REPORT

BUMBLEBEE (*BOMBUS TERRESTRIS* L.) ORAL TOXICITY STUDY IN THE LABORATORY WITH IMIDACLOPRID TECHN.

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Sponsor	**Bayer AG** **Institute for Environmental Biology** **D-51368 Leverkusen**
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REPORT APPROVAL

Study Director

[redacted]
Ambrosiushoeve

[redacted]

date: 22-09-1999

Principal Investigator

[redacted]
Ambrosiushoeve

[redacted]

date: 22-09-1999

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Quality Assurance Officer [redacted]

Date: 1999-09-22

ANNEX to STATEMENT OF GLP COMPLIANCE

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Quality Assurance Officer [redacted]

Date: 1999-09-22

STATEMENT OF GLP COMPLIANCE (Study Director)

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Study Director [redacted]

Date: 22 - 09 - 1999

SUMMARY

The purpose of the toxicity study was to examine the effects of imidacloprid techn. on bumblebees when applied in the laboratory.

Per concentration 30 bumblebees were fed individually with 10 µl sucrose solution 50%, containing a range of concentrations of imidacloprid techn.

A range finding test preceded the definitive test. It appeared that 1.1 µg imidacloprid techn, per bumblebee killed 97% of the bumblebees, within 24 hours. The oral intake of 0.1 µg imidacloprid techn. per bumblebee, affected 90% of the bumblebees within 24 hours. Mortality was 0% after 48 hours. No effects on behaviour or survival were observed for doses of 0.01 µg or less imidacloprid techn. Based on these data, 0.96 µg, 0.72 µg, 0.53 µg, 0.33 µg and 0.11 µg imidacloprid techn. per 10 µl was offered to the bumblebees.

All concentrations fed in the definitive test, resulted in effect on the bumblebees. The most significant effect was the "frozen behaviour" at which the bumblebees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus. Beside that, spasms and paralysis were observed as well. These effects lasted at least during the observation period of 72 hours. Most of the affected bumblebees which had taken in amounts of imidacloprid techn. of 0.33 µg / bumblebee or more died within 24 hours.
Amounts of imidacloprid techn. higher than 0.11 µg per bumblebee, cause effect and mortality of the bumblebees.
The LD_{50} of imidacloprid techn. based on the linear regression is:

- LD_{50} (24 hours): 0.33 µg imidacloprid techn. ($r^2 = 0.73$)
- LD_{50} (48 hours): 0.22 µg imidacloprid techn. ($r^2 = 0.53$)
- LD_{50} (72 hours): 0.15 µg imidacloprid techn. ($r^2 = 0.53$)

The effect of imidacloprid techn., in the concentrations higher than 0.1 µg / bumblebee is obvious. The ED_{50} is between 0.1 and 0.01 µg / bumblebee. The data provide no basis for an accurate ED_{50} calculation.

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.

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PREFACE

GENERAL

Title	Bumblebee (*Bombus terrestris* L.) oral toxicity study in the laboratory with imidacloprid techn.
Project nr	AH99.4.22.2
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	09-06-1999
End observations	18-06-1999

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

The study procedures described in this protocol are based on the guideline:
Methods to determine the acute toxicity of pesticides for bumblebees (*Bombus terrestris* L.) in the laboratory
Steen, J.J.M. van der, Gretenkord, C., Schaefer, H.
Proceedings of the 6th ICPBR International Symposium on Hazard of Pesticides to Bees. 17-19 Sept 1996 Braunschweig, Germany

DEFINITIONS

LD_{50} is the lethal dose value in µg product per bumblebee, which kills 50 % of the bumblebees within in a defined period of time.
ED_{50} is the dose value in µg product per bumblebee, which brings about an effect on 50 % of the bumblebees within in a defined period of time.
Mortality: bumblebees are considered to be dead when they don't respond on stimuli like touching with a pair of tweezers.
Effect: Effect is mortality + paralysis + spasm + "frozen behaviour" at which the bumblebees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus.

