

REPORT

HONEYBEE (*APIS MELLIFERA* L.) CONTACT TOXICITY STUDY IN THE LABORATORY WITH IMIDACLOPRID TECHN.

Study Director

Test facility

Sponsor

Study report completion

Number of report pages

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**Ambrosiushoeve
Hilvarenbeek
The Netherlands**

**Bayer AG
Institute for Environmental Biology
D-51368 Leverkusen**

02-09-1999

20



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REPORT APPROVAL

Study Director

[Redacted]
Ambrosiushoeve

[Redacted]

date: 22-09-1999

Principal Investigator

[Redacted]
Ambrosiushoeve

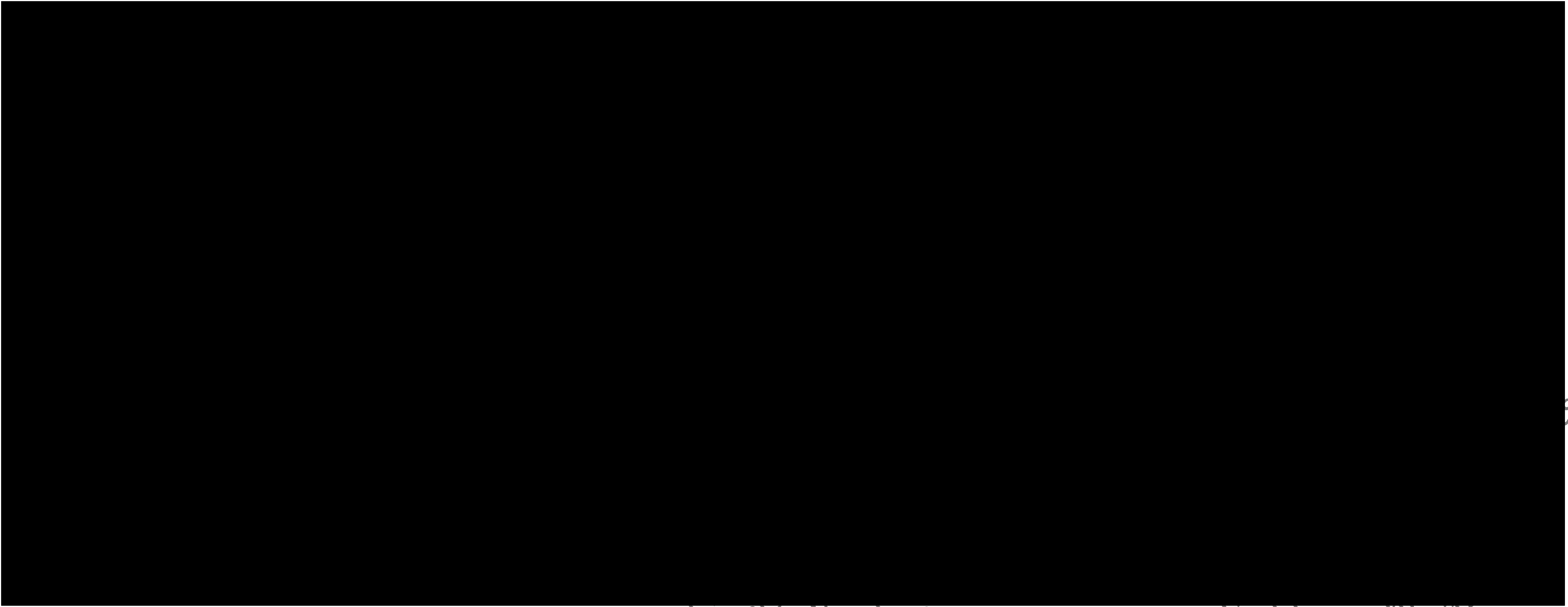
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STATEMENT OF GLP COMPLIANCE (Quality Assurance)

