity makes it impossible to assess oral LD_{50}. The oral LD_{50} of this active substance will not be assessed.
Instead, the limit test (100 micrograms of Azoxystrobin per bee) will be carried out separately for each of the four honeybee (HB) subspecies, using the commercial formulation.

- The agrochemical is tested as the commercial formulation AMISTAR which contains 250g of active ingredient per L of the formulation.
- The assessment of LD_{50} foresees five increasing doses with a 2-fold difference where at least two doses are higher and two lower than the presumed LD50, which should be below 100 µg/bee, since the limit test carried out at 100µg/bee produced a 100% morality.
- Moreover, one negative control (with water) and one reference chemical (Dimethoate) are also prepared. Thus 7 cages per replicate are needed.
- The stock solution S5 is prepared by dissolving AMISTAR in the sucrose solution (500g/L) at a rate of 4% (v/v), e.g. 1mL AMISTAR and 24mL syrup.
- The solution S4 is prepared by diluting the S5 in syrup 1:1
- The solution S3 is prepared by diluting the S4 in syrup 1:1
- The solution S2 is prepared by diluting the S3 in syrup 1:1
- The solution S1 is prepared by diluting the S2 in syrup 1:1
- The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 5mg/mL

After the exposure, the bees are fed with sucrose solution in distilled water (500g/L). The test feeding solutions (T1-T5) correspond to the stock solutions. Water control is pure feed. The reference chemical feeding solution (TD) is prepared by adding SD to the feed at a rate of 1% (v/v).

Glyphosate
The low solubility in water (about 10g/L) and low presumed toxicity make it impossible to assess oral LD_{50}. Instead, the limit test (100 µg of glyphosate per bee) is carried out, separately for each of the four HB subspecies:

- The agrochemical is tested as commercial formulation ROUNDUP PLATINUM which contains 480g of active ingredient per L of the formulation.
- Per replicate (=source hive) only one treated group is prepared.
- Moreover, one negative control (pure syrup) and one reference chemical (Dimethoate) will also be prepared. Thus 3 cages per replicate are needed.
- The stock solution (S) is ROUNDUP PLATINUM.
- The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 5mg/mL.

The bees are fed with sucrose solution in distilled water (500g/L). The test feeding solution (T) is prepared by adding S (pure ROUNDUP PLATINUM) to the sucrose solution at a rate of 2.1% (v/v). The reference chemical feeding solution (TD) is prepared by adding the relative stock solution (TD) to the feed at a rate of 1% (v/v). Water control is pure feed.

1.1.2. Assessment of acute contact LD_{50} on adult honey bees

The method is based on the OECD TG 214 (OECD, 1998b), which should be read in conjunction with the adapted protocol below. Following details are fixed:

- Flying bees (foragers) are obtained either one of the two methods described in Williams et al. (2013), par. 4.3.3.2 or 4.3.3.3 as appropriate (Collecting flying adult workers of an undefined age for laboratory experiments using a container, or, Collecting flying adult workers of an undefined age for laboratory experiments using an entrance trap, respectively).
- Bees are anaesthetised by CO2:air mixture (3:2 to 2:1 v/v) for a sufficient time interval, which should never exceed 1h.
- The bees are then randomly sampled, treated and allocated in test cages.
- Each replicate (cage) contains 20 bees originated from the same colony.
- A feeder with pure sucrose solution is provided *ad libitum* to each cage. A 5mL syringe with its tip cut away is used for this purpose.
- The bees are fed with sucrose solution in distilled water (500g/L) for the entire test duration (48h).
- Three replicates are carried out basing on the bees coming from three different colonies
- The test temperature is 25°C +/-1°C. A data logger is placed close to the test cages and the real temperature is recorded at least twice per hour.

Observations:

- The bee mortality in each cage is assessed at 24 and 48h after the exposure.

Validity criteria: the test is valid when:

- The water and solvent control mortality do not exceed 10% within 48h.
- The mortality in toxic standard is >50% at 24h.

Sulfoxaflor

- The agrochemical is tested in pure (active substance) form.

The assessment of LD_{50} foresees five increasing doses with a 2-fold difference with the presumed LD_{50} (0.38ug/bee) as the central one.

- Moreover two negative controls (one with water and one with acetone) and one reference chemical (Dimethoate) are also prepared. Thus 8 cages per replicate are needed.
- The treatment solution S5 is prepared by dissolving Sulfoxaflor in acetone at a rate of 1.5mg/mL.
- The solution S4 is prepared by diluting the S5 in acetone 1:1.
- The solution S3 is prepared by diluting the S4 in acetone 1:1.
- The solution S2 is prepared by diluting the S3 in acetone 1:1.
- The solution S1 is prepared by diluting the S2 in acetone 1:1.
- The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 0.5mg/mL.

The bees are treated individually with 1µL of treatment solution each (S1-S5, SD, water or acetone).

Azoxystrobin

The low solubility of Azoxystrobin in acetone (86g/L) does not allow for testing of doses higher than 86µg per bee. Its low toxicity makes it impossible to assess topical LD_{50}. Instead, the limit test (100 µg of Azoxystrobin per bee) is carried out separately for each of the four HB subspecies, using the commercial formulation.

- The agrochemical is tested as commercial formulation AMISTAR which contains 250g of active ingredient per L of the formulation.
- Per replicate (=source hive) only one treated group is prepared.
- Moreover, one negative control (distilled water) and one reference chemical (Dimethoate) will be also prepared. Thus 3 cages per replicate are needed.
- The treatment solution (S) is prepared by adding AMISTAR to distilled water at a rate of 40% (v/v). No wetting agent is needed since the formulation contains appropriate coadjuvants.
- The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 0.5mg/mL.

The bees are treated individually with 1µL of treatment solution each (S, SD, water)

Glyphosate

The low solubility of Glyphosate (10g/L in water) does not allow for testing of doses higher than 1µg per bee. Its low toxicity makes it impossible to assess topical LD_{50}. Instead, the limit test (100µg of Glyphosate per bee) is carried out separately for each of the four HB subspecies, using the commercial formulation.

- The agrochemical is tested as commercial formulation ROUNDUP PLATINUM which contains 480g of active ingredient per L of the formulation.
- Per replicate (=source hive) only one treated group is prepared.
- Moreover, one negative control (distilled water) and one reference chemical (Dimethoate) are also prepared. Thus 3 cages per replicate are needed.
- The treatment solution (S) is prepared by adding ROUNDUP PLATINUM to distilled water at a rate of 21% (v/v). No wetting agent is needed since the formulation contains appropriate coadjuvants.
- The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 0.5mg/mL.
- The bees are treated individually with 1µL of treatment solution each (S, SD, water).

1.1.3. Assessment of acute contact LD_{50} on honey bee larvae

The method is based on the OECD TG 237 (OECD, 2013), which should be read in conjunction with the adapted protocol below.