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OBJECTIVE

PURPOSE AND RATIONALE

The purpose of the toxicity study was to examine the effects of imidacloprid techn. on bumblebees when applied in the laboratory.
Per concentration 30 bumblebees were fed individually with 10 µl sucrose solution 50%, containing a range of concentrations of imidacloprid techn.
A range finding test preceded the definitive test. It appeared that 1.1 µg imidacloprid techn, per bumblebee killed 97% of the bumblebees fed, within 24 hours. The oral intake of 0.1 µg imidacloprid techn. per bumblebee, affects 90% of the bumblebees within 24 hours. Mortality was 0% after 48 hours.
Based on these data, 0.96 µg, 0.72 µg, 0.53 µg, 0.33 µg and 0.11 µg imidacloprid techn. per 10 µl was offered to the bumblebees.
The treatment was compared to a 50 % sucrose-solution (negative control) and a Dimethoate positive control.
This study provides a rational basis for the assessment of toxicological risk to bumblebees.

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MATERIAL AND METHODS

TEST SYSTEM

Test system	Bumblebees, *Bombus terrestris* L. (Hymenoptera: Apidae).
Source	Research Centre for Insect Pollination and Beekeeping. "Ambrosiushoeve" Per study, the bumblebees originate from a pool of bumblebee colonies, reared by Ambrosiushoeve
Sampling	Bumblebees used in the test were collected from different colonies and were randomised by chance
Number of animals per group	30 worker bumblebees
Number of groups	Nine five groups fed with 5 concentrations test substance in sucrose solution 50% one group fed with the negative control substance (sucrose solution 50%) three groups fed with three concentrations of the positive control substance (Dimethoate in sucrose solution 50 %)
Identification	Each cage used for testing was labelled uniquely.
Randomisation	Allocation of the bumblebees to any particular treatment by chance

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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

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HUSBANDERY / ENVIRONMENTAL CONDITIONS

The bumblebees were kept in groups of 30 in 10 cm wide, 4 cm deep and 10 cm high wooden cages with front walls made of glass for observation of the bumblebees.
Apart from oral dosing and during starvation, a 50% aqueous sucrose solution was provided ad libitum for food.
The bumblebees were kept in a dark room with a continuously monitored environment of 25±2°C.
Treatment of the bumblebees was carried out in the same room in the light. Observations were carried out using red light.

TEST ARTICLE PREPARATION

imidacloprid techn.
On 14-06-1999. 0.0095 gram imidacloprid techn was dissolved in 119.35 gram 50% sucrose-solution (0.96 µg / 10 µl). The solution was stirred overnight. This resulted in a clear solution.
On 15-06-1999 dilutions were made.

- 1.0117 gram imidacloprid solution (0.96 µg / 10 µl) + 0.3337 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 0.72 µg / 10 µl,
- 1.0148 gram imidacloprid solution (0.96 µg / 10 µl) + 0.8142 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 0.53 µg / 10 µl,
- 0.5187 gram imidacloprid solution (0.96 µg / 10 µl) + 1.0023 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 0.33 µg / 10 µl.
- 0.4975 gram imidacloprid solution (0.96 µg / 10 µl) + 3.9862 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 0.11 µg / 10 µl.

Dimethoate:
The specific range of the LD_{50} of Dimethoate is about 0.5 to 2.5 µg a.i. / bumblebee. A concentration range of approximately 1, 1.5 and 2 µg dimethoate a.i./ bumblebee = 2.5, 3.75 and 5 µg Dimethoate formulation was tested.

- 0.0522 gram Dimethoate was dissolved in 120.78 gram 50% sucrose-solution. This resulted in the concentration of 5.19 µg Dimethoate / 10 µl sucrose-solution 50%,
- 5.0556 gram Dimethoate (concentration 5.19 µg / 10 µl) + 1.6720 gram sucrose-solution 50% resulted in a Dimethoate 40 % concentration of 3.90 µg / 10 µl,
- 2.5029 gram Dimethoate (concentration 5.19 µg / 10 µl) + 2.5263 gram sucrose-solution 50% resulted in a Dimethoate 40 % concentration of 2.58 µg / 10 µl.

Sucrose-solution 50%:

- 198.58 gram sucrose was dissolved in 200.22 gram tapwater. This resulted in a sucrose-solution 50%. The density of the sucrose-solution was 1.2 g/ml.

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TREATMENT

The range finding test preceded the acute oral LD_{50} test.