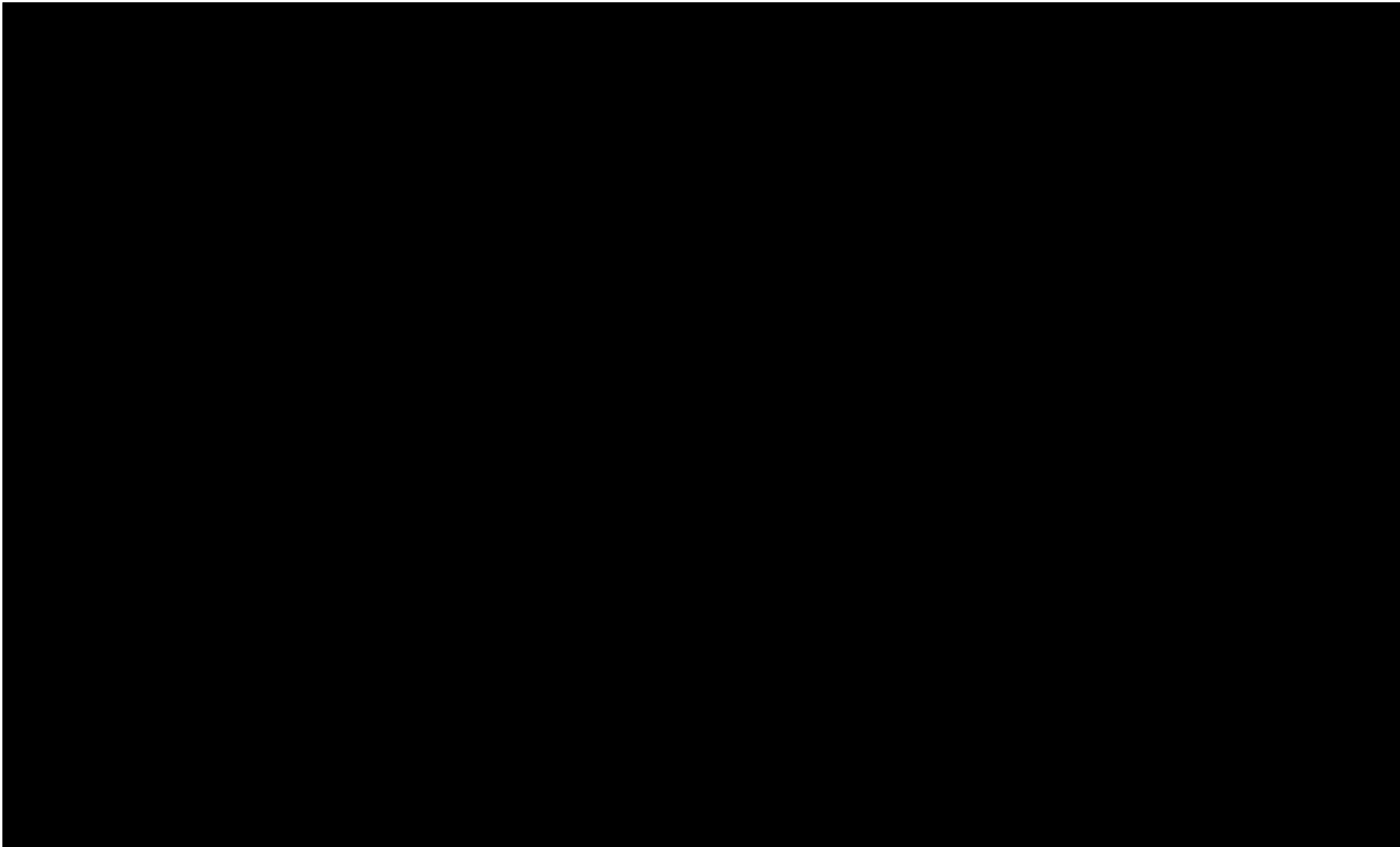


Quality Assurance Officer



Date: 1999-09-22

ANNEX to STATEMENT OF GLP COMPLIANCE



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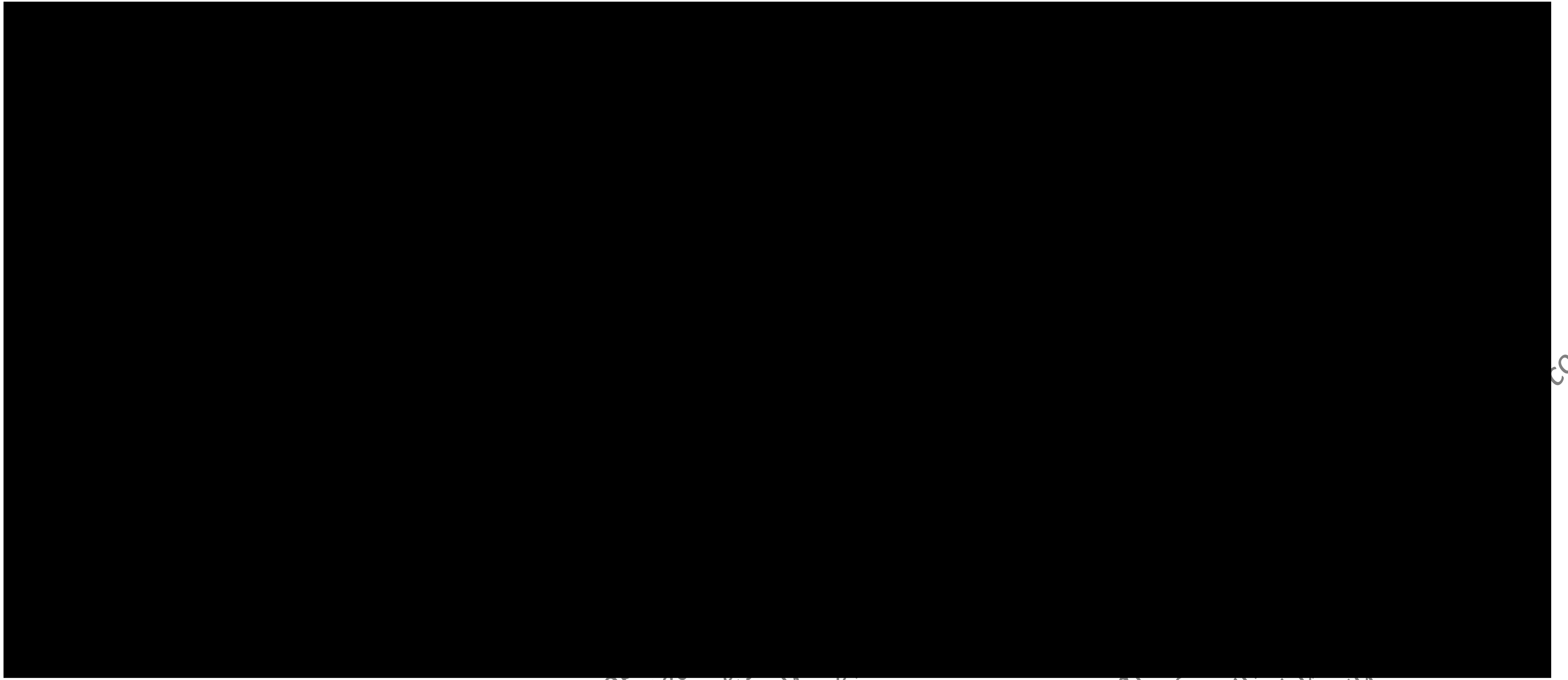
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Quality Assurance Officer



Date: 1999-07-22

STATEMENT OF GLP COMPLIANCE (Study Director)



contents

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Study Director



Date:.....22 - 09 - 1999

SUMMARY

The purpose of the toxicity study was to examine the effects of imidacloprid techn. on honeybees when applied in the laboratory. Individual honeybees were exposed to imidacloprid techn. by way of administration of imidacloprid techn., dissolved in acetone, on the ventral part of the thorax.