Following details are fixed:

- The toxic reference (Dimethoate), dissolved in water at a concentration of 2.9mg/mL is added to the diet C on D4 (day of exposure) at a rate of 10% (V/V). Alternatively the toxic reference may be dissolved in acetone at a rate of 29mg/mL and added to the diet C on D4 at a rate of 1% (V/V). The choice of one of the two methods is based on the active ingredient to be tested. Accordingly to OECD TG 237, the diet C, administered to the larvae on the day 4, 5 and 6 of the test, is prepared by mixing 50% weight of fresh royal jelly and 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose
- The grafting cells are sterilised by immersing for at least 30 minutes in ethanol.
- The source colonies providing young larvae will not be treated with synthetic acaricides for at least 3 months prior to the test. Oxalic acid and thymol are allowed until 4 weeks before the test.
- The number of required larvae for each replicate (minimum 12 per treatment) is referred to alive larvae at D4. Grafting more larvae is suggested in order to compensate for manipulation mortality.
- The solutions of Sulfoxaflor and Azoxystrobin are prepared in acetone. The test design requires both negative and solvent controls. The test diet (except for the negative control) is prepared by adding the test solution (in acetone) at a rate of 1% to the diet C.
- The solution of glyphosate is prepared in water. The test design requires one negative control only. The test diet (except for the negative control) is prepared by adding the test solution (in water) at a rate of 10% to the diet C.
- The test diets are prepared directly before exposure, i.e., the diet is prepared before the test but the test substances are added on D4.
- No warming plate is used during feeding of larvae. It is necessary to feed larvae in a warm room, with the air conditioning off.
- The agrochemical is tested in pure (active substance) form. No commercial formulation is used.
- The assessment of LD_{50} foresees five increasing doses with a 2-fold difference with the presumed LD_{50} as the central one.
- There is no data on larval LD_{50} of the tested chemicals. For this reason a range finding test is needed prior to the final test. For Glyphosate and Azoxystrobin, the dose resulting from the highest soluble concentration in the stock solution (i.e., water) is used, as well as the subsequent 4 lower doses with the fold of 5. For Sulfoxaflor a dose of 15µg/larva, as well as the subsequent 4 lower doses with the fold of 10 are used. The test diet is a dense emulsion, thus the precipitation of the test compound in the diet is of no importance.

Sulfoxaflor

- The stock solution (S5) is prepared by dissolving Sulfoxaflor in acetone at a rate of 50mg/mL.
- The solution S4 is prepared by diluting the S5 in acetone 1:10.
- The solution S3 is prepared by diluting the S4 in acetone 1:10.
- The solution S2 is prepared by diluting the S3 in acetone 1:10.
- The solution S1 is prepared by diluting the S2 in acetone 1:10.
  The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 29mg/mL.
- The test diets are prepared by adding the test solutions (S1-S5, SD) to the diet C at a range of 1% (v/v) directly prior the exposure.

- The solvent control diet is prepared by adding acetone to the diet C at a range of 1% (v/v) directly prior the exposure.
- The negative control diet is pure diet C.

Azoxystrobin

- The stock solution (S5) is prepared by dissolving Azoxystrobin in acetone at a rate of 80mg/mL.
- The solution S4 is prepared by diluting the S5 in acetone 1:5.
- The solution S3 is prepared by diluting the S4 in acetone 1:5.
- The solution S2 is prepared by diluting the S3 in acetone 1:5.
- The solution S1 is prepared by diluting the S2 in acetone 1:5.
- The SD is obtained by dissolving Dimethoate (toxic standard) in acetone at a rate of 29mg/mL.
- The test diets are prepared by adding the test solutions (S1-S5, SD) to the diet C at a range of 1% (v/v) directly prior the exposure.
- The solvent control diet is prepared by adding acetone to the diet C at a range of 1% (v/v) directly prior the exposure.
- The negative control diet is pure diet C.

Glyphosate

- The stock solution (S5) is prepared by dissolving Glyphosate in water at a rate of 10mg/mL.
- The solution S4 is prepared by diluting the S5 in water 1:5.
- The solution S3 is prepared by diluting the S4 in water 1:5.
- The solution S2 is prepared by diluting the S3 in water 1:5.
- The solution S1 is prepared by diluting the S2 in water 1:5.
- The SD is obtained by dissolving Dimethoate (toxic standard) in water at a rate of 2.9mg/mL.
- The test diets are prepared by adding the test solutions (S1-S5, SD) to the diet C at a range of 10% (v/v) directly prior the exposure.
- The solvent control diet is not required in this test.
- The negative control diet is pure diet C.

The series of 5 doses to be tested in the final test are defined basing on the results of the range-finding test.

References

OECD (1998a). Guideline for the Testing of Chemicals No. 213: Honey bee, Acute Oral Toxicity Test.

OECD (1998b). Guideline for the Testing of Chemicals No. 214: Honey bee, Acute Contact Toxicity Test.

OECD (2013). Guideline for the Testing of Chemicals No. 237: Honey bee (*Apis mellifera*) larval toxicity test, single exposure.

Williams, G. R., Alaux, C., Costa, C., Fries, I., Kuhn, R., McMahon, D. P., Medrzycki, P., Murray, T. E., Natsopoulou, M. E., Neumann, P., Oliver, R., Paxton, R. J., Pernal, S. F., Shutler, D., Tanner, G., van der Steen, J. J. M and Brodschneider, R. (2013). Standard methods for maintaining adult *Apis mellifera* in cages under in vitro laboratory conditions. Journal of Apicultural Research, 52:1, 1-36, DOI: 10.3896/IBRA.1.52.1.04.

1.2. Solitary bees *Osmia bicornis*

Sara Hellström
Martin Luther University (MLU), Halle, Germany

This test protocol is based on the test guidelines OECD 247 – Bumble bee, Acute Oral Toxicity Test, OECD 213 - Honey bee, Acute Oral Toxicity Test and OECD 2014 Honey bee, Acute Contact Toxicity Test. It contains information from the unpublished ring test protocols used for *Osmia bicornis* by the ICPPR non-*Apis* working group (Uhl et al. 2019, Roessink et al. 2018).

In the PoshBee project, WP3, task 3.1, MLU was tasked with determining dose-response for the sulfoximine insecticide Sulfoxaflor, the glycine herbicide Glyphosate and the methoxy-acrylate fungicide Azoxystrobin on the solitary Megachilid bee species *Osmia bicornis*.

Full oral and topical LD_{50} tests were done for analytic grade pure Sulfoxaflor, dissolved in acetone. In order to assess the LD_{50} value, a.k.a the Median Lethal Dose, a series of 5 doses was assessed, along with one positive control (Dimethoate) and two negative controls (water and acetone).

For the less toxic substances Azoxystrobin and Glyphosate, a Limit test was conducted, testing one high dose along with positive and negative controls. Due to solubility issues, Azoxystrobin was tested as the commercial product Amistar and Glyphosate in the commercial formulation Roundup ProActive. Males and females were tested separately. Mortality was assessed at timepoints 24, 48, 72 and 96 hours post-exposure.

Test timeline

The entire test process involves a number of steps, in order to obtain enough individuals to populate the test and to maximize the number of bees that feed on the substance in the oral exposure assays. The stages can be summarized as follows:

- Bee emergence from cocoons (2-5 days).
- “Training”, weighing and sorting bees into treatment groups (1 day).
- Administration of doses (1 day).
- Observation of survival (4 days).

The entire experiment thus takes between 7 to 11 days, if the aim is to observe mortality at timepoints 24, 48, 72 and 96 hours post-exposure.

Validity criteria

- Mortality in the control group <15% at the end of test (OEPP/EPPO, 2010).
- Mortality in the positive control group should be >50% after 24 hours.

Emergence

Cocoons of *Osmia bicornis* from commercial suppliers will arrived cooled down and are kept in a 4°C fridge to prolong hibernation until start of test. Larger cocoons generally contain females and smaller cocoons males, but there is a significant size overlap. Bees are to be taken from the 4°C fridge and placed one cocoon per vial (Eppendorf 2mL). Vials are closed with a cotton plug to prevent emerged bees from escaping. Calculate 10-20% more cocoons than needed for each experiment (i.e 240 + 50 = 290) to account for non-emerging bees and bees of the wrong sex.

