Institut für
Biologische Analytik und
Consulting IBACON GmbH

Final Report

Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 3772 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae)

(GLP compliant study based on EPPO 170 (1992))

Author: Dipl. Biol. [redacted]

Study Completion Date: August 26, 1999

Sponsor

Bayer AG
Crop Protection Division
Institute for Environmental Biology
Alfred-Nobel-Str. 50
40789 Monheim
Germany

Test Facility

Institut für Biologische Analytik
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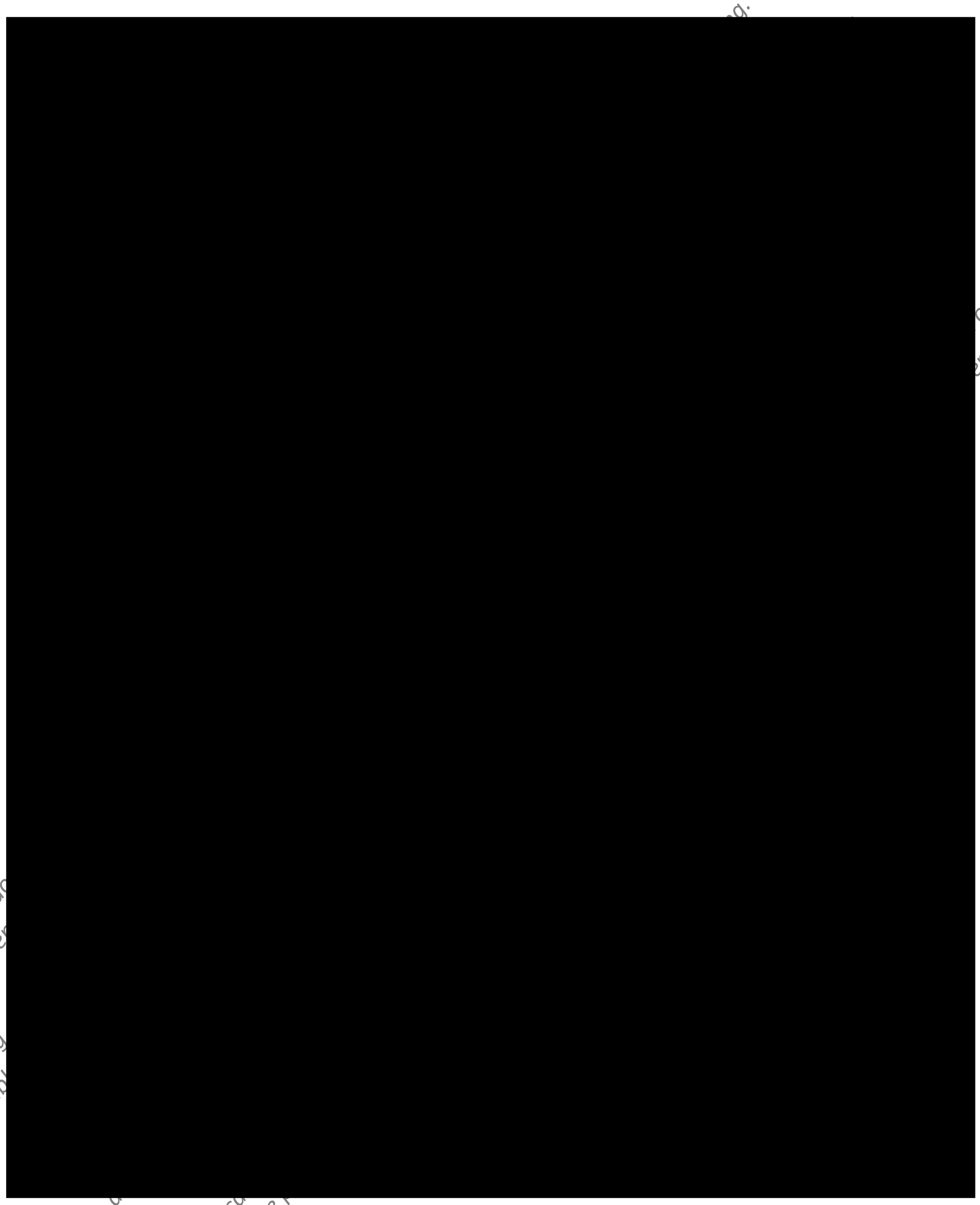
64380 Rossdorf
Germany

Project 6330036

Copy of GLP Certificate



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List of Tables

1. Summary

Report: [redacted] (1999): Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 3772 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae).
Source: IBACON, unpublished report No.: 6330036, August 26, 1999.

