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BioChem agrar
Labor für biologische und chemische Analytik GmbH

FINAL REPORT

Substance A

STUDY TITLE

Acute toxicity of Substance A to the honeybee *Apis mellifera* L. under laboratory conditions

Guidelines:

EPPO Standard PP 1/170(2) (1999); OECD 213 (1998), OECD 214 (1998)

Author

[redacted]

STUDY COMPLETED ON

July 07, 2000

PERFORMING LABORATORY

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LABORATORY PROJECT IDENTIFICATION

BioChem agrar project Number: 00 10 48 0501

SPONSOR

Bayer AG
Landwirtschaftszentrum Monheim
Institut für Ökobiologie
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STUDY MONITOR

[redacted]

Total number of pages: 16

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TABLE OF CONTENTS

SUMMARY

Study title:	Acute toxicity of Substance A to the honeybee *Apis mellifera* L. under laboratory conditions
Guideline(s):	EPPO Standard PP 1/170(2) (1999); OECD 213 (1998), OECD 214 (1998)
BioChem project No.:	00 10 48 501
Test substance:	Substance A
Formulation:	white powder
Test species:	*Apis mellifera carnica* L. stage: adults (worker bees) source: purchased from bee keeper [redacted]
Test system:	oral toxicity and contact toxicity test of Substance A on honeybees
Test conditions:	temperature: 24-26 °C relative humidity: 63-75 % lighting intensity: ca 100 lx photoperiod: constant darkness throughout the test (with the exception of diffuse artificial light for less than 8 hours (over the whole test period) for the treatment and observations (ca 100 lx))
Treatments:	controls, test substance concentrations
Test substance treatment levels:	the test substance was applied at the following rates: oral toxicity test: 1, 3, 9, 27, 81 ng/bee contact toxicity test: 40, 56, 78.4, 109.8 and 153.7 ng/bee
Replicates:	3 replicates per treatment, each with 10 bees
Duration:	48 hours (oral toxicity) 96 (contact toxicity)
Biological observations:	mortality: 24 and 48 hours after application

Results:

The test endpoints were mortality and behaviour of the honeybees in comparison with the control.

Contact exposure to substance A caused the following mortalities:

	Mortality/Corrected mortality according to Abbott (%)			
Substance A ng/bee	24h	48	72	96
control	3.3/-	3.3/-	6.7/-	6.7/-
153.7	76.6/75.9	80.0/79.3	80.0/78.6	80.0/78.6
109.8	60.0/58.6	73.3/72.4	73.3/71.4	80.0/78.6
78.4	26.7/24.2	50.0/48.3	50.0/46.4	56.6/53.6
56.0	23.3/20.7	30.0/27.6	30.0/25.0	36.6/32.2
40.0	30.0/27.6	33.3/31.1	33.3/28.6	36.6/32.2
LD_{50}(contact) ng/bee	**97.7**	**74.9**	**78.4**	**69.0**
Confidence limits lower	79.08	61.77	64.70	56.06
upper	120.73	90.90	94.99	85.0
Slope b	2.56	2.63	2.75	2.6

Therefore it is concluded that the LD_{50} for contact exposure was 74.9 ng substance A per bee in the contact toxicity test after 48 hours of exposure. The study was prolonged because mortality increased between 24 h and 48 h. The LD_{50} after 72 and 96 hours was 78.4 and 69.0 ng substance A per bee.
In all contact treatments apathy, discoordinated movements and immobility were observed after up to 48 hours after application. 72 h and 96 h after application the surviving bees had recovered and exhibited no further behavioural anomalies.

Oral uptake caused the following results:

The oral uptake of the test substance at doses of 81, 27, 9, 3 and 1 ng test substance per bee caused 46.7 %, 10 %, 20.0 %, 3.3 % and 6.7 % mortality after 48 h.

Therefore it is concluded that the LD_{50} (48 h) is 74.9 ng substance A per bee in the contact toxicity test and slightly higher as the highest provided dose of 81 (70.3 consumed) ng test substance A per bee in the oral toxicity test.

