Institut für
Biologische Analytik und
Consulting IBACON GmbH

Final Report

Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 4168 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: September 21, 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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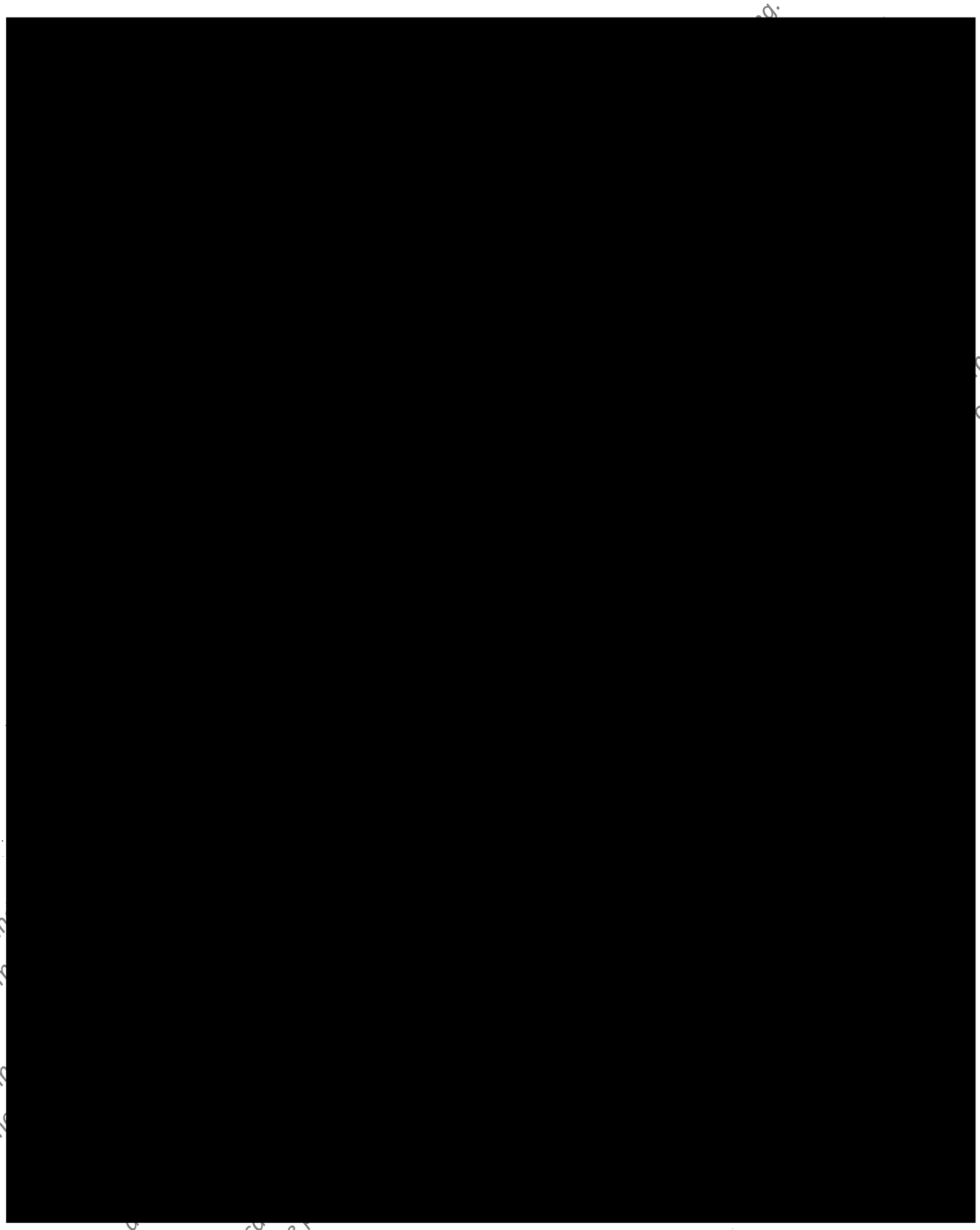
64380 Rossdorf
Germany

Project 6370036

IBACON 6370036 / MO-99-016005

Copy of GLP Certificate



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1. Summary

Report: [redacted] 1999): Laboratory Testing for Toxicity (Acute Oral LD_{50}) of WAK 4168 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae) -Limit Test-
Source: IBACON, unpublished report No.: 6370036, September 21, 1999.