Guidelines: EPPO No. 170
Deviations: temperature: 29 °C; relative humidity: 68 - 70 % instead of 25 ° C ± 2 ° C and relative humidity of 60 -70 % as indicated in the guideline

GLP: yes (certified laboratory)

Material and methods: test substance: WAK 3772, purity: 95%, batch number: M00136; under laboratory conditions, starved honey bees (*Apis mellifera*, 3 groups of 10 bees per dose) received a single oral dose of either 48.5, 23.9, 12.0, 6.0, 3.1, 1.5, 0.7 or 0.1 ng per bee in 20 mg sugar solution. Subsequently, honey bees were observed over a period of 48 hrs for behavioural impairments and survival rate. The reference treatment (0.2 µg dimethoate per bee) caused a 100 % mortality (the facility-specific LD_{50} dose for dimethoate is typically between 0.10 and 0.14 µg/bee).

Findings: Toxicity to Honey Bees, Laboratory Tests

Test substance	WAK 3772
Test object	*Apis mellifera*
Application rates ng product/bee	48.5*, 23.9*, 12.0*, 6.0*, 3.1*, 1.5*, 0.7* and 0.1*
Exposure	oral (sugar solution)
LD_{50} ng product/bee (24 and 48h)	> 48.5

* values based on actual intake of the test substance

Observations: One of 30 bees died after application of an oral dose with 23.9, 12.0 and 3.1 ng WAK 3772 per bee, respectively. No behavioural abnormalities were observed for the 48 hours of the experimental time.
Three of 30 bees died in the control and all bees died after treatment with Dimethoate.

2. Survey of the Study

2.1 General Information

Title:	Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 3772 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae)
Sponsor:	Bayer AG Crop Protection Division Institute for Environmental Biology Alfred-Nobel-Str. 50 40789 Monheim Germany
Monitoring:	[redacted]
Test Substance:	WAK 3772
Test Facility:	Institut für Biologische Analytik und Consulting IBACON GmbH Industriestrasse 1 64380 Rossdorf Germany
IBACON Project:	6330036

Project Staff:

Test Facility Management:	[redacted]
Study Director:	Dipl. Biol. [redacted]
Technical Coordination:	
Head of Quality Assurance Unit (QAU):	Dipl. Biol.
Quality Assurance Unit Manager:	Dipl. Biol.

Schedule:

Study Initiation Date:	June 14, 1999
Date of First Amendment to Study Protocol:	June 24, 1999
Experimental Starting Date:	July 6, 1999
Experimental Completion Date:	July 8, 1999
Draft Report Date:	July 26, 1999
Study Completion Date:	August 26, 1999

2.2 Good Laboratory Practice

This study was performed in compliance with:

- The OECD Principles of Good Laboratory Practice (as revised in 1997) and the
- Chemikaliengesetz ('*Chemicals Act*') der Bundesrepublik Deutschland (ChemG), Anhang 1 ('*Annex 1*'), 1994/97.

This study was assessed in compliance with the study protocol and the IBACON Standard Operating Procedures. This study and/or test facility were periodically inspected by the Quality Assurance Unit (QAU) and the dates and the phases of the inspections are included in this final report. The data contained within this final report were audited in comparison to the raw data.

A quality assurance statement, signed by the Quality Assurance Unit, is included in this final report.

2.3 Archiving

The following data / sample(s) will be archived

for 15 years:
- all raw data
- the study protocol
- the study protocol amendment
- one certified copy of the final report

for at least 2 years:
- one sample of the test substance and of the toxic standard

following the date on which the final report is audited by the Quality Assurance Unit at:

Institut für Biologische Analytik
und Consulting IBACON GmbH
Industriestrasse 1
64380 Rossdorf
Germany

No raw data or material relating to the study will be discarded without the sponsor's prior consent.