Vials are placed in an incubator set to 21°C with a 16:8 photoperiod (L:D). After 24 hrs, vials are checked for emerged bees, once in the morning, and once in the afternoon. A bee is counted as emerged if the cocoon is fully chewed open. Emerged bees are to be placed back in a 4°C fridge in a petri dish marked with the date, one for males and one for females. At the end of the day, record the

number of emerged bees. This process can continue until enough bees have emerged to populate the test. Bees can be stored in a fridge for a maximum of 5 days (ideally no more than 4) before being used in tests. Bees will take longer to emerge at the start of the season (March) compared to later (April-June).

Training, weighing and sorting bees

Each treatment group should have approximately the same mean weight and similar variance. Each group should also contain an even mix of bees with different emergence dates, since this factor might affect survival. To achieve this, all bees must be weighed and sorted by rank into treatments – enough time needs to be allocated for this task. Ideally, the weighing and sorting can be done at the start of the day prior to the start of the test, after which the bees can remain in the test cages. Note the start and end time of the weighing and sorting process.

At the start of the weighing/sorting day, or the night before, place 10-20 bees in larger transparent plastic boxes to allow for defecation and flight. Let the bees remain there for at least four hours under natural light conditions. One feeder (2mL Eppendorf tube with small hole) filled with 50% v/w sugar solution is provided per cage. The feeder is fitted with a visual cue in the form of a petal (preferably yellow Brassicaceae, e.g. *Brassica rapa*), glued close to the feeder's hole. This "training" stage increases the likelihood of feeding in oral exposure tests (Hodapp & Kimmel 2018).

The bees are housed in Nicot cages during the test. The Nicot cages consist of three plastic parts and one 1mm-mesh metal net. Each cage has a number written on the side of it. The cage number will be the ID number of the bee since the bee will remain in the cage throughout the test. The hoarding cages containing bees that have been allowed to fly can be put into a fridge to sedate bees to facilitate handling. Start by putting the cage on the scale and press "tare". Wait until the scale reads 0. Put a bee inside and weigh it again. Write down the weight in mg, rounded to one decimal place in a document that allows for sorting such as Excel (Table 1). When all bees are weighed, sort the sheet in order of Weight and Date, then assign treatments accordingly. Remove any obvious light or heavy outlier bees. Using this method, all treatment groups will have equal weight distribution and equal number of bees emerged on different days.

Table 1: Basic Data Sheet for *Osmia bicornis*

Bee ID (cage no)	Weight (mg)	Day post emerged (0-5)	Treatment(A-G)
18	45.6	1	C

1.2.1. Assessment of acute oral LD_{50} on *Osmia bicornis*

Administration of oral doses

Bees that will participate in the oral test are left to starve overnight in the Nicot cages after the weighing and sorting is complete. Dosing starts the following morning under daylight conditions. For each dose, 30% extra bees are needed, since not all bees will feed. Each bee will be fed the substance in 20µL 25% sugar solution.

The feeding methodology is based on the "flower method" presented by Ladurner et. al (2005), which utilizes the bee's visual attraction to petals. The dose is presented to each individual bee in a small ampoule fastened to a piece of tape. In our case, we use the cut-off tip of a 0.2 mL collection tube. This slows down evaporation and prevents the bee from disrupting the droplet by walking into it. A

section of a petal is presented as a visual cue, adjacent to the food source. A see-through plastic cup is placed over it, such as a medicine cup or similar.

These "feeding stations" can be prepared ahead of time, as you will need one for each bee in the test. Place a droplet containing the dose inside the ampoule, and transfer the bee from the Nicot cage to the cup. Bees can be chilled in a fridge for 5-10 minutes prior, to facilitate handling. Write the ID No. of the bee next to the assigned cup. Write down the time the dose was presented to the bee. Bees are left with the dose for 3 hours. Observe the vials every 30 minutes and assess visually if the droplet is consumed or not. If the droplet has reduced to around 20%, the bee is included in the test. All bees that have not consumed after 3 hours are excluded. One dose can be administered using a staggered method, so that not all cages have to be observed simultaneously. Note the time the dose was given in the data file, and round to nearest half hour. Always leave 5 additional feeding stations containing control solution out for the full exposure time, as an evaporation control. Weigh the ampoules before and again after 3 hours to assess mean evaporation rate.

- Sulfoxaflor LD_{50} tests are run separately for males and females.
- Axoxystrobin and Glyphosate treatments can be run simultaneously, together with control groups, separately for males and females (Table 2)

Oral doses
Dilutions from stock are to be made one day prior to, or on the same day as the dosing, and stored in 4°C fridge. **IMPORTANT:** Always save at least one 2 ml sample of the **diluted stock solution and the first and last dilution step** to be used for dose validation.

Sulfoxaflor
Pre-trials have indicated that the LD_{50} value should lie between 12-19 ng/bee. We therefore test 2-fold lower and higher dilutions from this value, for both males and females (Table 2).

- Stock solution = 1000 mg/L Sulfoxaflor analytic standard dissolved in acetone, stored in 1 mL aliquots at -20°C.
- Diluted stock solution (1:1) in acetone = 500 mg/L stored in 2 mL aliquots at -20°C.
- Doses F-B are prepared by serial dilution of the diluted stock solution 1:1 in acetone.
- Dose A is half water control, half 1% acetone solvent control.
- Dose G – Positive control is Dimethoate 1 µg/bee.
- Doses A-G are mixed 1:100 with 25% w/v sugar solution, creating a sugar test solution with 1% acetone content, for example 0.5mL acetone solution in 49.5mL sugar solution.

Table 2: Sulfoxaflor oral dosing regime

Name	Dose per bee (µg/bee)	Volume (µL)	Conc test solution (mg/L)	Conc diluted stock (mg/L
A (control)	0	20	0	0
B	0.003	20	0.15625	15.625
C	0.006	20	0.3125	31.25
D	0.0125	20	0.625	62.5
E	0.025	20	1.25	125
F	0.05	20	2.5	250
G (Dimethoate)	0.5	20	2.5	250

***A*zoxystrobin**

- One LIMIT dose of 100 µg/bee is to be given.
- 40 bees per treatment group.
- Stock solution = AMISTAR 250 g/L active ingredient.
- **Stock solution mixed (1:500) with 25% sugar solution** to create test solution with 500 mg/L concentration giving 5ug/µL = 100 µg in 20 µL final dose.

Glyphosate

- One LIMIT dose of 100 µg/bee is to be given. 40 bees per treatment group.
- Stock solution = Roundup 480 g/L active ingredient.
- Stock solution mixed (1:960) with 25% sugar solution creating test solution with 500 mg/L concentration giving 5ug/µL = 100 µg in 20 µL final dose. For example 0.5 ml stock solution in 480 ml sugar solution (Table 3).

Table 3: Oral Limit test set up

Name	Dose per bee (µg/bee)	Conc (mg/L)	Volume (µL)
A (control)	0	0	20
B Glyphosate	100	5	20
C Azoxystrobin	100	5	20
G (Dimethoate)	0.5	0.025	20

Positive control (Dimethoate)

- One dose of 1 µg/bee will be given.
- Stock solution: 10 g/L in acetone.
- Diluted stock solution mixed (1:200) with 25% sugar solution to create test solution with 25 mg/L concentration giving 0.05 µg/µL = 1 µg in 20 µL final dose.