The sponsor indicated that the contact LD₅₀ was between 40 and 200 ng / honeybee. That is why a concentration range of about 40 ng to 200 ng imidacloprid / honeybee was tested.

Per concentration honeybees were treated with 1 µl acetone containing respectively: 207 ng imidacloprid techn., 166 ng imidacloprid techn., 125 ng imidacloprid techn., 85 ng imidacloprid techn. and 42 ng imidacloprid techn. per 1 µl.

The treatment was compared to an acetone treatment (negative control) and a Dimethoate positive control.

The concentrations imidacloprid techn. administered to the honeybees in this test caused mortality of the honeybees. Mortality was preceded by effect. The most significant effect was the “frozen behaviour” at which the honeybees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus. The first signs of effect were observed within 30 minutes after administration of imidacloprid techn.

Mortality continued during the observation period.

The LD₅₀ of Imidacloprid techn. based on the linear regression is:

- LD₅₀ (72 hours): 129 ng imidacloprid techn. ($r^2 = 0.42$)

The ED₅₀, of Imidacloprid techn. based on the linear regression is:

- ED₅₀ (72 hours): 131 ng imidacloprid techn. ($r^2 = 0.38$)

The effect of imidacloprid techn. administered in the concentrations from 40 to 200 ng / honeybee is clear. The typical “frozen behaviour” is observed in all concentrations tested. Mortality continued during the test period and because honeybees that are immobilised for several days eventually die, after 72 hours the LD₅₀ and the ED₅₀ are in the same range.

ARCHIVING AND STORAGE

The original signed protocol, the original signed report and a copy of the raw data are archived by the sponsor.

A copy of the protocol, a copy of the report a reference sample of the test substance and the raw data are archived by Ambrosiushoeve.

PREFACE

GENERAL

Title Honeybee (*Apis mellifera* L.) contact toxicity study in the laboratory with imidacloprid techn.

Project nr AH99.4.22.3.

Sponsor Bayer AG
Agricultural Centre
Institute for Environmental Biology
Representative: [REDACTED]

Test facility Ambrosiushoeve
Ambrosiusweg 1
5081 NV Hilvarenbeek
The Netherlands

Study Director [REDACTED]
Ambrosiushoeve
Ambrosiusweg 1
5081 NV Hilvarenbeek
The Netherlands

Principal Investigator [REDACTED]
Ambrosiushoeve
Ambrosiusweg 1
5081 NV Hilvarenbeek
The Netherlands

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SCHEDULE

Start experiments	29-06-1999
End observations	02-07-1999

QUALITY ASSURANCE

This study is performed in compliance with the Ambrosiushoeve Standard Operating Procedures and with the most recent edition of:

OECD Principles of Good Laboratory Practise (as revised in 1997) ENV/MC/CHEM(98)17 (OECD Paris 1998)

GUIDELINE

Guideline on test methods for evaluating the side effects of plant protection products on honeybees.

European and Mediterranean plant protection organisation Bulletin EPPO 22, 203-215 (1992).

DEFINITIONS

LD₅₀ is the lethal dose value in ng product per honeybee, which kills 50% of the honeybees within in a defined period of time.

ED₅₀ is the dose value in ng product per honeybee, which brings about an effect on 50 % of the honeybees within in a defined period of time.

Mortality: honeybees are considered to be dead when they don't respond on stimuli like touching with a pair of tweezers.

Effect: Effect is mortality + paralysis + "frozen behaviour" at which the honeybees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus.