In all contact treatments and in the 81 ng a.i./bee and 27 ng a.i./bee oral treatments apathy, discoordinated movements and immobility were observed after 24 hours after application. 48 hours after application the surviving bees had recovered and exhibited no further behavioural anomalies.

The validity criterion - mortality in the control ≤ 10 % - was accomplished (being 3.3 % in the contact and 3.3 % in the oral toxicity tests after 48 hours).

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1. General information

1.1. Objective

The purpose of this study was to determine the acute toxicity of the "Substance A" on the honeybee *Apis mellifera* in a laboratory test after oral and contact exposure.
The selected test design corresponds to the recommendation of the EPPO Standard Guideline PP/1/170 (2) (1999). "Side-effects on honeybees" and the OECD Guidelines 213 and 214 (draft 1998).

1.2. Justification for selection of the test system

Data on the toxicity to *Apis mellifera* were generated to comply with the EU Registration Directive 91/414/EEC (amended by the Commission Directive 96/12/EC).

1.3. Project staff

Study Director: [redacted]

Personnel: [redacted]

1.4. Time schedule

Study initiation date:	28.04.00
Signature of the study plan by the sponsor:	02.05.00
Experimental start date:	31.06.00
Experimental termination date:	02.07.00
Study completion date:	07.07.99

1.5. Test guidelines

EPPO Standard PP1/170(2) (1999)
OECD Guideline No. 213 (draft 1998)
OECD Guideline No. 214 (draft 1998)

1.6. Archiving

Study plan and final report as well as the raw data will be kept in the archive of BioChem agrar GmbH Cunnersdorf.
Disposal of any data requires the sponsor's consent.

2. Performance of the test

2.1. Test substance

Name.:	Substance **A**
Received:	25.04.2000
Active ingredient/content: (according to certificate)	A (not available)
Appearance:	white powder
Stability:	chemically stable under standard conditions
Water solubility:	soluble (> 100 mg/l)
Storage conditions:	room temperature (max. 30 °C), dark and dry
Safety precautions:	designation according to "EU Classification": no information consideration of the safety measures in general use with handling of plant protection products
Field of use:	unknown
Dose applied in the test:	oral: 1, 3, 9, 27, 81 ng / bee (nominal) contact: 40, 56, 78.4, 109.8, 153.7 ng / bee (nominal)
Further details:	fax of: 12.04.00, 19.04.00 and 25.04.00

2.2. Reference substance (toxic standard)

was not required

2.3. Control

The oral and contact controls were treated with 50 % (w/v) sucrose solution and acetonic sucrose solution (0.005 % v/v), respectively, as well as acetone.

2.4. Test system

Test organism: honeybee - *Apis mellifera carnica* L.
(workerbees of a colony in good health condition)

Origin of the test organism: purchased from the bee-keeper [redacted]
on 03.05.00

All bees used in the test came from healthy, disease free and queen-right bee colonies. The bees were taken from a hive that had not received treatments with chemical substances for at least one month. The honeybees were reared in the hive until they were used for testing.
10 bees were transferred to each test cage without anaesthesia. Test bees were collected from an entrance located on the top of the bee hive using an automatic trapping device (carousel with 4 glass tubes divided into two sections: a) ∅ 2 cm and 25 cm long; b) ∅ 0.5 cm and 10 cm long). For collecting the bees the carousel with the glass tubes was placed on the top of the bee hive. The glass tubes were fixed in the carousel. Then the entrance on the top of the bee hive was opened. After a bee walked in the glass tube, the carousel was turned so that the next empty glass tube was located over the entrance. The tube containing the bee was removed and placed on the test cage entrance. The bee was put inside the test cage by gentle blowing in the thinner end of the glass tube. This process was repeated until sufficient bees were available within the test cages for use in the test. After transferring the bees in the test cages they had time for acclimatisation to the test room conditions for ca. 2 hours (starving period in the oral toxicity test or approximately 2 hours in the contact toxicity test before application of the bees).