2.4 Signatures

Study Director: Dipl. Biol. [redacted]

[redacted]

date: January 26, 1999

Test Facility Management: [redacted]

[redacted]

date: Jan. 27, 1999

3. Quality Assurance Unit Statement

[redacted]

4. Statement of Compliance

[redacted]

5. Objectives of the Study

5.1 Title

Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 3772 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae)

5.2 Purpose

If honey bees can be exposed to residues of WAK 3772, this study is useful for the registration of the pesticides in question. This study provides:

- the acute toxicity levels of the test substance to honey bees;
- toxicity information comparable to expected residues from standard rates, for assessment of the potential hazard to honey bees;
- informational support for precautionary label statements.

5.3 Guidelines/Recommendations

This study was designed to comply with the following method:

- EPPO 1992: Guideline on test methods for evaluating the side-effects of plant protection products on honey bees, Bulletin OEPP/EPPO Bulletin 22, 203-215 1992, No. 170

5.4 Justification for the Selection of the Test System

This test system is required by the Registration Directive 91/414/EEC and/or by the 'SETAC - Guidance document on regulatory testing procedures for pesticides with non-target arthropods' (Barrett et al. 1994) for the hazard assessment of pesticides.

6. Materials and Methods

6.1 Test Substance, Control and Toxic Standard

Test Substance

The test substance and the information concerning the test substance were provided by the sponsor:

Name:	WAK 3772
Batch No.: (Lot No.☺	M00136
Active Ingredient(s)/Purity:	95% according to certificate of analysis
Certificate of Analysis Ref. Code / Date:	July 7, 1998 (Date of Analysis)
Indication:	insecticide
Aggregate State at Room Temperature:	solid
Colour:	yellowish (according to IBACON personnel)
Solubility:	in water: not indicated
Stability:	pure: see expiry date in water: test substance must be considered as stable under test conditions
Expiry Date:	July 2000
Storage:	in original container, at room temperature, in the dark

Control

Oral Test:	tap water

Toxic Standard

The information concerning the toxic standard according to the substance container label:

Name:	Perfekthion EC
Batch No.:	98-1
Active Ingredient/Purity:	Dimethoate: 396 g/L
Chemical Structure of a.i.:	$CH_3NHCOCH_2SP(OCH_3)_2$ (S)
Manufacturer:	BASF AG, Unternehmensbereich Pflanzenschutz, D-67056 Ludwigshafen
Expiry Date:	October/2000
Storage:	at room temperature, in the dark, in original container
Amount Applied in this Study:	0.2 µg active ingredient per bee

6.2 Test System

Taxonomic Group:	worker honey bees (Insecta, Hymenoptera)
Species:	adult *Apis mellifera carnica* L.
Age and Sex:	4 - 6 week old female bees
Origin:	honey bee colonies, disease-free and queen-right, bred by IBACON
Collection:	with glass tubes, from the flight board without anaesthetics
Bee Maintenance:	conformed to proper cultural practices

6.3 Test Units

Type:	stainless steel chambers
Size:	10 cm x 8.5 cm x 5.5 cm (length x width x height)
Front Side:	removable glass sheet
Bottom:	perforated with 98 ventilation holes; ø 1 mm
Inner Walls:	lined with filter paper (Co. Macherey & Nagel, D-52355 Düren, Art. No. 68)
No. Of Individuals:	10 per test unit
Replicates:	3 per dosage/control
Identification:	test units were uniquely identified with study number, application date, treatment, concentration and replicate number
Bee Assignment:	assigned to test levels impartially

6.4 Test Conditions

Surrounding Type:	incubators
Temperature:	29 °C[1]
Relative Humidity:	68 -70 %[1]
Light:	darkness (except during observation)
Ventilation:	ventilation to avoid possible accumulation of pesticide vapour
Recording:	test conditions were recorded with suitable instruments and documented in the raw data

6.5 Food

Food:	commercial ready-to-use syrup (Apiinvert; 30 % Saccharose, 31 % Glucose, 39 % Fructose) *ad libitum*

[1] in deviation from the guidelines which recommend a temperature of 25 ° C ± 2 ° C and a relative humidity of 60 - 70 %. Experience of IBACON has shown that this deviation to the guideline will have no adverse effect on the study.