A replicate is the same test substance in a certain concentration applied to 10 honeybees from another hive

OBJECTIVE

PURPOSE AND RATIONALE

The purpose of the toxicity study was to examine the effects of imidacloprid techn. on honeybees when applied in the laboratory. Individual honeybees were exposed to imidacloprid techn. by way of administration of imidacloprid techn., dissolved in acetone, on the ventral part of the thorax. Per concentration honeybees were treated with 1 μ l acetone containing respectively: 207 ng imidacloprid techn., 166 ng imidacloprid techn, 125 ng imidacloprid techn, 85 ng imidacloprid techn and 42 ng imidacloprid techn per 1 μ l.

The treatment was compared to an acetone treatment (negative control) and a Dimethoate positive control.

This study provides a rational basis for the assessment of toxicological risk to honeybees.

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MATERIAL AND METHODS

TEST SYSTEM

Test system	Honeybees, <i>Apis mellifera mellifera</i> L. (Hymenoptera: Apidae).
Rationale	Recognised by the cited EPPO guideline as the recommended test system.
Source	Research Centre for Insect Pollination and Beekeeping. "Ambrosiushoeve"; responsible beekeeper: [REDACTED]
Sampling	Bees were collected from combs without brood.
Number of animals per cage used for testing	10 worker honeybees.
Number of animals treated per concentration	3 x 10 worker honeybees (three cages of 10 honeybees = 3 replicates)
Randomisation	Allocation of the honeybees to any particular treatment was by chance.

TEST SUBSTANCE

Identification: imidacloprid techn.
 Description: solid powder
 colour: beige
 Batch: PT 230824088
 Composition: 1-((6-chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine
 CAS no.: 138261-41-3
 Article no.: 04145852
 Tox. no.: 4941-00
 Purity: 98.6%,
 Analytical method: HPLC, ext. Std.
 Expiry date: 03-09-1999
 Stability for at least
 48 Hours in vehicle:

water	stable
acetone	stable

REFERENCE SUBSTANCE

Identification: Dimethoate
 Description: blue liquid
 Batch: 92511812
 Composition: 400 g dimethoate / litre
 Expiry date: December 2000

HUSBANDERY / ENVIRONMENTAL CONDITIONS

The honeybees were kept in groups of 10, in 10 cm wide, 5.5 cm deep and 8.5 cm high stainless steel cages with front walls made of glass for observation of the honeybees and perforated bottoms for ventilation. The stainless steel parts were lined with white paper (No. 68 Macher-Nagel & Co. D-5165 Düren).

Apart from oral dosing and during starvation, a 50% aqueous sucrose-solution was provided ad libitum for food.

The honeybees were kept in a dark room with a continuously monitored environment of $25 \pm 2^\circ\text{C}$. During feeding, the honeybees are in the same room in the light. Observations were carried out using red light.

TEST ARTICLE PREPARATION

Imidacloprid techn.

- Stock solution: 0.0207 gram imidacloprid techn. dissolved in 79.9672 gram acetone which resulted in a concentration of 207 ng / 1 μl . From this stock solution a dilution range was made:
- 4.0013 gram stock solution + 0.9902 gram acetone resulted in an imidacloprid techn. concentration of 166 ng / 1 μl ,
- 3.0020 gram stock solution + 1.9837 gram acetone resulted in an imidacloprid techn. concentration of 125 ng / 1 μl ,
- 2.0846 gram stock solution + 3.0154 gram acetone resulted in an imidacloprid techn. concentration of 85 ng / 1 μl ,
- 1.0194 gram stock solution + 4.0009 gram acetone resulted in an imidacloprid techn. concentration of 42 ng / 1 μl .

Dimethoate:

The LD_{50} of Dimethoate is about 0.2 μg a.i. / honeybee. A concentration range of approximately 0.04, 0.2 and 0.4 μg dimethoate a.i./ honeybee = 1.0, 0.5 and 0.1 μg Dimethoate formulation was tested.