1.2.2. Assessment of acute topical LD_{50} on *Osmia bicornis*

Administration of topical doses

After weighing/sorting following step 1-4, bees can be directly put back into a 4°C fridge for around 15 minutes of cold anaesthesia, in batches of 20-30 bees at a time. Keep the bees in cages on ice and take them out one by one for dose administration, holding the anesthetized bee's legs with a tweezer. The dose is given in a 1 µL droplet placed directly on the thorax between the wing bases, using a dedicated 1-10 µL pipette. The droplet dries up quickly, and the bee can be put back in its cage, provided with sugar and placed in an incubator.

Sulfoxaflor

The LD_{50} value for honey bees is ca. 40 ng/bee (unpublished data). We therefore test 2-fold lower and higher dilutions from a slightly lower value, for both males and females (Table 3). (The validity of this scale can first be assessed in a pre-trial with 3 bees/dose.) Bees should be housed individually throughout the experiment. Since acetone is used as solvent, the solutions should be kept on ice during dosing to prevent excess evaporation.

- Stock solution = 1000 mg/L Sulfoxaflor analytic standard dissolved in acetone, stored in 1 mL aliquots at -20C.
- Dose F is created by mixing stock solution (1:8) in acetone creating a 125 mg/L test solution.
- Doses E-B are prepared by serial dilution 1:1 in acetone (Table 4).
- Dose A is half water control, half acetone control (20 bees each).
- Dose G – Positive control is Dimethoate 1 µg/bee.

Table 4: Sulfoxaflor Topical LD_{50} Doses

Name	Dose per bee (µg/bee)	Conc. (mg/L)	Volume (µL)
A (Control)	0	0	1
B	0.0078	7.8125	1
C	0.0156	15.625	1
D	0.0313	31.25	1
E	0.0625	62.5	1
F	0.1250	125	1
G (Dimethoate)	1	1000	1

Azoxystrobin (Amistar)
The non-toxicity of Azoxystrobin 100µg pure a.i has been determined in a previous Limit test, but it was re-run with Amistar commercial formulation using the herein described protocol. One LIMIT dose of 100 µg/bee is to be given with 40 bees per treatment group.

- Stock solution = AMISTAR 250 g/L active ingredient.
- Test solution is stock solution mixed (1:1.5) in water, for example 1mL AMISTAR and 1.5mL water.

Glyphosate (Roundup)
One LIMIT dose of 100 µg/bee is to be given with 40 bees per treatment group.

- Stock solution = Roundup ProActive 480 g/L active ingredient.
- Test solution is stock solution (4:6) in water = for example 2.08mL Roundup and 7.92mL water.

Positive control (Dimethoate)
Presumed *Osmia bicornis* LD_{50} topical value is 1 µg/bee (unpublished data ICPPR).

- Stock solution: 10 g/L in acetone.
- Test solution (1:10) in acetone = 1000 mg/L = 1 µg/uL.

1.2.3. Housing and observation after exposure

After dosing, bees are kept in the assigned Nicot cage throughout the test. The feeding cup is filled with 50% sugar solution using a pipette. This can be done through the net of the cage wall, without opening the cage. Place Nicot cages upright in racks, and place in an incubator (22±1 C, 40-60% humidity, 16:8 h light-dark cycle). Mortality is checked at 24 and 48 h after consumption (±1 hour). Fill cups with new sugar solution if necessary. If mortality rises by >10% between 24 and 48 hours while control mortality remains under 15%, observation should continue until 96 hrs.

1.2.4. Data Analysis

Probit analysis based on mortality result is reported as the LD_{50} value at 24, 48, 72 and 96 hours in ng/bee and ng/g body weight with 95% confidence intervals. Control mortality correction is to be performed if control mortality exceeds 10% at 48 hours post-exposure using the Schneider-Orreli formula (Medrzycki et al. 2013).

Table 5: Data Sheet for Analyses

Weight	Day post emergence	Treatment	Dead 4 h (0/1)	Dead 24h (0/1)	Dead 48h (0/1)	ID

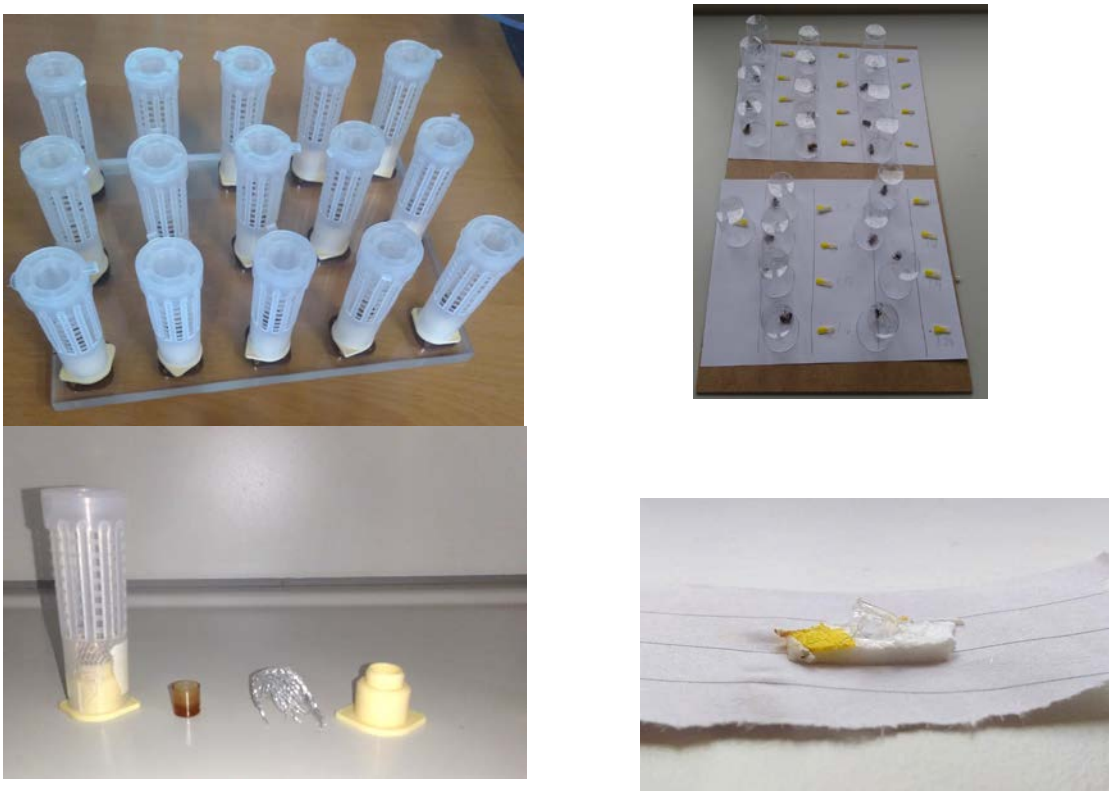

Figure 1: Pictures of setup. *Nicot individual housing setup (left and below) and feeding station using petal (right).*

References

Hodapp, B. & Kimmel, S. (2018). Oral toxicity test with solitary bees: Experiences on the acute feeding test. Hazards of pesticides to bees - 13th international symposium of the ICP-PR Bee protection group, October 18 – 20 2017, Valencia (Spain). Julius-Kühn-Archiv 462, 158-160.

Ladurner, E., Bosch, J., Kemp, W. P. & Maini, S. (2005). Evaluation of a standard artificial flower design to feed individual bees known amounts of pesticides. Apidologie 36, 379–287.

Solitary bee, Acute Contact Toxicity Test, version from 2016 (Contact) and 2017 (Oral). ICPPR workgroup non-Apis bees.

OEPP/EPPO guideline No. 170. OEPP/EPPO (2010).

Medrzycki, P. et al. (2013). Standard methods for toxicology research in *Apis mellifera*. Journal of Apicultural Research 52, 1–60.