Worker bees of average size were taken from young colonies. In order to select the bumblebees, only bumblebees between 0.15 grams to 0.23 grams were used for the test. The bumblebees were anaesthetised with CO_2 and weighed individually

The bumblebees remained together, deprived of food, for 2 to 3 hours in test cages. After this period the bumblebees were individually housed in test cups of 4 centimetres diameter and 3 centimetres height.

The bumblebees were activated to search for the test solution by pipetting 10 µl sucrose solution (50%) next to the little hole through which the bumblebee could reach the test substance.

The test solutions were offered to 40 bumblebees. After the starvation period, 10 µl test solution was pipetted on microscope glass cover slips outside the test cup. The test cup was placed next to the drop of test solution in a way the bumblebee could swallow the test solution through the little hole.

The bumblebees had two hours to take in the test solution.

As soon as 30 bumblebees had taken in the test solution, the feeding period stopped

After the intake period, the bumblebees fed with the same concentration of the test substance, were housed together and fed with sucrose solution ad libitum. The sucrose-solution was changed daily.

In the test, 5 concentrations of imidacloprid techn.: 0.96, 0.72, 0.53, 0.33 and 0.11 µg/10µl were fed to the bumblebees. In the positive control 3 concentrations: 5.16, 3.90 and 2.58 µg / 10 µl Dimethoate were fed to the bumblebees. In the negative control a 50% sucrose solution was offered to the bumblebees.

The test was carried out once.

OBSERVATIONS

Behaviour abnormalities, e.g. paralysis, uncoordinated movements or any locomotor disabilities as well as mortality were recorded 26 hours, 51 hours and 73 hours after feeding started.

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RESULTS

DATA / TABLES

Table 1
Mortality and effect during the observation period of the bumblebees fed with 0.96 µg imidacloprid techn. / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	26:10	30	100%	30	100%

Table 2
Mortality and effect during the observation period of the bumblebees fed with 0.72 µg imidacloprid techn. / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	26:33	26	87%	30	100%
17-06-1999	52:37	29	97%	30	100%
18-06-1999	74:14	30	100%	30	100%

Table 3
Mortality and effect during the observation period of the bumblebees fed with 0.53 µg imidacloprid techn. / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	26:08	26	87%	30	100%
17-06-1999	52:10	29	97%	30	100%
18-06-1999	73:48	30	100%	30	100%

Table 5
Mortality and effect during the observation period of the bumblebees fed with 0.33 µg imidacloprid techn. / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	25:50	22	73%	30	100%
17-06-1999	51:52	29	97%	30	100%
18-06-1999	73:29	29	97%	30	100%

Table 6
Mortality and effect during the observation period of the bumblebees fed with 0.11 µg imidacloprid techn. / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	25:29	1	3%	30	100%
17-06-1999	51:29	1	3%	30	100%
18-06-1999	73:05	4	13%	30	100%

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Table 7
Mortality and effect during the observation period of the bumblebees fed with 5.19 µg Dimethoate / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	25:05	29	97%	29	97%
17-06-1999	51:05	29	97%	29	97%
18-06-1999	72:44	30	100%	30	100%

Table 8
Mortality and effect during the observation period of the bumblebees fed with 3.90 µg Dimethoate / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	24:47	26	87%	26	87%
17-06-1999	50:54	26	87%	26	87%
18-06-1999	72:25	26	87%	26	87%

Table 9
Mortality and effect during the observation period of the bumblebees fed with 2.58 µg Dimethoate / 10µl sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	24:23	9	30%	9	30%
17-06-1999	50:21	9	30%	9	30%
18-06-1999	71:55	10	33%	10	33%

Table 10
Sucrose solution 50%
Mortality and other effects during the observation period of the bumblebees fed with sucrose-solution 50%.

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
16-06-1999	24:00	0	0%	0	0%
17-06-1999	49:56	0	0%	0	0%
18-06-1999	71:36	0	0%	0	0%

MORTALITY AND OTHER EFFECTS

All concentrations fed in the definitive test, resulted in effect on the bumblebees. The most significant effect was the "frozen behaviour" at which the bumblebees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus. Beside that, spasms and paralysis were observed as well. These effects lasted at least during the observation period of 72 hours.
Most affected bumblebees which had taken in amounts of imidacloprid 0.33 µg / bumblebee or more died within 24 hours.
Amounts of imidacloprid techn. 0.11 µg per bumblebee or higher, cause effect and mortality of the bumblebees. Amounts of imidacloprid techn. of 0.01 µg per bumblebee and less, do not cause effect and mortality of the bumblebees (result range finding test).