- Stock solution: 0.0125 gram Dimethoate was dissolved in 8.3159 gram acetone. This resulted in the concentration of 1.20 μg Dimethoate / 1 μl acetone.
- 1.9988 gram stock solution was dissolved in 1.9795 gram acetone. This resulted in the concentration of 0.60 μg Dimethoate / 1 μl acetone.
- 0.9853 gram 0.0125 stock solution was dissolved in 8.9158 gram acetone. This resulted in the concentration of 0.12 μg Dimethoate / 1 μl acetone.

Acetone:

- Acetone. The density of acetone is 0.8 g/ml.

TREATMENT

Naive honeybees were taken from combs without brood and transferred to the test cages (10 honeybees per cage). Per cage the honeybees were narcotised with CO₂. Acetone with imidacloprid techn. in different concentrations was administered on the ventral part of the thorax (1 µl / honeybee). After administration of the test substance, the honeybees were provided with sucrose-solution 50%.

The sucrose-solution was changed daily.

The sponsor indicated that the contact LD₅₀ was between 40 and 200 ng / honeybee. That is why a concentration range of about 40 ng to 200 ng imidacloprid / honeybee was tested.

In the test, 5 concentrations of imidacloprid techn.: 207, 166, 125, 85 and 42 ng/1µl were administered to the honeybees. Each concentration was administered to 3 cages (3 x 10 honeybees = 3 replicates).

In the positive control 3 concentrations: 1.20, 0.60 and 0.12 µg Dimethoate / 1 µl were administered to the honeybees

Each concentration was administered to 3 cages (3 x 10 honeybees).

In the negative control acetone was administered to the honeybees.

The percentage of mortality per cage of both the test substance and the positive control were calculated per cage.

The test was carried out once.

OBSERVATIONS

Behaviour abnormalities, e.g. paralysis, uncoordinated movements or any locomotor disabilities as well as mortality were recorded several times directly after treatment and 24, 48 and 72 hours after treatment.

RESULTS

DATA / TABLES

Table 1
Imidacloprid techn.; mortality and other effects during the observation period of the honeybees treated with 207 ng / 1µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		1	2	3	1	2	3	1	2	3
29-06-1999	4:30	0	0	0	9	10	9	0	0	0
30-06-1999	25:19	2	1	4	7	8	6	0	0	0
01-07-1999	48:49	7	4	6	3	2	2	0	0	0
02-07-1999	73:11	9	8	8	1	0	0	0	0	0

Table 2
Imidacloprid techn.; Percentages mortality and percentages effect of the honeybees, treated with 207 ng / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		1	2	3	1	2	3
29-06-1999	4:30	0%	0%	0%	90%	100%	90%
30-06-1999	25:19	20%	10%	40%	90%	90%	100%
01-07-1999	48:49	70%	40%	60%	100%	60%	80%
02-07-1999	73:11	90%	80%	80%	100%	80%	80%

table 3
Imidacloprid techn.; mortality and other effects during the observation period of the honeybees treated with 166 ng / 1µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		1	2	3	1	2	3	1	2	3
29-06-1999	4:34	0	0	7	4	2	1	5	8	5
30-06-1999	25:21	0	4	1	1	3	1	0	0	0
01-07-1999	48:50	4	6	1	4	1	1	0	0	0
02-07-1999	73:13	10	8	1	0	0	0	0	0	0

Table 4
Imidacloprid techn.; Percentages mortality and percentages effect of the honeybees, treated with 166 ng / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		1	2	3	1	2	3
29-06-1999	4:34	0%	0%	10%	90%	100%	70%
30-06-1999	25:21	0%	40%	10%	10%	70%	20%
01-07-1999	48:50	40%	60%	10%	80%	70%	20%
02-07-1999	73:13	100%	80%	10%	100%	80%	10%

table 5

Imidacloprid techn.; mortality and other effects during the observation period of the honeybees treated with 125 ng / 1µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3
29-06-1999	4:37	0	0	0	7	4	2	3	5	6
30-06-1999	25:19	2	3	0	2	2	1	0	3	0
01-07-1999	48:52	3	5	2	0	2	0	0	0	0
02-07-1999	73:15	4	9	2	0	0	0	0	0	0