Uhl, P., Awanbor, O., Schulz, R. S. & Brühl, C. A. (2019). Is *Osmia bicornis* an adequate regulatory surrogate? Comparing its acute contact sensitivity to *Apis mellifera*. PLoS One 14(8): e0201081. (2019).

Roessink, I., Hanewald, N., Schneider, C., Exeler, N., Schnurr, A., Molitor, A.-M., Soler, E., Kimmel, S., Molitor, C., Smagghe, G., Van der Steen, S. (2018). A method for a solitary bee (*Osmia* sp.) first tier acute contact and oral laboratory test: an update. Hazards of pesticides to bees - 13th international symposium of the ICP-PR Bee protection group, October 18 – 20 2017, Valencia (Spain) 158 Julius-Kuhn-Archiv, 462.

1.3. Bumble bees

1.3.1. Assessment of acute oral LD_{50} on bumble bees

Edward A. Straw, Alberto Linguadoca, Mark J. F. Brown
Royal Holloway University of London (RHUL), UK

This protocol is a supplement to OECD 247, a protocol for the acute oral toxicity testing of bumble bees published in 2017.

The following document is divided according to the sub-headings of OECD 247. Where an improvement is a supplement to, or contradiction of, a pre-existing point, this is noted.

Collection and randomization of bumble bees

- A day after colony delivery, 10 workers per colony should be removed and their faeces screened for micro-parasites as visible under 400x magnification. If any infections are detected, the whole source colony should be removed from the experiment.
- It is recommended for ease of access that colonies without cotton wool are purchased. Additional care should be given to the choice of test subject to avoid collecting males or callows, with supplementary red-light aiding the discrimination here.
- (Supplementation of point 13 in OECD 247) Workers should be excluded if they fall outside the range of 0.1-0.4 grams. An explicit bounding removes investigator bias and improves repeatability across organisations.
- Bumble bees should be housed a day in advance of pesticide exposure to aid in reducing deaths in the treatments owing to manual handling, not chemical exposure.
- (Contradiction of point 14 of OECD 247) Bees should not be randomly allocated to treatments, random allocation does not ensure a lack of bias and can lead to statistically significant differences in start weight between treatments. Instead, within each source colony bees should be rank ordered by weight. Then bees should be distributed to treatment groups in weight order, one treatment at a time, working down the weight list (i.e. 1^{st} heaviest to Treatment A, 2^{nd} heaviest to Treatment B, 3^{rd} heaviest to Treatment C, 4^{th} heaviest to Treatment A, 5^{th} heaviest to Treatment B etc.). This can then be repeated for all source colonies, with the 1^{st} treatment distributed to shifted by 1 (i.e. 1^{st} heaviest to Treatment B, 2^{nd} heaviest to Treatment C, 3^{rd} heaviest to Treatment A etc.). This creates an even ranked weight distribution by treatment, and an even colony distribution as required by point 25 of OECD 247.
- The interaction term treatment*colony*weight should be included in the analysis.

Preparation of test doses

- (Supplementation of point 40 in OECD 247) Where a commercial pesticide product is used, such as a formulation or adjuvant, the following information, if available, should be reported either in the main text or supplementary materials: national authorisation number (such as MAPP in the UK), producer name, producer identification number, active ingredient(s) identity, active ingredient(s) concentration(s), active ingredient(s) CAS number(s), production lot number, production date, business purchased from, listed co-formulant(s) identity, listed co-formulant(s) concentration(s), and listed co-formulant(s) CAS number(s). The aim of recording this level of detail is to enable the test to be repeated with verifiably the same substance. This level of information is required because of regional and temporal differences between products with the same name.
- Where a commercial pesticide product is used, such as a formulation or adjuvant, the following information, if available, should be reported either in the main text or supplementary materials: the values of doses trialled reported as equivalent doses of formulation (as a volume).

Test and control groups

- (Contradiction of point 23 of OECD 247) When performing a limit test, 30 bees per treatment is sufficient to allow detection of lethal effects. Using 50 bees per treatment is unnecessary and thus breaches the Replace, Reduce and Refine principle of animal testing.

Exposure (feeding)

- (Contradiction of point 28 of OECD 247) Weighing the syringes used to expose the bees to the pesticides is an unnecessary time constraint. >90% of all bees will consume the whole droplet within 4 hours. Visual confirmation of the consumption can be performed under supplemental red light. Investigators should be trained against syringes with known quantities pipetted in to help define the 80% threshold. This is more protective and accurate than the OECD measure, as only bees who consumed >80% are included, which is particularly important when working with chemicals that could elicit food avoidance.

Observations and measurements

- (Contradiction of point 36 of OECD 247) The 'sublethal' metrics of 'affected' and 'moribund' are poorly defined and of little practical purpose. The range of normal bumble bee behaviour covers 'affected' entirely, and 'moribund' bees almost always die within hours. Given the data collected using these metrics is not suitable for analysis, nor sufficiently defined to be repeatable, *ad hoc* observations are recommended instead.

Sublethal measurements (additional subheading)

- (Supplement of point 28 of OECD 247) *Ad libitum* sucrose consumption can be recorded by weighing the syringe prior to plugging it into the Nicot cage and again at the end of the experiment. This reveals any effects on appetite with minimal additional efforts, thus adding a sublethal metric to the test. For a more granular approach syringes can be weighed periodically (every 12 hours) over the course of the experiment.
- At the end of the experiment, or (for dead bees) at the first time point at which death is recorded, bees should be weighed. This allows for changes in body mass to be incorporated into the experiment with minimal additional effort. 2ml reaction tubes can be labelled and weighed, then a bee added and weighed again, to derive the weight of the bee. These reaction tubes should be moved to a freezer periodically (no longer than every 30 minutes, ideally a -80 degrees freezer) to euthanise the bees and retain them for later dissection. Depending on the time period between checks, it may be appropriate to disaggregate dead bee weights and live bee weights in statistical analyses.
- If later dissections are to occur the period between mortality checks should be no longer than every 12 hours to prevent degradation of the corpse.

Dissection (additional subheading)

- Speculative pilot work should be performed to generate *a priori* hypothesises of how the substance tested could impact the bumble bees. If impacts are seen on feeding and body mass, then dissection of the gut should occur to check for visible impacts.
- To dissect the gut out and allow for quantification of any damage, bees should be removed from the freezer in batches of 8, placed on ice and slowly allowed to defrost before dissection. The abdomen should be cut off and pinned to a black wax plate. The abdomen should then be cut on one side and pinned open. 100µL of 0.8% Ringers solution should be pipetted directly onto the gut and another 100µL onto the wax to the side of the body to prevent desiccation. The honey crop should be cut, and the gut transferred to the droplet on the wax.
- To record any visible damage a dissecting scope camera can be used to take images of the midgut at 10x-60x magnification using supplementary light. Calibration of the image scale should occur, using a standard or a measuring device.

References
OECD (2017). OECD 247 Guideline for The Testing Of Chemicals Bumble bee, Acute Oral Toxicity Test.

1.3.2. Assessment of acute contact LD_{50} on bumble bees

Margret Jürison, Reet Karise, Marika Mänd
Estonian University of Life Sciences (EMU), Tartu, Estonia

This test protocol is based on the test guidelines OECD 246 – Bumble bee, Acute Contact Toxicity Test and on the guidelines worked out by PoshBee RHUL group - Straw E.A., Linguadoca A. and Brown M.J.F. (see par. 1.3.1).