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STATISTICAL ANALYSIS

As there is no mortality in the negative control, the data are not corrected for control mortality.
The LD_{50} of Imidacloprid techn. based on the linear regression is:

- LD_{50} (24 hours): 0.33 µg imidacloprid techn. ($r^2 = 0.73$)
- LD_{50} (48 hours): 0.22 µg imidacloprid techn. ($r^2 = 0.53$)
- LD_{50} (72 hours): 0.15 µg imidacloprid techn. ($r^2 = 0.53$)

The ED_{50} is between 0.1 and 0.01 µg/bumblebee. The data provide no basis to calculate an accurate ED_{50}.

EVALUATION OF THE RESULTS

Within the range of concentrations of imidacloprid techn. which caused effect, higher concentrations did not result automatically in more mortality.
Affected bumblebees may die of starvation.
The effect of imidacloprid techn., in the concentrations of 0.1 µg / bumblebee and more is obvious.
The ED_{50} is between 0.1 and 0.01. The data provide no basis for an accurate ED_{50} calculation.
The NOEL, based on the range finding test is 0.01 µg / bumblebee.
The LD_{50} values of Dimethoate, determined in the test is 3.06 µg / bumblebee (= 1.22 µg a.i. / bumblebee).

VALIDY OF THE TEST

The LD_{50} (24 hours) value of Dimethoate, determined in this test was 3.06 µg / bumblebee (= 1.22 µg a.i. / bumblebee). The acceptable range of the LD_{50} of Dimethoate is 0.5 to 2.5 µg a.i. / bumblebee. 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 bumblebees, fed with imidacloprid techn. can be considered as caused by imidacloprid techn.

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APPENDIX

DATA OF THE RANGE FINDING TEST

A range finding test preceeds the definitive test in order to determinate the range of concentrations to be tested in the definitive test. A range finding test is performed in the same way as the definitive test but is not included in the quality assurance program of this test.

Table 1
imidacloprid techn.
Mortality and effects during the observation period of the bumblebees fed with 1.1 µg / 10µl sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
11-06-1999	27:57	29	97%	30	100%
12-06-1999	54:14	30	100%	30	100%
13-06-1999	80:43	30	100%	30	100%

Table 2
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees fed with 0.1 µg / 10µl sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
11-06-1999	27:40	0	0%	27	90%
12-06-1999	53:57	0	0%	27	90%
13-06-1999	80:23	16	53%	28	93%

Table 3
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees fed with 0.01 µg / 10µl sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
11-06-1999	27:20	0	0%	0	0%
12-06-1999	53:39	0	0%	0	0%
13-06-1999	80:06	0	0%	0	0%

Table 4
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees fed with 0.001 µg / 10µl sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
11-06-1999	26:46	0	0%	0	0%
12-06-1999	53:05	0	0%	0	0%
13-06-1999	79:32	0	0%	0	0%

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Table 5
Dimethoate
Mortality and effect during the observation period of the bumblebees fed with 5.54 µg / 10µl sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
11-06-1999	26:07	21	70%	21	70%
12-06-1999	52:26	21	70%	21	70%
13-06-1999	78:53	22	73%	22	73%

Table 6
Dimethoate
Mortality and effect during the observation period of the bumblebees fed with 4.16 µg / 10µl sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	Effect (n bumblebees)	percentage
11-06-1999	25:50	16	53%	16	53%
12-06-1999	52:07	21	70%	21	70%
13-06-1999	78:33	21	70%	21	70%

Table 7
Dimethoate
Mortality and other effect during the observation period of the bumblebees fed with 2.77 µg / 10µl sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
11-06-1999	25:30	2	7%	2	7%
12-06-1999	51:50	2	7%	3	7%
13-06-1999	78:14	3	10%	3	70%

Table 8
Sucrose solution 50%
Mortality and effect during the observation period of the bumblebees fed with sucrose-solution 50%. (range finding test)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
11-06-1999	25:16	0	0%	0	0%
12-06-1999	51:30	0	0%	0	0%
13-06-1999	77:55	0	0%	0	0%

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