Test conditions

Test cage:	disposable cage of cardboard with holes in the bottom for ventilation and a glass plate in front for observation of the bees (dimensions inside: 80 mm x 45 mm x 65 mm)
Number of honeybees/cage:	10
Number of cages/concentration:	3
Number of honeybees/ concentration:	30
Feeding:	continuously during the test
Food:	50 % (w/v) aqueous sucrose solution

Climatic conditions (test room)

Temperature:	24-26 °C (according to EPPO Guideline and study plan: (25±2) °C)
Relative humidity:	63-75 % (according to EPPO Guideline and study plan: about 60-70 %)
Both registered:	continuously by a thermohygrograph Manufacturer: Feingerätebau Drebach type: 456
Illumination:	constant darkness throughout the test (with the exception of diffuse artificial light for less than 8 hours (over the whole test period) for the treatment and observations (ca 100 lx))
Test duration:	48 hours (oral toxicity) 96 hours (contact toxicity)

2.5. Experimental procedure

2.5.1. Preparation of the test solutions and method of application

Table 1: Test concentrations used in the test

treatment group	product	concentration (ng/bee)
1a control (oral)	50 % sucrose solution	-
1b control (oral)	50 % sucrose solution (containing 0.005 % v/v acetone)	-
1c control (contact)	acetone	-
2 test substance	Substance A	oral toxicity test (sucrose solution/0.005 % acetone) 81 27 9 3 1 contact toxicity test (acetone) 153.7 109.8 78.4 56.0 40.0

The 50 % (w/v) sucrose solution was prepared with deionised water, just prior to application.

The exactly weighed out amounts of the substance were dispersed in sucrose solution (after dissolving in acetone) or in acetone (see appendix 1).

2.5.2. Course of the trial

Workerbees of the honeybee *Apis mellifera* L. were exposed to different doses of the test substance. The treated bees were kept under controlled climatic conditions and assessed for toxic effects for up to 48 hours (oral toxicity) and 96 h (contact toxicity) . Two different routes of exposure were used permitting the evaluation of both toxic feeding effects and contact effects of the test substance. For both routes of exposure control treatments were included in the test design. If appropriate mortality values were used to provide a regression line and calculate the median lethal dose value (LD_{50}) expressed in ng of the substance per bee. At the beginning of the test, 10 healthy workerbees per replicate (3 replicates/test concentration) were transferred individually in glass tubes from the hive (i.e. from the trapping device) to the test cage without anaesthesia. For both the oral and contact toxicity tests the groups of 10 healthy bees for use in different treatment groups were chosen without bias.
In the oral toxicity test, the bees were starved for a period of ca. 2 hours prior to test initiation in order that the bees were equal in terms of their gut contents at the start of the test. In the contact toxicity test the bees were fed after application and this was done within ca. 2 hours after collecting bees from the hive.
The preparation of the test solutions was performed according to point 2.5.1.
After 3 hours in the oral toxicity test and immediately after application in the contact toxicity test, untreated 50 % (w/v) aqueous sucrose solution was used as food in both the tests. Food was provided *ad libitum* using a feeding tube (described in the section below). Twice a day the feeding tube was filled up with new sucrose solution before the bees had consumed the whole sucrose solution.
The number of dead and affected bees were counted at 24 and 48 hours. At these times any behavioural abnormalities of the bees were also recorded.

Oral toxicity test

The bees were fed with a defined quantity of a 50 % aqueous sucrose solution that contained the test substance (in a concentration series). The control treatments were fed with 50 % aqueous sucrose solution alone and with 50 % aqueous sucrose + acetone (0.005 % v/v).
Before the sucrose solution was filled in the feeding tubes these feeding tubes were pre-weighed. In the test, groups of 10 bees were provided with 0.2 ml (= 0.251 g) of the test solution which was presented to the bees in a glass ampoule (half-open on his longitudinal axis and 5 cm long). The feeding tubes were introduced through a hole in the roof of the cage.
Due to their social feeding habit, the honeybees of a distinct group are assumed to receive approximately the same amount of the test substance (i.e. ca. 20 µl/bee). Maximally 3 hours after test beginning, the feeding tube was reweighed to determine the exact quantity of the test solution consumed. At this time the feeding tube was replaced with a feeding tube containing untreated 50 % aqueous sucrose solution.

