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Institut für
Biologische Analytik und
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Final Report

Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 5074 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae) -Limit Test-

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

Author: Dipl. Biol. [redacted]

Study Completion Date: January 14, 2000

Sponsor

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

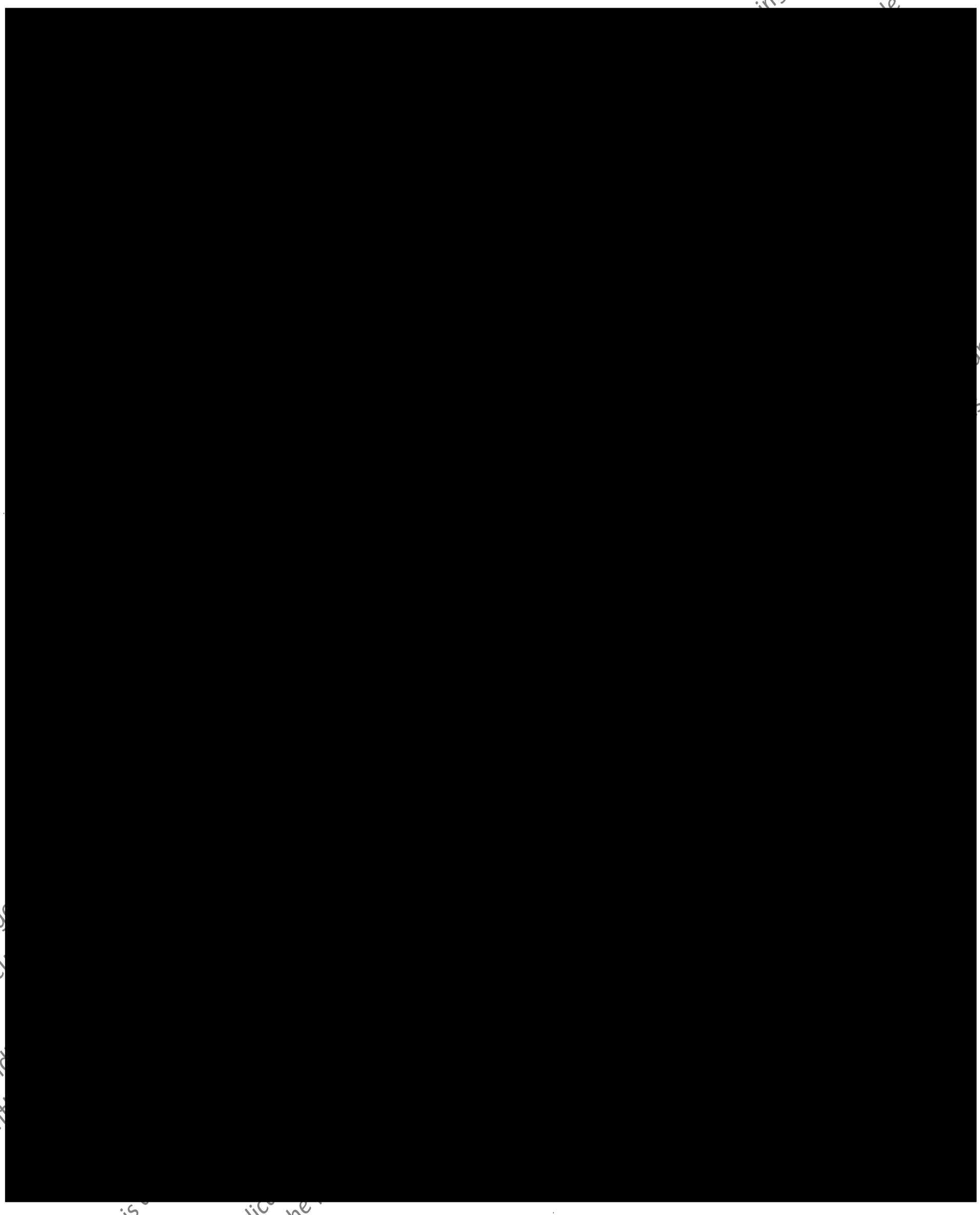
Test Facility

Institut für Biologische Analytik
und Consulting IBACON GmbH
Arheilger Weg 17
64380 Rossdorf
Germany

Project 7150036

7150036 / MO-00-000908

Copy of GLP Certificate



Contents

List of Tables

1. Summary

Report: [redacted] (1999): Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 5074 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae) -Limit Test-
Source: IBACON, unpublished report No.: 7150036, January 14, 2000.