Collection and randomization of bumble bees (see also see par. 1.3.1)

- Workers should be collected from several commercially acquired medium-sized queen-right colonies, which are without cotton wool insulation and contain brood at all stages of development. Worker bumble bees should be selected by size and weight and should remain in the range of 0.1-0.4 grams.
- Males and queens can be grown from normal queen-right colonies or ordered directly form the producer. Males should be selected by weight, with a minimum weight threshold of 0.2g. Queen bumble bees should be selected by weight, with a minimum weight threshold of 0.5g.
- Bumble bees should be housed for one day in experimental conditions before pesticide exposure to reduce occasional deaths in the treatments coming from mishandling, not chemical exposure.
- All bumble bees used in the experiment must originate from several colonies, need to be weighed individually and allocated to different treatment groups by even weight to avoid any colony or individual size effect within a treatment group (see par. 1.3.1 for detailed methodology).

Exposure (contact droplet)

- Topical application can be performed under supplemental red light.
- When using acetone as a solvent, the stock and solutions should be kept on ice during handling, since acetone evaporates easily at room temperature.
- For males, a volume of 2 µl of solution containing the suitable dosage of test chemical or control material should be applied with a micro-applicator or pipette to the dorsal side of the thorax of each bumble bee. After application, the bumble bees are returned to their individual test cages and supplied with aqueous 50 % (w/v) sucrose solution *ad libitum*.
- For queens, a volume of 4 µl of solution containing the suitable dosage of test chemical or controls should be applied with a micro-applicator or pipette to the dorsal side of the thorax of each bumble bee. After application, the bumble bees are returned to their individual test cages and supplied with aqueous 50 % (w/v) sucrose solution *ad libitum*.

References
OECD (2017). OECD 246 Guideline for The Testing Of Chemicals, Acute Contact Toxicity Test.

2. Task 3.2 Protocols: toxicokinetic assessment of acute oral and contact exposure

2.1. Honey bees

Piotr Medrzycki, Gennaro Diprisco, Cecilia Costa
Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA-AA), Bologna, Italy

The objective is to expose young bees to sublethal doses of the tested chemicals and subsequently sample groups of bees at different time points following the exposure, in order to estimate the dynamics of the active ingredient's metabolisation. A test group consists of 30 bees in one cage.

- Three agrochemicals are selected for the tests: Sulfoxaflor, Azoxystrobin, Glyphosate.
- Only one *Apis mellifera* subspecies (*ligustica*) is considered.
- The toxicokinetics of the selected agrochemicals in *Apis mellifera* are assessed in adult bees (oral and contact exposure), through the following methods:

2.1.1. Toxicokinetic assessment of oral acute exposure

The method is based on OECD TG 245 (OECD, 2017), which should be read in conjunction with the adapted protocol below.

Following details are fixed:

- Newly emerged bees are obtained through the method described in Williams et al. (2013), par. 4.2.2. or 4.2.3. Brood combs taken from queen-right colonies are incubated at 33-34°C for this purpose. After one day the emerged bees are collected from the combs and distributed into the test cages. The newly emerged bees are incubated at 33 °C for about 4 days (after a hatching period of one day) and fed with sucrose solution *ad libitum*. The bees used for the test are 5 days old at the moment of the exposure.
- After the incubation period, bees are anaesthetised by CO2:air mixture (3:2 to 2:1 v/v) for a sufficient time interval, which should never exceed 1h.
- Bees are then randomly allocated to the test cages. Each cage contains 30 bees that originated from the same colony.
- The number of cages in one replicate equals the number of time points to be assessed. Three replicates are prepared for each time point. Each cage is assigned to a specific time point and replicate from the beginning of the test.
- One aliquot is then sampled for the analysis of the background (BG) level of the tested compound in the unexposed bees. Simultaneously a sample of the test syrup is collected as well, in order to carry out the chemical analyses.
- In the exposure phase, the 30 bees in each cage receive a feeder containing a total of 300 microliters of test feeding solution (=10 µL per bee). Caps of 2mL Eppendorf tubes are used for this purpose.
- Once the test feed is finished (not later than 4 hours), a feeder with pure sucrose solution is provided *ad libitum*. A 5mL syringe with its tip cut away is used for this purpose.
- After the exposure the bees are kept alive until they are sampled.
- Each sampling is carried out as follows: the feeder is withdrawn from the cage and bees must be starved for 2 hours (in order to avoid regurgitation) and then the whole cage is frozen at -

18°C or lower. After 1h the bees are moved to a 50mL falcon vial and kept frozen (-18°C or lower) until the chemical analysis.

- The oral exposure is bulk acute (10 µg of pure substance per bee), which is a necessary modification of OECD TG 245, which foresees *ad libitum* chronic exposure. In this study the acute exposure with a known amount is required.
- It is also necessary that the bees consume the test syrup quickly, which might not happen with younger bees (1-2 days, as foreseen by TG 245); for this reason 5 days old bees are used.
- Bee mortality in each cage is assessed and recorded after the exposure and each time a sample is collected.

Sulfoxaflor

- The agrochemical is tested in pure (active substance) form. No commercial formulation is used.
- The test syrup is prepared by adding a 50mg/L Sulfoxaflor solution in acetone to the sucrose solution (50%w/v), at a rate of 1%. Thus 10µL (dose per one bee) of this diet contains 5 ng of the chemical.

The testing protocol of Sulfoxaflor follows the one described above (par 2.1.1.) with some modifications:

- Due to the expected rapid metabolisation (within 72h) and the necessity to reduce the exposure time to a minimum (4 hours is not acceptable), foragers are used instead of young bees. The bees are obtained using either one of the two methods described in Williams et al. (2013), par. 4.3.3.2 or 4.3.3.3 as appropriate (Collecting flying adult workers of an undefined age for laboratory experiments using a container, or, Collecting flying adult workers of an undefined age for laboratory experiments using an entrance trap, respectively). The mentioned methods aim to collect flying bees, which assures their adequate age. In fact it was observed in past studies that young bees often show a tendency to not consume provided food quickly, but to pass different durations of time without eating.
- Sampling time points of the exposed bees are: 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h after the bees have finished the test syrup.

Azoxystrobin

- The agrochemical is tested as commercial formulation AMISTAR which contains 250g of active ingredient per L of the formulation.
- The test syrup is prepared by adding Amistar (diluted 1/100 in water) to the sucrose solution (50%w/v), at a rate of 40mL/L. Thus 10µL (dose per one bee) of this diet contains 1µg of the chemical.
- The expected metabolisation rate is slow, thus the sampling time points of the exposed bees are: 0 h, 6 d, 10 d, 14 d, 17 d, 20 d and 24 d after the bees have finished the test syrup.

Glyphosate

- The agrochemical is tested as commercial formulation ROUNDUP PLATINUM which contains 480 g of active ingredient per L of the formulation.
- The test syrup is prepared by adding Roundup Platinum (diluted 1/100 in water) to the sucrose solution (50%w/v), at a rate of 20.83 mL/L. Thus 10µL of this diet contain 1µg of the chemical.
- The expected metabolisation rate is slow, thus the sampling time points of the exposed bees are: 0 h (immediately after the exposure) 2 d, 4 d, 6 d, 8 d and 10 d after the bees have finished the test syrup.

2.1.2. Toxicokinetic assessment of contact acute exposure

This protocol is based on OECD TG 214 (OECD, 2013) purely in regards to preparation and administration of doses.