Contact toxicity test

The test compound was dissolved in acetone.
Bees anaesthetised with CO_2 were treated individually by topical application of the test solution with an Eppendorf Micropipette. 1 µl of the test substance solution were applied to the ventral thorax of each bee.
After application, the treated bees were returned to the test cages which were supplied with a feeding tube containing 50 % aqueous sucrose solution.

Order of application and cleaning procedure

The order of feeding or topical application was the following:
- control
- test substance (from the lowest to the highest concentration)

Following application of the treatments, the equipment used was cleaned according to the respective SOP. Cleaning agents were "Essigreiniger", washing-up liquid, tap water and deionised water for glass equipment.

Chronological test schedule (abstract)

- Transfer of the bee hive to the test room
- Preparation of the test cages
- Transfer of 10 bees to each cage (without anaesthesia) and acclimatisation for approximately 2 hours in both tests
- In the acclimatisation phase of the oral toxicity test no food was provided before application (i.e. the bees were starved for ca. 2 hours)
- In the contact toxicity test no food was provided before application (the food was provided immediately after application, i.e. within 2 hours after collecting bees from the hive)
- Preparation of the test solutions (up to 1 h before application)
- Anaesthesia of the bees with CO_2 for ca 1 min (contact toxicity test only)
- Application of the test solutions (both oral and contact administration)
- Placing of food tubes with 50 % sucrose solution into the contact treatment cages
- 3 hours after the oral application, feeding tubes reweighed and replaced with tubes containing untreated 50 % sucrose solution
- Observation of the bees throughout the experiment (including 24 hours) and feeding as required
- Final assessment 48 h after application (oral toxicity) respectively 96 h (contact toxicity)

2.5.3. Assessment of the effects

Time and frequency of assessments:
- 24 and 48 hours after application (oral toxicity)
- 24, 48, 72 and 96 hours after application (contact toxicity)

Evaluation parameters:

· mortality:	number of dead bees per cage
· behaviour:	poisoning symptoms and behavioural anomalies in comparison with the control bees

Validity criterion:
- mortality in the control: ≤ 10 %

2.6. Calculation and evaluation

The corrected mortality according to ABBOTT was calculated for each concentration following the formula

$$M\,(\%) = \frac{c - t}{c} \cdot 100$$

M	=	corrected mortality (%)
c	=	number of surviving bees in the control treatment group
t	=	number of surviving bees in the treated treatment group

The determination of the LD_{50} and the analysis of the statistical significance was carried out by Probit analysis and the FISCHER exact-test (EASY ASSAY Critical Values Ver. 3.01, 1998 by RATTE).

3. Results

The findings are summarized in the tables 2 and 3 and the detailed set of results are presented in Appendices 3 and 4.

3.3 % and 6.6 % mortality were observed in the oral and contact control treatments during 48 hours and 96 hours. Thus, the test accomplished the validity criterion (mortality in the control ≤ 10 %).

In the oral toxicity test statistically significant mortality was observed at the dose of 81 ng test substance per bee (46.7 % mortality during 48 hours). The tested doses of 1, 3, 9 and 27 ng test substance per bee resulted in 6.7, 3.3, 20.0 and 10 % mortality with no statistically significant differences compared to the control mortality. In the oral toxicity test most of the contaminated sucrose solutions were completely consumed at each dose level within 3 hours of dose administration. In those that sucrose solutions were not fully consumed within 3 hours the vast majority of the dose was consumed. The results are presented in terms of the actual measured consumed dose (see appendix 3). The test period was not extended beyond 48 h as there was no evidence of progressive mortality between 24 and 48 hours.
Therefore it is concluded that the LD_{50} (48 h) is > 81 ng test substance A (70.3 ng consumed) per bee in the oral toxicity test
Abnormal behaviour (apathy, discoordinated movements and immobility) of the bees were observed 4 and 24 h after oral application of the 81 and 27 ng test substance per bee in comparison with the control bees.