Guidelines: EPPO No. 170
Deviations: temperature: 29 °C; relative humidity: 52 -86 % 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 5074, purity: 98%, batch number: DIJ11371; under laboratory conditions, starved honey bees (*Apis mellifera*, 3 groups of 10 bees per dose) received a single oral dose of either 119.8 or 1.2 µg per bee in ca. 25 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 5074
Test object	*Apis mellifera*
Application rates µg product/bee	119.8* and 1.2*
Exposure	oral (sugar solution)
LD_{50} µg product/bee (24 and 48h)	> 119.8

* values based on actual intake of the test substance

Observations: None of 30 bees died after application of an oral dose with 119.8 µg or 1.2 µg WAK 5074 per bee. No behavioural abnormalities were observed for the 48 hours of the experimental time.
No bee died in neither the water, nor in the pure syrup control. 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 5074 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae) - Limit Test -

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

Monitoring: [redacted]

Test Substance: WAK 5074

Test Facility:
Institut für Biologische Analytik
und Consulting IBACON GmbH
Arheilger Weg 17
64380 Rossdorf
Germany

IBACON Project: 7150036

Project Staff:

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

Schedule:

Study Initiation Date:	September 13, 1999
Experimental Starting Date:	September 16, 1999
Experimental Completion Date:	September 18, 1999
Draft Report Date:	December 1, 1999
Study Completion Date:	January 14, 2000

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
- 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
Arheilger Weg 17
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]

date: January 19, 2000

Test Facility Management: Dr. [redacted]

date: January 14, 2000

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 5074 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae) - Limit Test -

5.2 Purpose

If honey bees can be exposed to residues of WAK 5074, 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 5074
Batch No.: (Original Sample)	DIJ11371
Active Ingredient(s)/Purity:	98 % according to the sponsor
Indication:	insecticide
Aggregate State at Room Temperature:	liquid
Colour:	colourless (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:	04/2002
Storage:	in original container, at approximately - 15 °C, in the dark

Controls

Controls:	1) pure syrup 2) tap water + syrup

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:	52 - 86 %[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 21.

Application in the Oral Test:	*ca.* 25 mg of WAK 5074 contaminated food (for the 100 µg/bee nominal dose the test substance was distributed directly in the food and for the 1 µg/bee nominal dosages 1 part solvent (=water) including the test substance + 19 parts syrup were used) offered in syringes which were weighed before and after introduction into the cages (duration of uptake did not exceed 3 hrs)
Dosages of the Test Substance in the Oral Test:	119.8 and 1.2 µg/bee (values based on actual intake of the test substance)
Dosage of the Toxic Standard:	0.2 µg a.i. Dimethoate per bee
Controls:	1) WAK 5074 free sugar solution (used as the control for the 100µg/bee (nominal) dosage group) 2) WAK 5074 free sugar solution with water as solvent (used as the control for the 1µg/bee (nominal) dosage group)

6.7 Course of the Test

Treatment Groups:	controls, 2 dosages of test substance, toxic standard
Replicates:	3 per treatment group
Individuals:	10 per unit, 30 individuals per treatment group
Starvation Time:	60 minutes
Exposure Time:	48 hours

6.8 Test Parameters

Mortality:	number of dead bees after 1, 2, 4 hours (first day); 24 and 48 hours
Behavioural Abnormalities:	behavioural abnormalities (vomiting, apathy, intensive cleaning, discoordinated movements) after 1, 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:	no control mortality at experimental end (48 hours)
Toxic Standard Mortality:	resulted in 100 % mortality

6.11 Deviations to the Study Protocol

Concerning:	2.4 Test Conditions
According to the Study Protocol:	40 - 70 % relative humidity
Deviation to the Study Protocol:	the relative humidity remained in the range of 52 - 86 % instead of 40 - 70% as indicated in the study protocol
Reason for the Deviation:	because of technical reasons (climatic) the relative humidity had a broader range than expected
Presumed Effect on the Study:	none, the broader humidity range reflects natural conditions and has no adverse effect on the outcome of the study

7. Results and Discussion

7.1 Oral Toxicity Test

After a starving period of 60 minutes, no mortality occurred after ingestion of 119.8 and 1.2 µg WAK 5074 per bee (Table 1, Appendix Table 2 - 4).