Guidelines: EPPO No. 170
Deviations: temperature: 29 °C; relative humidity: 64 -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 4168, purity: 99.0%, batch number: 960118ELB01; under laboratory conditions, starved honey bees (*Apis mellifera*, 3 groups of 10 bees per dose) received a single oral dose of either 99.5 or 1.2 µg 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 4168
Test object	*Apis mellifera*
Application rates µg product/bee	99.5* and 1.2*
Exposure	oral (sugar solution)
LD_{50} µg product/bee (24 and 48h)	> 99.5

* values based on actual intake of the test substance

Observations: Obviously the test substance appeared to have a repellent effect in the 99.5 µg/bee dosage group indicated by the long period of uptake by the bees in this dosage group, although bees were previously starved for 60 minutes. 11 of 30 (36.7 %) bees died after application of an oral dose with 99.5 µg WAK 4168 per bee. One of the 30 (3.3 %) bees died after application of an oral dose with 1.2 µg WAK 4168 per bee. Behavioural abnormalities like apathy or discoordinated movements occurred in the 99.5 µg/bee dosage group. No behavioural abnormalities were observed in the 1.2 µg/bee dosage group for the 48 hours of the experimental time.
No bee died in both, pure syrup and water/syrup control groups. 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 4168 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 4168
Test Facility:	Institut für Biologische Analytik und Consulting IBACON GmbH Industriestrasse 1 64380 Rossdorf Germany
IBACON Project:	6370036

Project Staff:

Test Facility Management:	Dr. [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:	June 14, 1999
Experimental Starting Date:	July 7, 1999
Experimental Completion Date:	July 9, 1999
Date of 1st Study Protocol Amendment:	June 24, 1999
Draft Report Date:	August 25, 1999
Study Completion Date:	September 21, 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]

date: September 21, 1999

Test Facility Management: Dr. [redacted]

date: September 21, 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 4168 on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae) - Limit Test -

5.2 Purpose

If honey bees can be exposed to residues of WAK 4168, 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 4168
Batch No.: (Lot No.:)	960118ELB01
Active Ingredient(s)/Purity:	99.0 % according to certificate of analysis
Certificate of Analysis Ref. Code / Date:	January 12, 1996
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:	January 2001
Storage:	in original container, at room temperature, 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-I
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:	64 - 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 21.

Application in the Oral Test:	*ca.* 20 mg of WAK 4168 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 was 27 hours in the 100 µg/bee (nominal) dosage group)
Dosages of the Test Substance in the Oral Test:	99.5 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 4168 free sugar solution (used as the control for the 100µg/bee (nominal) dosage group) 2) WAK 4168 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 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:	no control mortality at experimental end (48 hours)
Toxic Standard Mortality:	resulted in 100 % mortality

6.11 Deviations to the Study Protocol

Concerning:	duration of uptake of the test substance
According to the Study Protocol:	duration of uptake of the test substance should not exceed 3 hours
Deviation to the Study Protocol:	uptake of test substance in the 100 µg/bee (nominal) dosage group took 27 hours and not 3 hours as indicated in the protocol
Reason for the Deviation:	because the bees needed more than the expected 3 hours for the uptake of the contaminated food
Presumed Effect on the Study:	none, because bees ingested enough amount of treated food and similar intake of solution in treated and control groups

7. Results and Discussion

7.1 Oral Toxicity Test

Although the bees were starved for 60 minutes, the bees needed 27 hours for the complete uptake of the contaminated food in the 99.5 µg/bee (nominal) dosage group. The test substance appeared to have a repellent effect on the bees at least at 99.5 µg/bee (nominal). No repellent effect was observed in the 1.2 µg/bee (nominal) dosage group.