6.6 Application of the Test Substance, the Control and the Toxic Standard

The dosage applied was not adjusted to reflect the percentage a.i.

The procedure of preparation of the test substance solution is described in detail in the Attachment page 20.

Application in the Oral Test:	*ca.* 20 mg of WAK 3772 contaminated food (1 part solvent + 19 parts syrup) and offered in syringes which were weighed before and after introduction into the cages (duration of uptake did not exceed 3 hours)
Dosages of the Test Substance in the Oral Test:	48.5, 23.9, 12.0, 6.0, 3.1, 1.5, 0.7 and 0.1 ng/bee (values based on actual intake of the test substance)
Dosage of the Toxic Standard:	0.2 µg a.i. Dimethoate per bee (oral test)
Control:	WAK 3772 free sugar solution

6.7 Course of the Test

Treatment Groups:	control, 8 dosages of test substance, toxic standard
Replicates:	3 per treatment group
Individuals:	10 per unit, 30 individuals per treatment group
Starvation Time:	70 minutes
Exposure Time:	48 hours

6.8 Test Parameters

Mortality:	number of dead bees after 60 minutes; 2, 4 hours (first day); 24 and 48 hours
Behavioural Abnormalities:	behavioural abnormalities (vomiting, apathy, intensive cleaning, discoordinated movements) after 60 minutes; 2, 4 hours (first day); 24 and 48 hours

6.9 Result Evaluation

Mortality: results obtained from the bees treated with test substance are compared to those obtained from the toxic standard and the controls. Due to the results it was not necessary to conduct statistical analysis.

6.10 Validity Criteria of the Study

Control Mortality:	10 % at experimental end (48 hours)
Toxic Standard Mortality:	resulted in 100 % mortality

6.11 Deviations to the Study Protocol

No deviations to the Study Protocol occurred.

7. Results and Discussion

7.1 Oral Toxicity Test

After a starving period of 70 minutes, mortality occurred after ingestion of 23.9 (3.3 %), 12.0 (3.3 %) and 3.1 ng (3.3 %) WAK 3772 per bee. No mortality occurred in the other test substance treated groups. Behavioural abnormalities were not observed (Table 1, Appendix Table 2 - 4).

Due to the results of this study (no mortality > 50 % in the treatment groups) it does not seem to be reasonable to calculate the LD_{50}.

In the controls three of the 30 bees (10 %) bee died within the whole experiment.

100 % mortality occurred after ingestion of 0.2 µg Dimethoate per bee in the toxic standard group .

Table 1. Mortality[a] and behavioural abnormalities[a] of the bees in the oral toxicity test [b]

	after 1 hour		after 4 hours		after 24 hours		after 48 hours	
ingested test substance ng/bee	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %
48.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23.9	3.3	0.0	3.3	0.0	3.3	0.0	3.3	0.0
12.0	3.3	0.0	3.3	0.0	3.3	0.0	3.3	0.0
6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3.1	0.0	0.0	0.0	0.0	0.0	0.0	3.3	0.0
1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
contr.	0.0	6.7	3.3	3.3	6.7	0.0	10.0	0.0
toxic st.	0.0	0.0	80.0	13.3	100.0	0.0	100.0	0.0

[a] results are averages from three replicates (ten bees each) per dosage/control

[b] see Appendix for details

behav. abnorm. = behavioural abnormalities; contr. = solvent/sugar control; toxic st. = toxic standard

7.2 Conclusions

Mortality:	Oral $LD_{50\ (24\ and\ 48h)}$ of WAK 3772: > 48.5 ng/bee
Behavioural Abnormalities:	No behavioural abnormalities occurred in the test substance treatment groups.