Following details are fixed:

- Newly emerged bees are obtained through the method described in Williams et al. (2013), par. 4.2.2 or 4.2.3. Brood combs taken from queen-right colonies are incubated at 33-34°C for this purpose. After one day the emerged bees are collected from the combs and distributed into the test cages. The newly emerged bees are incubated at 33 °C for about 4 days (after a hatching period of one day) and fed with sucrose solution ad libitum. The bees used for the test are 5 days old at the moment of the exposure.
- After the incubation period, bees are anaesthetised by CO2:air mixture (3:2 to 2:1 v/v) for a sufficient time interval, which should never exceed 1h.
- 1 µL of the tested chemical (solved in acetone or water at a definite concentration) is applied on the dorsal side of the thorax of each bee.
- Bees are then randomly allocated to the test cages. Each cage contains 30 bees that originated from the same colony.
- The number of cages in one replicate equals the number of time points to be assessed. Three replicates are prepared for each time point. Each cage is assigned to a specific time point and replicate from the beginning of the test.
- One aliquot is then sampled for the analysis of the background (BG) level of the tested compound in the unexposed bees. Simultaneously a sample of the test syrup is collected as well, in order to carry out the chemical analyses.
- A feeder with pure sucrose solution is provided *ad libitum* for the entire test duration. A 5mL syringe with its tip cut away is used for this purpose.
- After the treatment the bees are kept alive until they are sampled.
- Each sampling is carried out as follows: the feeder is withdrawn from the cage and bees must be starved for 2 hours (in order to avoid regurgitation) and then the whole cage is frozen at -18°C or lower. After 1h the bees are then moved to a falcon vial and kept frozen until the chemical analysis.
- Bee mortality in each cage is assessed and recorded after the exposure and each time a sample is collected.

Sulfoxaflor

- The agrochemical is tested in pure (active substance) form. No commercial formulation is used.
- Each bee is treated with 1 µL of acetone solution of Sulfoxaflor (5 mg/L) and thus exposed to 5 ng of the chemical.
- Sampling time points of the treated bees are: 0 h (immediately after the exposure) 1 h, 3 h, 5 h, 12 h, 24 h, 48 h, 72 and 96 h after the treatment.

Azoxystrobin

- The agrochemical is tested in pure (active substance) form. No commercial formulation is used.
- Each bee is treated with 1 µL of acetone solution of Azoxystrobin (1g/L) and thus exposed to 1µg of the chemical.
- The expected metabolisation rate is slow, thus the sampling time points of the treated bees are: 0 h (immediately after the exposure) 3 d, 5 d and 10 d after the treatment.

Glyphosate

- The agrochemical is tested as commercial formulation ROUNDUP PLATINUM which contains 480 g of active ingredient per L of the formulation.
- Each bee is treated with 1µL of water solution of Roundup Platinum (2.083 mL/L) and thus exposed to 1µg of the chemical. To assure correct adsorption 1% of TRITON is added to the treatment solution.
- The expected metabolisation rate is slow, thus the sampling time points of the treated bees are: 0 h (immediately after the exposure) 3 d, 5 d, 7 d and 10 d after the treatment.

References

OECD (1998). Guideline for the Testing of Chemicals No. 214: Honey bee, Acute Contact Toxicity Test

OECD (2017). Guideline for the Testing of Chemicals No. 245: Honey bee (*Apis mellifera* L.), Chronic Oral Toxicity Test (10-day feeding)

Williams, G. R., Alaux, C., Costa, C., Fries, I., Kuhn, R., McMahon, D. P., Medrzycki, P., Murray, T. E., Natsopoulou, M. E., Neumann, P., Oliver, R., Paxton, R. J., Pernal, S. F., Shutler, D., Tanner, G., van der Steen, J. J. M and Brodschneider, R. (2013). Standard methods for maintaining adult *Apis mellifera* in cages under in vitro laboratory conditions. Journal of Apicultural Research, 52:1, 1-36, DOI: 10.3896/IBRA.1.52.1.04.

2.2. Bumble bees

Margret Jürison, Reet Karise, Marika Mänd
Estonian University of Life Sciences (EMU), Tartu Estonia

The objective is to expose bumble bees to sublethal doses of the tested chemicals and subsequently sample groups of bumble bees at different time points following the exposure, in order to estimate the dynamics of the active ingredient's metabolization. The target is to collect a minimum of 2g of bee material.

- A worker test group consists of 20 individually caged bumble bees for each time point.
- A male test group consists of 20 individually caged bumble bees for each time point.
- A queen test group consists of 6 individually caged bumble bees for each time point.
- Three agrochemicals are selected for the tests: Sulfoxaflor, Azoxystrobin and Glyphosate.
- The toxicokinetics of the selected agrochemicals in *Bombus terrestris* is assessed on workers, males and queens (oral and contact exposure), through the following methods.

2.2.1. Toxicokinetic assessment of oral acute exposure

The method is based on OECD 247 (2017), which should be read in conjunction with the adapted protocol below.

The following details are fixed:

- Bumble bees are obtained from a commercial supplier.
- Bees are randomly selected from the colonies and allocated individually to the test cages.
- The bumble bees from each group for different time point sampling are evenly distributed by weight (see Section 1.3.1 above).
- The Sulfoxaflor and Azoxystrobin dosages used for toxicokinetic experiments are no observed effect level (NOEL) calculated from dose response tests.
- The glyphosate dosage for toxicokinetic experiments comes from the limit test dose.

- Each time group consists of 20 (workers, males) bees or 6 (queen) bees.
- Bumble bees are acclimatized 24 hours before the experiment in experimental conditions (25 ± 1 °C, ~60% relative humidity and permanent darkness) with feed available *ad libitum*.
- Bees need to consume the test syrup quickly.
- Each treatment is carried out as follows: the feeder is withdrawn from the cage and worker bees must be starved for 2-4 hours before exposure to the chemical feed. The queens must be starved for 6-8h before treatment. Males must be starved 4-6h before treatment.
- In the exposure phase, the bees in each cage receive a feeder containing a total of 40 microliters of test feeding solution. A 2ml syringe with its tip cut away is used for this purpose.
- If the bees do not consume the test feed within 4h, they will be excluded from the test. Only bees who consumed >80% are included.
- Once the test feed is consumed, a feeder with pure sucrose solution is provided *ad libitum*. A 5ml syringe with its tip cut away is used for this purpose.
- After the exposure the bees are kept alive until they are sampled.
- Bee mortality is assessed and recorded after the exposure every time a sample is collected.

Sulfoxaflor

- The agrochemical is tested in pure (active substance) form. No commercial formulation is used.
- Sulfoxaflor stock solution 25ml of distilled water + 10mg of Sulfoxaflor = concentration 400mg/L.

Workers:

- Tested Dose per bee 0.08µg/bee = Final concentration 2mg/L.
- The test syrup is prepared by adding Sulfoxaflor stock solution to the sucrose solution (50%w/v). Thus 40µL (dose per one bumble bee) of this diet contains 0.08µg of the chemical.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Males:

- Tested Dose per bee 0.02µg/bee = Final concentration 0.5 mg/L.
- The test syrup is prepared by adding 18.75µL Sulfoxaflor stock solution to 7481.25µl of the distilled water and 7500µL of the sucrose solution (50%w/v). Thus 40 µL (dose per one bumble bee) of this diet contains 0.02µg of the chemical.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Queens:

- Tested Dose per bee 0.18µg/bee = Final concentration mg/L.
- The test syrup is prepared by adding a 5000µL Sulfoxaflor stock solution to 5000µL of the sucrose solution (50%w/v). Thus 40 µL (dose per one bumble bee) of this diet contains 0.18 µg of the chemical.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Azoxystrobin

The agrochemical is tested as the commercial formulation Amistar which contains 250g of active ingredient (Azoxystrobin) per L of the formulation.