Table 6

Imidacloprid techn.; Percentages mortality and percentages effect of the honeybees, treated with 125 ng / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		replicate			replicate		
		1	2	3	1	2	3
29-06-1999	4:37	0%	0%	0%	100%	90%	80%
30-06-1999	25:19	20%	30%	0%	40%	80%	10%
01-07-1999	48:52	30%	50%	20%	30%	70%	20%
02-07-1999	73:15	40%	90%	20%	80%	90%	20%

table 7

Imidacloprid techn.; mortality and other effects during the observation period of the honeybees treated with 85 ng / 1µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3
29-06-1999	4:42	0	0	0	2	0	2	7	10	5
30-06-1999	25:23	2	0	0	1	6	0	0	0	0
01-07-1999	48:56	6	4	2	0	1	0	0	0	0
02-07-1999	73:17	6	4	2	0	0	0	0	0	0

Table 8

Imidacloprid techn.; Percentages mortality and percentages effect of the honeybees, treated with 85 ng / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		replicate			replicate		
		1	2	3	1	2	3
29-06-1999	4:42	0%	0%	0%	90%	100%	70%
30-06-1999	25:23	20%	0%	0%	30%	60%	0%
01-07-1999	48:56	60%	40%	20%	60%	50%	20%
02-07-1999	73:17	60%	40%	20%	60%	40%	20%

table 9

Imidacloprid techn.; mortality and other effects during the observation period of the honeybees treated with 42 ng / 1 µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3
29-06-1999	4:44	0	0	0	2	0	0	5	8	6
30-06-1999	25:21	1	2	0	1	0	1	0	0	0
01-07-1999	48:56	4	2	1	0	0	0	0	0	0
02-07-1999	73:15	4	2	1	0	0	0	0	0	0

Table 10

Imidacloprid techn.; Percentages mortality and percentages effect of the honeybees, treated with 42 ng / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		replicate			replicate		
		1	2	3	1	2	3
29-06-1999	4:44	0%	0%	0%	70%	80%	60%
30-06-1999	25:21	10%	20%	0%	20%	20%	10%
01-07-1999	48:56	40%	20%	10%	40%	20%	10%
02-07-1999	73:15	40%	20%	10%	40%	20%	10%

table 11

Dimethoate; administered and mortality and other effects during the observation period of the honeybees treated with 1.20 µg / 1 µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3
29-06-1999	2:36	1	0	0	0	1	1	0	0	0
30-06-1999	26:29	10	10	10	0	0	0	0	0	0
01-07-1999										
02-07-1999										

Table 12

Dimethoate; Percentages mortality and percentages effect of the honeybees, treated with 1.20 µg / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		replicate			replicate		
		1	2	3	1	2	3
29-06-1999	2:36	10%	0%	0%	10%	10%	10%
30-06-1999	26:29	100%	100%	100%	100%	100%	100%
01-07-1999							
02-07-1999							

table 13

Dimethoate; mortality and other effects during the observation period of the honeybees treated with 0.60 µg / 1µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3
29-06-1999	2:34	0	0	1	0	0	0	0	0	0
30-06-1999	26:35	7	10	10	0	0	0	0	0	0
01-07-1999	48:05	7	10	10	0	0	0	0	0	0
02-07-1999	72:51	7	10	10	0	0	0	0	0	0

Table 14

Dimethoate; Percentages mortality and percentages effect of the honeybees, treated with 0.60 µg / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		replicate			replicate		
		1	2	3	1	2	3
29-06-1999	2:34	0%	0%	10%	0%	0%	10%
30-06-1999	26:35	70%	100%	100%	70%	100%	100%
01-07-1999	48:05	70%	100%	100%	70%	100%	100%
02-07-1999	72:51	70%	100%	100%	70%	100%	100%

table 15

Dimethoate; mortality and other effects during the observation period of the honeybees treated with 0.12 µg / 1µl acetone