8. References

Barrett K.L., Grandy N., Harrison E.G., Hassan S.A. & Oomen P. 1994: SETAC - Guidance document on regulatory testing procedures for pesticides with non-target arthropods. 28-30 March 1994, IAC Wageningen, The Netherlands

Chemikaliengesetz der Bundesrepublik Deutschland (ChemG), Anhang 1, in der Fassung der Bekanntmachung vom 25. Juli 1994 (BGBl. I S. 1703) mit Änderungen vom 27. September 1994 (BGBl. I S. 2705) und 14. Mai 1997 (BGBl I S. 1060)

EC Agrochemical Registration Directive (DS65) (Directive 91/414/EEC). ANNEX III. Requirements for the dossier to be submitted for the authorization of a plant production product

EPPO 1992: Guideline on test methods for evaluating the side-effects of plant protection products on honey bees, Bulletin OEPP/EPPO Bulletin 22, 203-215 1992, No. 170

OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997 [C(97)186/Final], Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

9. Distribution of the Final Report

Sponsor:	1x (the original final report)
IBACON:	1x (one certified copy of the original final report)

Appendix

Table 2. (Exact Data). Definitive oral toxicity test; mortality and behavioural abnormalities of the bees

	test substance	after 1 hour			after 2 hours			after 4 hours			after 24 hours			after 48 hours		
unit #	dosage ng/bee	dead #	beh. #	abnor. symp.	dead #	beh. #	abnor. symp.	dead #	beh. #	abnor. symp.	dead #	beh. #	abnor. symp.	dead #	beh. #	abnor. symp.
1	47.8	0	0		0	0		0	0		0	0		0	0	
2	48.4	0	0		0	0		0	0		0	0		0	0	
3	49.2	0	0		0	0		0	0		0	0		0	0	
1	24.9	0	0		0	0		0	0		0	0		0	0	
2	23.8	1	0		1	0		1	0		1	0		1	0	
3	22.9	0	0		0	0		0	0		0	0		0	0	
1	12.6	0	0		0	0		0	0		0	0		0	0	
2	11.6	1	0		1	0		1	0		1	0		1	0	
3	11.8	0	0		0	0		0	0		0	0		0	0	
1	5.8	0	0		0	0		0	0		0	0		0	0	
2	6.1	0	0		0	0		0	0		0	0		0	0	
3	6.0	0	0		0	0		0	0		0	0		0	0	
1	3.1	0	0		0	0		0	0		0	0		0	0	
2	3.1	0	0		0	0		0	0		0	0		1	0	
3	3.2	0	0		0	0		0	0		0	0		0	0	
1	1.5	0	0		0	0		0	0		0	0		0	0	
2	1.5	0	0		0	0		0	0		0	0		0	0	
3	1.5	0	0		0	0		0	0		0	0		0	0	
1	0.7	0	0		0	0		0	0		0	0		0	0	
2	0.8	0	0		0	0		0	0		0	0		0	0	
3	0.7	0	0		0	0		0	0		0	0		0	0	
1	0.1	0	0		0	0		0	0		0	0		0	0	
2	0.1	0	0		0	0		0	0		0	0		0	0	
3	0.1	0	0		0	0		0	0		0	0		0	0	
1	control	0	2	c	1	1	c	1	1	c	2	0		3	0	
2	control	0	0		0	0		0	0		0	0		0	0	
3	control	0	0		0	0		0	0		0	0		0	0	
1	toxic standard	0	0		0	1	b	10	0		10	0		10	0	
2	standard	0	0		1	0		7	3	c	10	0		10	0	
3	standard	0	0		2	3	b	7	1	c	10	0		10	0	

= number of individuals; beh. abnor. = behavioural abnormalities

symp. = observed symptoms according to the following key: a = food refusal/vomiting; b = moving coordination problems; c = apathy; d= intensive cleaning; e=nervous

