

2.2 Proposal for a new OECD guideline for the testing of chemicals on adult honey bees (*Apis mellifera* L.) in a 10 day chronic feeding test in the laboratory and results of the recent ring test 2014

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Abstract

Background - Even though the evaluation of potential chronic oral effects on adult honey bees (*Apis mellifera* L.) is an integral part of the risk assessment according to e.g. the EC Regulation 1107/2009 and the EFSA Guidance Document, (EFSA 2013), there is no validated guideline available for this test system, yet. To address this new requirement and to develop a new test guideline an international ring test group was founded and a ring test was carried out in summer 2014. The ring test was carried out on the basis of a test protocol, which followed the recommendation for the proposed guideline.

Results - A validity criterion for the control mortality of $\leq 15\%$ was met for the untreated control group in all tests and laboratories within the first run. However, for the solvent group this validity criterion could not be met in 7 out of 17 labs. In the reference item treatment group clear dose-response correlation could be observed with the tested concentration levels and the mean LC₅₀ and LDD₅₀ values could be calculated, as well as the NOEC and NOEDD levels.

Conclusion - The results gained in these tests indicate the suitability and reproducibility of the described test method which could serve as a basis for an official test guideline. However, the use of acetone as solvent at the tested concentration level is still questioned.

Key words: chronic toxicity, honey bee, laboratory test

1. Introduction

Recent developments in the risk assessment of plant protection products (PPP) on bees require the evaluation of potential chronic oral effects on adult honey bees (*Apis mellifera* L.). There are already publications available, describing possible methods for this new testing procedure such as Decourtye *et al.* (2005)², Suchail *et al.* (2001)⁴ and CEB (2012)¹. However, none of these procedures/methods have been ring tested and validated yet. Therefore an OECD ring test group was founded in spring 2014. In a first meeting a test protocol was agreed based on the TG OECD 213³, recent publications and the experiences of the participating labs. In summer 2014, 17 laboratories from 8 countries including two bee institutes, two industry laboratories and 13 contract labs conducted the ring test in order to harmonize the current test procedures with the objective of the development of a Test Guideline for the evaluation of the chronic toxicity of PPP's on adult honey bees in the laboratory.

2. Experimental Methods

Young adult honey bees (1 to 4 days old) from healthy, untreated colonies were used in the test. To obtain the bees for the test, brood combs containing capped cells with an expected hatch on the same day from one or more colonies were either incubated in a climatic chamber or placed into an excluder cage and returned to the hives for the hatching period. After collection without anaesthetisation, the bees were acclimatized for about one day before test start. During the acclimatisation period the bees were fed with 50 % aqueous sugar solution *ad libitum*; no additional feeding of pollen and water was supplied during acclimatization and test period.

The conditions during the hatching, acclimatisation and test period were $33 \pm 2^\circ\text{C}$ with a relative humidity of 50 – 70 %.

The cages used were well-ventilated and made of material which was either easy to clean (e.g. reusable stainless steel) or disposable.

The test design was a dose-response test with two control groups (untreated and solvent control) and five different concentrations of the reference item. The untreated control group was fed with untreated 50 % aqueous sucrose solution and the solvent control group was fed with 50 % aqueous sucrose solution containing 5 % acetone. The reference item Perfekthion / BAS 152 11 I was tested at the concentration levels of 0.2, 0.4, 0.6, 0.8 and 1.0 mg a.i. (dimethoate)/kg food. A number of 30 honey bees were tested per treatment group, divided in 3 replicates, each containing 10 bees.

The stock solution for the reference item treatment was prepared only once for the whole test period by using deionized water as solvent and stored in the refrigerator at $4\text{ }^{\circ}\text{C} \pm 4\text{ }^{\circ}\text{C}$ for up to 10 days. The final feeding solutions (control and reference item groups) were prepared at least every 4 days with 50 % aqueous sugar solution and stored in the refrigerator as well.

The treated and untreated feeding solutions were offered *ad libitum* to the test organisms via feeders introduced into each test unit (e.g. plastic syringes, approx. 10 mL). The bees in one test unit shared the feeding solution and thus received similar doses (trophallaxis). Every day the feeders containing the respective feeding solutions were replaced by fresh feeders (one application interval). The amount of feeding solution(s) consumed was determined by weighing the feeders before and after feeding, using calibrated equipment.

Mortality and behavioural abnormalities were assessed and recorded daily at about the same time of the day for a period of 10 days starting 24 ± 2 hours after start of the test period until test end.