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3
29-06-1999	2:49	0	0	0	0	0	0	0	0	0
30-06-1999	26:39	0	0	0	0	1	0	0	0	0
01-07-1999	48:09	0	1	0	0	0	0	0	0	0
02-07-1999	72:55	0	1	0	0	0	0	0	0	0

Table 16

Dimethoate; Percentages mortality and percentages effect of the honeybees, treated with 0.12 µg / 1 µl acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		replicate			replicate		
		1	2	3	1	2	3
29-06-1999	2:49	0%	0%	0%	0%	0%	0%
30-06-1999	26:39	0%	0%	0%	0%	10%	0%
01-07-1999	48:09	0%	10%	0%	0%	10%	0%
02-07-1999	72:55	0%	10%	0%	0%	10%	0%

table 17

Acetone; mortality and effect during the observation period.

Date	observation after (hours)	mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		replicate			replicate			replicate		
		1	2	3	1	2	3	1	2	3
29-06-1999	4:55	0	0	0	0	0	0	0	0	0
30-06-1999	25:32	0	1	0	0	0	0	0	0	0
01-07-1999	49:07	0	1	0	0	0	0	0	0	0
02-07-1999	73:21	1	1	0	0	0	0	0	0	0

Table 18

Acetone; Percentages mortality and percentages effect of the honeybees treated with acetone

Date	observation after (hours)	percentage mortality			percentage effect		
		replicate			replicate		
		1	2	3	1	2	3
29-06-1999	4:55	0%	0%	0%	0%	0%	0%
30-06-1999	25:32	0%	10%	0%	0%	10%	0%
01-07-1999	49:07	0%	10%	0%	0%	10%	0%
02-07-1999	73:21	10%	10%	0%	0%	10%	0%

MORTALITY AND OTHER EFFECTS

The concentrations imidacloprid techn., administered to the honeybees in this test, caused mortality of the honeybees. Mortality was preceded by effect. The most significant effect is the “frozen behaviour” at which the honeybees are motionless except a little trembling of body parts like abdomen, antennae or tarsus. The first signs of effect were observed within 30 minutes after administration of imidacloprid techn.

Mortality continued during the observation period.

STATISTICAL ANALYSIS

For the statistical analysis, the data were corrected for control mortality (mean mortality of the three cages treated with acetone).

For the correction, the formula of Schneider Orelli: %effect = ((b-k) / (100-k)) x 100

b = percentage mortality / test cage

k = percentage mortality of negative control (mean of the 3 test cages).

The LD₅₀ of Imidacloprid techn. based on the linear regression is:

- LD₅₀ (72 hours): 129 ng imidacloprid techn. (r² = 0.42)

The ED₅₀ of Imidacloprid techn. based on the linear regression is:

- LD₅₀ (72 hours): 131 ng imidacloprid techn. (r² = 0.38)

EVALUATION OF THE RESULTS

The effect of imidacloprid techn., administered in the range from 40 to 200 ng / honeybee is clear. The typical “frozen behaviour” is observed at all concentrations tested. Mortality increased during the test period and after 72 hours the LD₅₀ and the ED₅₀ are in the same range.

VALIDY OF THE TEST

The LD₅₀ (24 hours) of Dimethoate, based on the data obtained in this test is 0.52 µg / honeybee (= 0.21 µg a.i. / honeybee). The acceptable range of the LD₅₀ of Dimethoate in 24 hours is 0.05 to 0.4 µg a.i. / honeybee. The mortality in the control does not exceed 15%. The results in the positive and negative control meet the standards. The test can be considered as valid and the effects observed in the honeybees treated with imidacloprid techn. can be considered as caused by imidacloprid techn.

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