Workers:

- Tested Dose - NOEL dose calculated from dose response test.
- The test syrup is prepared by adding a suitable amount of Amistar to sucrose solution (50%w/v) and distilled water.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Males:

- Tested Dose per bee 80µg/bee = Final concentration 2000mg/L.
- The test syrup is prepared by adding a 120µL Amistar to 7500µL of the sucrose solution (50%w/v) and 7380µL of distilled water. Thus 40µL (dose per one bumble bee) of this diet contains 80µg of the Azoxystrobin.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Queens:

- Tested Dose per bee 350µg/bee = Final concentration 8750mg/L.
- The test syrup is prepared by adding a 350µL Amistar to 5000µL of the sucrose solution (50%w/v) and 4650µL of distilled water. Thus 40µL (dose per one bumble bee) of this diet contains 350µg of the Azoxystrobin.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Glyphosate

- The agrochemical is tested in pure (active substance) form.
- Glyphosate stock solution 25ml of distilled water + 250mg glyphosate = concentration 10000mg/L.
- Tested Dose per bee 200µg/bee = Final concentration 5000 mg/L.
- The test syrup is prepared by adding a 5000µL of glyphosate stock solution to 5000µL of the sucrose solution (50%w/v). Thus 40 µL (dose per one bumble bee) of this diet contains 200µg of the chemical
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

2.2.2. Toxicokinetic assessment of contact acute exposure

This protocol is based on OECD 246 (OCDE 2017) which should be read in conjunction with the adapted protocol below

The following details are fixed:

- Bumble bees are obtained from a commercial supplier.
- Bees are randomly selected from the colonies and allocated individually to the test cages.
- The bumble bees from each group for different time point sampling are evenly distributed by weight (see Section 1.3.1 above).
- The Sulfoxaflor dosages for toxicokinetics experiments are no observed effect level (NOEL) calculated from dose response tests.

- The Azoxystrobin and glyphosate dosage for toxicokinetic experiments come from the limit test dose.
- Each time group consists of 20 (workers, males) bees or 6 (queen) bees.
- Bumble bees are acclimatized 24 hours before the experiment in a controlled environment (25 ± 1 °C, ~60% relative humidity and permanent darkness).
- A feeder with pure sucrose solution is provided *ad libitum* for the entire test duration. A 5ml syringe with its tip cut away is used for this purpose.
- After the treatment the bees are kept alive until they are sampled.
- Bee mortality is assessed and recorded after the exposure every time a sample is collected.

Sulfoxaflor

- The agrochemical is tested in pure (active substance) form. No commercial formulation is used.
- Sulfoxaflor stock solution 0.2ml of acetone + 10mg of Sulfoxaflor = concentration 50000mg/L.

Workers:

- Tested Dose per bee 1µg/bee = Final concentration 500mg/L.
- Each bumble bee is treated with **2**µL of Sulfoxaflor solution (500 mg/L) and thus exposed to 1µg of the Sulfoxaflor. To assure correct adsorption the final treatment solution should contain 0.1% of TRITON.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Males:

- Tested Dose per bee 0.5µg/bee = Final concentration 250mg/L.
- Each bumble bee is treated with **2**µL of acetone solution of Sulfoxaflor (250 mg/L) and thus exposed to 0.5µg of the Sulfoxaflor. To assure correct adsorption the final treatment solution should contain 0.1% of TRITON.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Queens:

- Tested Dose per bee 20µg/bee = Final concentration 5000mg/L.
- Each bumble bee is treated with 4µL of acetone solution of Sulfoxaflor (5000 mg/L) and thus exposed to 20µg of the Sulfoxaflor. To assure correct adsorption the final treatment solution should contain 0.1% of TRITON.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Azoxystrobin

- The agrochemical is tested in pure (active substance) form.
- Each worker and male bee is treated with 2µL of acetone solution of Azoxystrobin (50000 mg/L) and thus exposed to 100µg of the chemical.
- Each queen bee is treated with 4µL of acetone solution of Azoxystrobin (25000 mg/L) and thus exposed to 100µg of the Azoxystrobin. To assure correct adsorption the final treatment solution should contain 0.1% of TRITON.
- Sampling time points of the treated bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have consumed the test syrup.

Roundup Flex

- The agrochemical is tested as the commercial formulation ROUNDUP FLEX which contains 450 g of active ingredient (Glyphosate) per L of the formulation.
- Each bee is treated with 2µL of water solution of Roundup Flex by adding 44.4µL of Roundup flex formulation to 155.6µL of diluted water) and thus exposed to 200µg of the glyphosate. To assure correct adsorption the final treatment solution should contain 0.1% of TRITON.
- Sampling time points of the exposed bees are: Pre-exposure (without chemical), 0 h (immediately after the exposure) 2 h, 4 h, 6 h, 8 h, 24 h, 30 h, 48 h, and 72 h after the bees have finished the test syrup.

References

OECD (2017). OECD 246 Guideline for The Testing Of Chemicals, Acute Contact Toxicity Test.

3. Task 3.3 Protocols: chronic exposure of bees to Sulfoxaflor, Glyphosate and Azoxystrobin

3.1. Chronic exposure of honey bee workers to Sulfoxaflor, Glyphosate and Azoxystrobin

Cedric Alaux, Yves Le-Conte, Lena Barascou
Institut National De La Recherche Agronomique (INRA), Paris, France

- Newly-emerged bees are collected and placed in plastic cages (30 bees per cage).
- To obtain one-day-old bees, brood frames containing late-stage pupae are removed from 8 colonies and kept overnight in an incubator under controlled conditions (34°C, 50-70% relative humidity). The next day, newly-emerged bees (less than 1 day old) are collected, mixed and placed in cages (10.5 cm x 7.5 cm x 11.5 cm). To simulate as much as possible colony rearing conditions, cages are equipped with a Beeboost® (Pherotech, Delta, BC, Canada), releasing one queen-equivalent of queen mandibular pheromone per day. Bees are provided *ad libitum* with candy (Apifonda) for 2 days and then exposed for 10 days to one of the pesticides via contaminated sugar syrup (50% sucrose). The concentrations of the pure pesticide compounds are based on environmental contaminations:
    - Sulfoxaflor (S1 : 0,01, S2 : 0,1 et S3 : 1 mg/kg)
    - Glyphosate (G1 : 5 et G2 : 30 mg/kg)
    - Azoxystrobin (A1 : 0,2 et A2 : 2 mg/kg).

We also exposed groups of bees to ternary pesticide mixtures as follows:

- M1: 0,1 mg/kg Sulfoxaflor, 5 mg/kg glyphosate 0,2 mg/kg Azoxystrobin
- M2: 0,1 mg/kg Sulfoxaflor, 30 mg/kg glyphosate, 2 mg/kg Azoxystrobin
- M3 : 0,01 mg/kg Sulfoxaflor, 5 mg/kg glyphosate, 0,2 mg/kg Azoxystrobin
- M4 : 0,01 mg/kg Sulfoxaflor, 30 mg/kg glyphosate, 2 mg/kg Azoxystrobin

- Dead bees are counted daily and removed. Daily mortality is recorded until day 33.
- Survival data from the chronic toxicity tests are analysed with a Cox proportional hazards regression model (*coxph* function of the *survival* package in R).
- The Cox model is used to calculate the Hazard Ratio (HR).

Author's contributions

PM developed honeybee protocols, SH solitary bee protocols, ES and AL bumblebee oral protocols, and MJ bumblebee contact protocols. CA elaborated the protocols for chronic exposure of honeybee worker. All authors contributed to the discussion of the protocols, and drafting the deliverable.