Behavioural abnormalities such as symptoms of poisoning or any abnormal behaviour in comparison to the control were recorded according to the following categories:

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation; e.g. light or blowing; bees may recover but usually die),

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination),

c = cramps (bees contracting abdomen or entire body),

ap = apathy (bees show only low or delayed reactions to stimulation e.g. light or blowing).

v = vomiting

The consumption of feeding solution per bee was calculated by the number of living bees at start of each feeding interval and the amount of feeding solution consumed until the following day

As endpoints the LC_{50} (expressed in mg a.i./kg feeding solution) and LDD_{50} (expressed in μg a.i./bee/day) as well as the NOEC and NOEDD values based on mortality were determined for all tests.

The validity criterion for the control mortality was set to $\leq 15\%$, adopted from the validity criterion of the EPPO 170 guideline ($\leq 15\%$) and OECD TG 213 ($\leq 10\%$), by taking into consideration the prolonged test period of 10 days.

3. Results

3.1 Mortality Results of the Reference Item

At the tested concentration levels a clear dose-response correlation could be observed in the reference item treatment in all 17 laboratories. The mean mortality levels over all labs were 6.9, 37.3, 68.8, 90.2 and 98.4 % following treatment with dimethoate concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg a.i./kg feeding solutions, respectively.

Table 1 Cumulative mortality [%] in the reference item treatment group during the 10-day test period

Cumulative mortality [%]																		
Treatment [mg a.i./Kg]	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Mean
Untreated control*																		
0	6.7	0.0	6.7	0.0	3.3	0.0	3.3	0.0	0.0	6.7	0.0	0.0	3.3	0.0	3.3	0.0	0.0	2.0
Reference item: Perfekthion																		
0.2	3.3	6.7	0.0	0.0	0.0	0.0	6.7	0.0	10.0	26.7	0.0	36.7	10.0	6.7	6.7	0.0	3.3	6.9
0.4	0.0	13.3	16.7	20.0	60.0	80.0	50.0	26.7	80.0	70.0	26.7	100	10.0	36.7	13.3	0.0	30.0	37.3
0.6	40.0	40.0	73.3	56.7	93.3	100	100	33.3	100	73.3	100	100	20.0	96.7	26.7	16.7	100	68.8
0.8	100	100	100	66.7	100	100	90.6	93.3	100	100	100	100	73.3	100	76.7	33.3	100	90.2
1.0	100	100	100	100	100	100	100	100	100	100	100	100	93.3	100	100	80.0	100	98.4

* untreated control group was fed with 50 % aqueous sucrose solution

3.2 Mortality Results of the Control Group

For the untreated control, all 17 labs met the internal validity criterion of ≤ 15 % mortality within the first run. Mortality levels for the untreated control fed with pure 50 % w/v aqueous sugar solution ranged from 0.0 % to 6.7 %, resulting in a mean mortality level over all labs of 2.0 %.

Table 2 Cumulative mortality [%] in the untreated and the solvent control group during the 10-day test period

Cumulative mortality [%]																			
Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Mean	Min	Max
Untreated control*																			
6.7	0.0	6.7	0.0	3.3	0.0	3.3	0.0	0.0	6.7	0.0	0.0	3.3	0.0	3.3	0.0	0.0	2.0	0.0	6.7
Solvent control**																			
3.3	6.7	3.3	6.7	20.0	6.7	90.0	3.3	6.7	16.7	3.3	80.0	0.0	16.7	33.3	0.0	23.3	18.8	0.0	90.0

* untreated control group was fed with 50 % aqueous sucrose solution

** solvent control group was fed with 50 % aqueous sucrose solution containing 5 % acetone

Since many test items are of low water solubility a suitable solvent should be available for this kind of test. Therefore, an additional solvent control group was included in the ring test in order to show that acetone is a suitable solvent for chronic toxicity tests and that a concentration of 5 % in the final feeding solution over a period of 10 days does not harm the bees. The mortality levels in the solvent control group ranged from 0.0 % to 90.0 %, resulting in a mean value over the labs of 18.8 %. In 7 out of 17 labs the mortality was over the defined control mortality level of ≤ 15 %.

3.3 Consumption of Feeding Solution

There was a distinct difference in food consumption of the bees among the laboratories, which ranged from 27.5 to 64.0 mg/bee/day for the untreated control group. The mean value over all 17 labs was 40.9 mg/bee ± 9.2. The same was observed for the acetone control. Here, a mean value of 40.6 mg/bee ± 9.4 was found.

A clear relationship could be demonstrated between the food consumption which is resulting in a corresponding dose and the mortality.