Table 3. (Relative Data). Definitive oral toxicity test; mortality and behavioural abnormalities of the bees

test substance dosage	after 1 hour				after 4 hours				after 24 hours				after 48 hours			
	mortality		beh.abnor.[a]		mortality		beh.abnor.[a]		mortality		beh.abnor.[a]		mortality		beh.abnor.[a]	
	mean	±SD	mean	±SD	mean	±SD	mean	±SD	mean	±SD	mean	±SD	mean	±SD	mean	±SD
ng/bee	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
48.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23.9	3.3	5.8	0.0	0.0	3.3	5.8	0.0	0.0	3.3	5.8	0.0	0.0	3.3	5.8	0.0	0.0
12.0	3.3	5.8	0.0	0.0	3.3	5.8	0.0	0.0	3.3	5.8	0.0	0.0	3.3	5.8	0.0	0.0
6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.3	5.8	0.0	0.0
1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
contr.	0.0	0.0	6.7	11.5	3.3	5.8	3.3	5.8	6.7	11.5	0.0	0.0	10.0	17.3	0.0	0.0
toxic st.	0.0	0.0	0.0	0.0	80.0	17.3	13.3	15.3	100.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0

[a]beh. abnor. = behavioural abnormalities; mean = mean of three replicates; ±SD = standard deviation from three replications
contr. = solvent/sugar treated control; toxic st. = toxic standard

Table 4. Definitive oral intake

substance dosage nominal	substance concentrations in food ng / mg	weight of syringes start[a] mg	end[b] mg	difference mg	ingested solution mg / bee	ingested substance ng / bee[d]	ø[c] ng / bee
40 ng/bee							
1	2	11184	10945	239	24	47.8	
2	2	11261	11019	242	24	48.4	48.5
3	2	11238	10992	246	25	49.2	
20 ng/bee							
1	1	11275	11026	249	25	24.9	
2	1	11236	10998	238	24	23.8	23.9
3	1	11265	11036	229	23	22.9	
10 ng / bee							
1	0.5	11248	10997	251	25	12.6	
2	0.5	11215	10984	231	23	11.6	12.0
3	0.5	11226	10991	235	24	11.8	
5 ng / bee							
1	0.25	11305	11073	232	23	5.8	
2	0.25	11219	10974	245	25	6.1	6.0
3	0.25	11220	10981	239	24	6.0	
2.5 ng / bee							
1	0.125	11248	11002	246	25	3.1	
2	0.125	11242	10994	248	25	3.1	3.1
3	0.125	11232	10975	257	26	3.2	
1.3 ng / bee							
1	0.0625	11216	10974	242	24	1.5	
2	0.0625	11257	11011	246	25	1.5	1.5
3	0.0625	11081	10841	240	24	1.5	
0.6 ng / bee							
1	0.031	11225	10992	233	23	0.7	
2	0.031	11237	10997	240	24	0.8	0.7
3	0.031	11205	10970	235	24	0.7	
0.1 ng / bee							
1	0.005	11186	10941	245	25	0.1	
2	0.005	11246	11002	244	24	0.1	0.1
3	0.005	11264	11037	227	23	0.1	
control							
1	0	11235	10982	253	25	0.0	
2	0	11213	10967	246	25	0.0	0.0
3	0	11204	10957	247	25	0.0	
toxic standard							
Dimethoate							
0.2 µg/bee							
1	0.01	11212	10968	244	24	0.2	
2	0.01	11248	11003	245	25	0.2	0.2
3	0.01	11238	10992	246	25	0.2	

[a] weight of syringes at the start of the experiment, [b] after removing from the test cages;
[c] ingested solution as calculated average, [d] results are rounded results, calculated from the exact data

Attachment: Preparation of the Test Substance Solutions

Oral Test

Prior to the dilution process, the stock solution was a clear fluid.

20 mg of the test substance was dissolved ad 500 g tap water (stock solution). This was done one day before the application. The stock solution was stirred overnight in a refrigerator.

Six 1 :2 dilutions of the stock solution were done step by step.

The 0.1 ng / bee concentration was obtained by diluting the 10 ng/bee dilution 1 : 100.

500 mg of each 1:2 dilution, 500 mg of the 1:100 dilution of the 10 ng dose and 500 mg of the stock solution were added to 9.5 g syrup (1:20) for preparation of the 8 different treatment solutions (reference not included).

40, 20, 10, 5, 2.5, 1.3, 0.6 ng/bee should be obtained when 20 mg of the contaminated sugar solution per bee were administered.

48.5, 23.9, 12.0, 6.0, 3.1, 1.5, 0.7 and 0.1 ng/bee were obtained, because the bees ingested between 23 and 26 mg contaminated food per bee.