Due to the results of this study (no mortality > 50 % in the highest dosage group) the LD_{50} must be > 119.8 µg/bee.

Two different controls were used for this test:

The pure syrup control was used for the 119.8 µg/bee group, because the test substance was mixed directly in the syrup. In the 1.2 µg/bee dosage group water was used as solvent, respectively.

No mortality occurred in the control groups 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	
uptaken test substance µg/bee	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %
119.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
syrup	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
toxic st.	0.0	0.0	76.7	23.3	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
syrup = syrup control; water = water control; toxic st. = toxic standard

7.2 Conclusions

Mortality:	Oral $LD_{50\ (24\ and\ 48h)}$ of WAK 5074: > 119.8 µg/bee
Behavioural Abnormalities:	No behavioural abnormalities occurred.

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 I, 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	dead	beh. abnor.		dead	beh. abnor.		dead	beh. abnor.		dead	beh. abnor.		dead	beh. abnor.	
#	µg/bee	#	#	symp.	#	#	symp.	#	#	symp.	#	#	symp.	#	#	symp.
1	119.0	0	0		0	0		0	0		0	0		0	0	
2	119.0	0	0		0	0		0	0		0	0		0	0	
3	121.5	0	0		0	0		0	0		0	0		0	0	
1	1.2	0	0		0	0		0	0		0	0		0	0	
2	1.2	0	0		0	0		0	0		0	0		0	0	
3	1.3	0	0		0	0		0	0		0	0		0	0	
	syrup															
1	control	0	0		0	0		0	0		0	0		0	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	
	water															
1	control	0	0		0	0		0	0		0	0		0	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	
	toxic															
1	standard	0	0		0	4	b,e	7	3	b,c	10	0		10	0	
2	standard	0	0		4	6	b,e	7	3	c	10	0		10	0	
3	standard	0	0		1	6	b,e	9	1	e	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
µg/bee	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
119.8	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
1.2	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
syrup	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
water	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
toxic st.	0.0	0.0	0.0	0.0	76.7	11.5	23.3	11.5	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
syrup = pure syrup control; water = water treated control; toxic st. = toxic standard

Table 4. Definitive oral intake

substance dosage nominal	substance concentrations in food µg/mg	weight of syringes start[a] mg	weight of syringes end[b] mg	weight of syringes difference mg	uptaken solution mg / bee	uptaken substance µg / bee[d]	uptaken substance ø[c] µg / bee
100 µg/bee							
1	5	11257	11019	238	24	119.0	
2	5	11204	10966	238	24	119.0	119.8
3	5	11223	10980	243	24	121.5	
1 µg/bee							
1	0.05	11200	10955	245	25	1.2	
2	0.05	11173	10933	240	24	1.2	1.2
3	0.05	11259	11004	255	26	1.3	
syrup control							
1	0	11208	10952	256	26	0.0	
2	0	11215	10957	258	26	0.0	0.0
3	0	11317	11069	248	25	0.0	
water control							
1	0	11234	10975	259	26	0.0	
2	0	11187	10946	241	24	0.0	0.0
3	0	11244	10999	245	25	0.0	
toxic standard							
Dimethoate							
0.2 µg/bee							
1	0.01	11227	10978	249	25	0.2	
2	0.01	11260	11027	233	23	0.2	0.2
3	0.01	11154	10905	249	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

100 µg/bee dosage (nominal):

20 mg of the test substance was mixed with 4 g syrup and overnight stirred at room temperature.

Control: pure syrup. The test substance was obviously completely dissolved in the syrup.

1 µg/bee dosage (nominal):

51 mg of the test substance was dissolved ad 50 g water (stock solution), treated ultra sonic for 20 minutes and overnight stirred in a refrigerator. After stirring and letting stand for a while a white sediment were visible. Therefore the stock solution was taken out under permanent stirring.

500 mg of the stock solution was added to 9.5 g syrup (1:20).

Control: 500 mg water and 9.5 g syrup.

100 and 1 µg/bee should be obtained when 20 mg of the contaminated sugar solution per bee were administered.

119.8 and 1.2 µg/bee were obtained, because the bees ingested between 24 and 26 mg contaminated food per bee.