Table 3 Mean consumption of feeding solution over the 10-day test period [mg/bee/day]

Consumption of feeding solution [mg/bee/day]																				
Treatment [mg a.i./kg]	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Mean	SD	
Untreated control	0	33.4	49.0	32.0	44.5	50.1	37.0	40.0	46.7	46.7	35.2	33.6	38.4	38.8	64.0	27.5	27.9	49.7	40.9	9.2
Solvent control**	0	32.7	44.4	34.3	40.7	58.0	36.5	58.0	39.6	42.2	42.5	39.5	50.0	33.4	50.9	34.4	19.7	34.0	40.6	9.4
Reference item: Perfekthion	0.2	33.0	40.5	28.8	35.7	42.2	33.4	36.1	39.7	42.0	36.0	26.7	39.0	31.2	46.7	29.1	29.0	34.6	35.5	5.4
	0.4	27.0	30.2	28.5	29.3	38.9	33.2	32.1	36.7	43.5	36.9	22.6	36.5	27.0	38.9	23.5	26.4	29.6	31.8	5.8
	0.6	23.8	29.3	27.8	28.0	51.2	41.2	33.4	32.6	50.2	28.6	27.6	43.3	26.6	41.7	20.5	20.6	38.0	33.2	9.2
	0.8	32.4	38.8	37.5	31.5	48.1	32.1	35.4	37.9	40.9	30.7	25.4	24.8	30.1	35.3	28.1	19.4	55.5	34.3	8.4
	1.0	23.7	30.9	34.0	35.7	41.2	32.8	41.3	40.3	50.9	31.5	24.7	36.5	29.4	35.9	26.2	18.7	49.7	34.3	8.5

* untreated control group was fed with 50 % aqueous sucrose solution

** solvent control group was fed with 50 % aqueous sucrose solution containing 5 % acetone

3.4 LC₅₀, LDD₅₀ NOEC and NOEDD

The LC₅₀ of dimethoate after 10 days was similar among the labs and ranged from 0.23 to 0.85 mg a.i./kg over the participating labs. The resulting mean LC₅₀ value was 0.48 ± 0.15 mg a.i./kg. The mean LDD₅₀ based on the mean daily uptake per bee was 0.015 µg a.i./bee ranging from 0.01 to 0.02 µg a.i./bee.

NOEC and NOEDD values could be determined for all studies. Mean NOEC for dimethoate was 0.28 ± 0.15 mg a.i./kg and the mean NOEDD was 0.009 ± 0.0026 µg a.i./bee.

Table 4 LC₅₀, LDD₅₀ and NOEC/NOEDD values of dimethoate

Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Mean	SD
LC₅₀ [mg a.i./kg]																		
0.59	0.54	0.50	0.58	0.38	0.34	0.39	0.59	0.30	0.30	0.44	0.23	0.64	0.42	0.65	0.85	0.41	0.48	0.15
Lower confidence limit																		
n.d.	n.d.	0.46	0.51	0.34	0.22	0.34	0.27	0.26	n.d.	0.41	0.20	n.d.	0.04	0.07	0.79	n.d.	0.33	0.20
Upper confidence limit																		
n.d.	n.d.	0.55	0.64	0.42	0.38	0.43	0.75	0.34	n.d.	0.51	0.40	n.d.	0.46	0.87	0.91	n.d.	0.56	0.19
NOEC [mg a.i./kg]																		
0.40	0.40	0.40	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.60	0.20	0.40	0.60	0.20	0.29	0.14
LDD₅₀ [µg a.i./bee/day]																		
0.015	0.019	0.014	0.018	0.016	0.011	0.011	0.02	0.013	0.011	0.01	0.009	0.019	0.017	0.017	0.017	0.013	0.015	0.003
Lower confidence limit																		
n.d.	0.017	0.013	0.016	0.014	n.d.	0.010	0.018	0.011	0.009	0.009	0.008	0.005	0.015	0.015	0.016	0.012	0.013	0.0036
Upper confidence limit																		
n.d.	0.021	0.016	0.020	0.018	n.d.	0.013	0.022	0.014	0.012	0.013	0.015	0.027	0.019	0.018	0.018	0.015	0.017	0.0039
NOEDD [µg a.i./bee/day]																		
0.011	0.012	0.011	0.007	0.008	0.007	0.007	0.008	0.008	0.007	0.005	0.008	0.016	0.009	0.009	0.012	0.007	0.009	0.0026
n.d. = not determined																		

3.5 Behavioural abnormalities

Related to the effects caused by the dimethoate treatment, behavioural abnormalities occurred mainly at the higher concentration/dose levels. Most of these bees were categorised as affected, apathetic or moribund. Hence, the chronic toxicity test can also be used to detect behavioural effects in a qualitative and quantitative manner.