In the contact toxicity test, an exposure to substance A resulted in mortality (after 96 h) of 36.6 %, 36.6 %, 56.6 %. 80.0 % and 80.0 % at doses of 40.0 ng, 56.0 ng, 78.4 ng, 109.8 ng up to 153.7 ng test substance A per bee. The LD_{50} (contact) of the test substance A was 97.7 ng, 74.9 ng, 78.4 ng and 69.0 ng test substance A per bee after 24 h, 48 h, 72h and 96 hours. Apathy, discoordinated movements and immobility were observed before bees died. 72 h and 96 h after application the surviving bees had recovered and exhibited no further behavioural anomalies.

Table 2: Mortality of bees in the oral toxicity test with Substance A (group feeding)

treatment	Dose (ng a.i./bee)	mortality (%)		corrected mortality (%) (according to ABBOTT)	
		time after application			
		24 h	48 h	24 h	48 h
Control (sucrose solution)		0	3.3	-	-
Control sucrose solution + acetone (0.005 % v/v)		0	3.3	-	-
Substance **A**	81(70.3)*)	46.7*	46.7*	46.7	44.8
	27 (26.9)*)	10.0	10.0	10.0	6.9
	9 (9)	20.0	20.0	20.0	17.2
	3 (3)	3.3	3.3	3.3	0
	1 (1)	6.7	6.7	6.7	3.5

*) calculated from the quantity of test solution remaining after 3 hours of dose administration (see appendix 2.1)
* statistically significant compared to control ($p \leq 0.05$)

Table 3: Mortality of bees in the contact toxicity test with Substance A (topical application)

treatment	dose (ng a.i./bee)	mortality (%)				corrected mortality (%) (according to ABBOTT)			
		time after application							
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Control (acetone)		3.3	3.3	6.7	6.7	-	-	-	-
Substance A	153.7	76.6*	80.0*	80.0*	80.0*	75.8	79.3	78.6	78.6
	109.8	60.0*	73.3*	73.3*	80.0*	58.6	72.4	71.4	78.6
	78.4	26.7*	50.0*	50.0*	56.6*	24.2	48.3	46.4	53.6
	56.0	23.3*	30.0*	30.0*	36.6*	20.7	27.6	25.0	32.2
	40.0	30.0*	33.3*	33.3*	36.6*	27.6	31.1	28.6	32.2

* statistically significant compared to control ($p \leq 0.05$)

4. Interpretation

Exposure to Substance A resulted in significant effects on honeybee mortality at a dose of 81 ng test substance A (70.3 ng consumed) per bee in the oral toxicity test and significant mortality for 40.0 ng test substance A up to a dose of 153.7 ng test substance A per bee in the contact toxicity test.
The LD_{50} for the oral toxicity could not be determined because the observed mortality was below 50 % up to a dose of 81 ng test substance A.
The LD_{50} for the contact exposure was 74.9 ng test substance A per bee after 48 hours.

The validity criterion - mortality in the control ≤ 10 % - was accomplished (being 6.6 % in the contact and 3.3 % in the oral toxicity tests after 48 hours).

5. References

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6. Signatures

Confirmation of the final report

[redacted]

Study Director: ..... Date

Testing Facility Management: 07.07.09 Date

Sponsor/Monitor: ..... Date

Appendix 1: **Preparation of test solutions - oral toxicity test**

1. *Preparation of sucrose solution (= control)*
- Mixing of sucrose and deionized water in a ratio of 1:1 (w/v); density of the 50 % aqueous sucrose solution: 1.257 g/cm^3

2. *Preparation of test substance solutions*
- Weighed: 0.081 g Substance A
- Dissolving in 1 ml acetone and made up to 24.764 g with 50 % sucrose solution, which gave stock solution (A)
- Food consumption/bee: 20 µl/bee

Preparation of dilutions:

volume of solution	name	+ 50 % sucrose solution	total volume of solution		concentration (ng/bee)
1 ml	A in acetone	19 ml	=20 ml	(A)	4050 ng/µl (81 000 ng/20 µl)
0.25 ml	A	Made up to	=250 ml	(B)	81
3.3 ml	B	+ 6.7 ml	= 10 ml	(D)	27
1.1 ml	B	+ 8.9 ml	= 10 ml	(E)	9
0.37 ml	B	+ 9.63 ml	= 10 ml	(F)	3
0.12 ml	B	+ 9.88 ml	= 10 ml	(G)	1

The solutions B to G were used for dosing.