Mortality occurred after ingestion of 99.5 (36.7 %) µg WAK 4168 per bee. One of the 30 bees (3.3 %) died after application of 1.2 µg WAK 4168.

After ingestion of 99.5 µg/bee WAK 4168, behavioural abnormalities (discoordinated movements and apathy) were observed two hours after application (56.7 %). During the 4 hours check 90.0 % of the bees were lazy/apathetic or showed discoordinated movements. 24 hours after the application 16.7 % of the bees had moving coordination problems. All bees recovered during the test after 48 hours (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 considered as > 99.5 µg/bee.

Two different controls were used for this test:

The pure syrup control was used for the 99.5 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 as it was for the control.

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	
ingested test substance µg/bee	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %
99.5	0.0	0.0	10.0	90.0	36.7	16.7	36.7	0.0
1.2	0.0	0.0	0.0	0.0	0.0	0.0	3.3	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	96.7	3.3	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 = pure syrup control; water =water/syrup control; toxic st. = toxic standard

7.2 Conclusions

Mortality:	Oral $LD_{50\ (24\ and\ 48h)}$ of WAK 4168: > 99.5 µg/bee Test substance presented a repellent effect in the 99.5 µg/bee dosage group.
Behavioural Abnormalities:	Behavioural abnormalities like apathy or discoordinated movements occurred in the 99.5 µg/bee dosage group.

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 µg/bee	dead #	beh. abnor. #	symp.	dead #	beh. abnor. #	symp.	dead #	beh. abnor. #	symp.	dead #	beh. abnor. #	symp.	dead #	beh. abnor. #	symp.
1	102.0	0	0		0	7	f	1	9	f	3	0		3	0	
2	104.5	0	0		0	8	f	0	10	f	1	2	b	1	0	
3	92.0	0	0		0	2	c	2	8	b,f	7	3	b	7	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		1	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	8	c	9	1	e	10	0		10	0	
2	standard	0	0		1	6	b,c	10	0		10	0		10	0	
3	standard	0	0		0	10	b,c	10	0		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; f = lethargic
syrup control = pure syrup control; water control = water/syrup control

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	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
99.5	0.0	0.0	0.0	0.0	10.0	10.0	90.0	10.0	36.7	30.6	16.7	15.3	36.7	30.6	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	3.3	5.8	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	96.7	5.8	3.3	5.8	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/syrup 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	end[b] mg	difference mg	ingested solution mg / bee	ingested substance µg / bee[d]	ø[c] µg / bee
100 µg/bee							
1	5	11216	11012	204	20	102.0	
2	5	11165	10956	209	21	104.5	99.5
3	5	11165	10981	184	18	92.0	
1µg/bee							
1	0.05	11182	10935	247	25	1.2	
2	0.05	11182	10937	245	25	1.2	1.2
3	0.05	11189	10939	250	25	1.3	
pure syrup control							
1	0	11177	10944	233	23	0.0	
2	0	11161	10899	262	26	0.0	0.0
3	0	11162	10891	271	27	0.0	
water/syrup control							
1	0	11125	10870	255	26	0.0	
2	0	11186	10942	244	24	0.0	0.0
3	0	11180	10937	243	24	0.0	
toxic standard Dimethoate							
0.2 µg/bee							
1	0.01	11084	10844	240	24	0.2	
2	0.01	11197	10957	240	24	0.2	0.2
3	0.01	11128	10889	239	24	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 treated with ultra sonic for 30 minutes and overnight stored in a refrigerator until the application.

Control: pure syrup.

1 µg/bee dosage (nominal):

50 mg of the test substance was dissolved ad 50 g water (stock solution) and overnight stirred in a refrigerator. The test substance was completely dissolved in water. The stock solution was clearly visible.

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.

99.5 and 1.2 µg/bee were obtained, because the bees ingested between 18 and 25 mg contaminated food per bee.