4. Discussion

The results of the untreated control group showed that a control mortality of $\leq 15\%$ is a feasible validity criterion for this kind of tests.

However, the results of the solvent control showed a great variability over the labs and in 7 out of 17 labs the mortality was over the accepted control mortality.

By searching for the reasons of this unexpected high mortality in the solvent control a detailed look at the major parameters led to the following conclusions:

- There is no indication of a bee race-related effect
- The acetone used had a high purity in all labs and was mostly of analytical quality
- No indication of an effect related to the age of the tested bees
- No country specific reasons could be detected

In some labs a relation between the food consumption and the increased mortality was found. Four labs having the highest mean food consumption also showed an increased mortality beyond the validity criterion. Furthermore it was observed that in the respective labs the mortality did not continuously increase but started to rise mainly from day 6 onwards. These two observations led to the assumption that there could be a certain threshold for the testing of acetone in some labs. This would mean that the bees are able to metabolize the acetone up to a specific level, but are affected or die as soon as this level has been exceeded.

In the reference item treatment a clear dose-response correlation could be observed in all 17 laboratories and the LC_{50} and LDD_{50} values of dimethoate were similar among the labs after 10 days. Due to the long test period of 10 days a timely dose response correlation can be observed at concentration levels causing more than 50% mortality. As to a standardized test method, this observation justifies the testing of only one concentration of the reference item which results in a mortality of $\geq 50\%$ at the end of the test.

During the conduct of contracted studies outside the ring test, some participating labs reported severe problems concerning the solubility of technical compound in feeding solutions at higher concentration (limited solubility in acetone and water; precipitation upon dilution with sucrose solution). Some formulations tend to sediment in the feeding solution throughout one feeding interval. Therefore, it has to be guaranteed that the feeding solutions are as homogenous as possible throughout one feeding interval. To ensure this requirement, preliminary tests for solubility and homogeneity might be necessary. For most test items of low toxicity to honey bees which have to be tested in a chronic feeding test for the risk assessment the highest possible tested concentration might be dictated by a limited solubility or homogeneity in the final feeding solution.

5. Conclusions

The results of the ring test showed that the validity criterion which was set for the untreated control mortality ($\leq 15\%$) is reasonable and feasible. Regarding the reference item treatment the testing of one concentration resulting in a mortality of $\geq 50\%$ at the end of the test is justified. Both validity criteria could be used in a standardised test guideline.

Acetone can be used as a solvent as long as the above mentioned control mortality validity criterion is met.

The results gained in the untreated control group and the reference item treatment indicate that the presented method was proved as suitable to generate stable and reproducible results on possible chronic effects of PPPs or other chemicals on honey bees and the described test method could serve as a basis for an official test guideline.

Acknowledgements

Thanks to the ring test participants who worked and are still working on this project:

Anne Sindermann and Hank Krueger (Wildlife International); Claire Molitor (Testapi SARL); Claudia Volles Heimo (WBF, Zentrum für Bienenforschung); David Gladbach (Bayer CropScience AG); Dorothee Lüken (LAVES); Frank Bakker and Josep Roig (Eurofins MITOX); Julie Fourrier (ICB – VetAgroSup); Katharina Kleebaum and Saskia Ruhland (BioChem agrar GmbH); Marcus Bicker (IBACON GmbH); Ming Hua Huang and Guillermo Fernandez (Eurofins EAS); Monica Colli and Simone Venturi (Biotecnologie BT S.r.l.); Piotr Medrzycki (CRA-API); Selwyn Wilkins (Fera); Stefan Kimmel (IES Ltd); Stefanie Niederdrenk and Dagmar Sack (BASF SE), Jens Pistorius (JKI)

References

1. CEB (Commission des Essais Biologiques), 2012. Méthode d'évaluation des effets de toxicité aiguë et à court terme des préparations phytopharmaceutiques sur l'abeille domestique (*Apis mellifera* L.). AFPP method n°230.
2. DECOURTYE, A. ET AL., 2005: Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honey bee *Apis mellifera*. *Archives of Environmental Contamination and Toxicology*, 48, 242-250.
3. OECD 213, 1998 Guideline for the testing of chemicals; Honey bees; acute oral toxicity test.
4. SUCHAIL, S., GUEZ, D. AND BELZUNCES, L.P., (2001): Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environmental Toxicology and Chemistry*, 20, 2482-2486.