Appendix 2: **Preparation of test solutions - contact toxicity test**

2. *Preparation of test substance solutions*

- Weighed: 0.1537 g Substance A
- Made up to 100 ml with acetone which gave a (stock solution A): 153.7 mg/100 ml = 1537 ng/µl

volume of solution	name	acetone	total volume of solution		concentration (ng/µl)
10 ml	A	Made up to	= 100 ml	(B)	153.7
7.1 ml	B	+ 2.9 ml	= 10 ml	(C)	109.8
5.1 ml	B	+ 4.9 ml	= 10 ml	(D)	78.4
3.6 ml	B	+ 6.4 ml	= 10 ml	(E)	56.0
2.6 ml	B	+ 7.4 ml	= 10 ml	(F)	40.0

The solutions B to F were used for dosing.

Appendix 3: **Table of results – oral toxicity test**

treatment	replicate	consumed test solution per 10 bees (g)*	a.i. per bee (ng)**	Dead bees per cage (number) after application	
				24 h	48 h
control (sucrose solution)	1	0.251		0	0
	2	0.251		0	1
	3	0.251		0	0
	mean			Σ 0	1
Control (sucrose solution + acetone 0.005 % v/v)	1	0.250		0	1
	2	0.251		0	0
	3	0.251		0	0
	mean			Σ 0	1
Substance **A**	1	0.251	1	1	1
	2	0.251	1	0	0
	3	0.251	1	1	1
	mean		1	Σ 2	2
	1	0.251	3	0	0
	2	0.251	3	0	0
	3	0.251	3	1	1
	mean		3	Σ 1	1
	1	0.251	9	2	2
	2	0.251	9	3	3
	3	0.251	9	1	1
	mean		9	Σ 6	6
	1	0.249	26.8	3	3
	2	0.251	27.0	0	0
	3	0.249	26.8	0	0
	mean		26.9	Σ 3	3
	1	0.234	75.5	6	6
	2	0.218	70.4	2	2
	3	0.201	64.9	6	6
	mean		70.3	Σ 14	14

* all test solutions administered to each replicate as 0.251 g

** calculated from the quantity of test solution remaining after 3 hours of dose administration

Appendix 4: **Table of results – contact toxicity test**

treatment	substance per bee (ng)	replicate	Dead bees per cage (number) after application			
			24 h	48 h	72 h	96 h
control (sucrose solution)		1	1	1	1	1
		2	0	0	1	1
		3	0	0	0	0
			Σ 1	1	2	2
Substance **A**	40.0	1	3	4	4	5
		2	4	4	4	4
		3	2	2	2	2
			Σ 9	10	10	11
	56.0	1	4	5	5	5
		2	1	1	1	3
		3	2	3	3	3
			Σ 7	9	9	11
	78.4	1	3	7	7	7
		2	2	4	4	5
		3	3	4	4	5
			Σ 8	15	15	17
	109.8	1	8	9	9	10
		2	7	8	8	9
		3	3	5	5	5
			Σ 18	22	22	24
	153.7	1	8	8	8	8
		2	8	9	9	9
		3	7	7	7	7
			Σ 23	24	24	24

Amendment to Report No. 00 10 48 501

Identification of test substance

Code name in report:	Test substance A
Name of test substance:	NTN33893 (a.i.)
Origin of test substance:	Bayer AG, Leverkusen PF-Production
Specification	
Tox. no.:	5255
Article no.:	04145852
Batch no:	230924394
a.i. content:	98.6 %
Date of analysis:	30.3.2000
Expiry date:	30.10.2000
Delivered to:	Bayer AG Institute for Environmental Biology Laboratory for non-target arthropods Internal laboratory no. 218
Date of reception:	13.4.2000
Contract laboratory:	Biochem GmbH, Cunnersdorf
Date of delivery as substance A:	18.4.2000
Delivered amount:	1.13 g
Order no.:	347517E0

Leverkusen, 21.6.00

[redacted]