REPORT

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

Study Director [redacted]

Test facility

Ambrosiushoeve
Hilvarenbeek
The Netherlands

Sponsor

Bayer AG
Institute for Environmental Biology
D-51368 Leverkusen

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CONTENT

REPORT APPROVAL

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Principal Investigator [redacted]
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STATEMENT OF GLP COMPLIANCE (Quality Assurance)

[redacted]

Quality Assurance Officer

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ANNEX to STATEMENT OF GLP COMPLIANCE

[redacted]

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Date: 1999-09-22

STATEMENT OF GLP COMPLIANCE (Study Director)

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Study Director

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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 exposed individually to imidacloprid techn. by way of administration on the ventral part of the thorax with 1 µl acetone, containing a range of concentrations of imidacloprid techn.

A range finding test preceded the definitive test. In order to determine a toxic concentration, based on the data, obtained in the range finding test and in consultation with the sponsor, six concentrations: 101 µg, 65 µg, 31 µg, 8 µg, 4 µg and 0.1 µg imidacloprid techn. per 1 µl acetone were administered to the bumblebees.

All concentrations tested 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. Besides that, spasms and paralysis were observed. These effects lasted at least during the observation period of 72 hours.
There was no correlation between the amount of imidacloprid techn. and the number of dead and affected bumblebees, whether the bumblebees were treated with 4 µg imidacloprid techn. or with 101 µg imidacloprid techn. Within 72 hours these treatments resulted in 90% to 100% dead or affected bumblebees. Imidacloprid techn., administered to bumblebees in the amount of 0.1 µg resulted in 47% mortality and 60% effect. Concentrations imidacloprid techn. of 0.05 µg / 1 µl or less, administered per bumblebee, did not cause effect and mortality (result range finding test). Mortality continued during the observation period. Bumblebees that were affected may have died of starvation.

The data provide no basis for an accurate LD_{50} and ED_{50} but it is obvious that the exposure of 0.1 µg imidacloprid techn. or more per bumblebees does seriously affect bumblebees.

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	Bumblebee (*Bombus terrestris* L.) contact toxicity study in the laboratory with imidacloprid techn.
Project nr	AH99.4.22.1
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

SCHEDULE

Start experiments	02-06-1999
End observations	11-06-1999

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 in 50 % of the bumblebees within 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.

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 exposed individually to imidacloprid techn. by way of administration on the ventral part of the thorax with 1 µl acetone containing a range of concentrations of imidacloprid techn.
A range finding test preceded the definitive test. In order to determine the toxic concentration, based on the data obtained in the range finding test and in consultation with the sponsor, six concentrations: 101 µg, 65 µg, 31 µg, 8 µg, 4 µg and 0.1 µg imidacloprid techn. per 1 µl acetone were administered to the bumblebees.
The treatment was compared to an acetone (negative control) treatment and a Dimethoate positive control.
This study provides a rational basis for the assessment of toxicological risk to bumblebees.

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	Ten (LD_{50} / ED_{50} test of 08-06-1999) six groups treated with 6 concentrations test substance in acetone one group treated with the negative control (acetone) three groups treated with three concentrations of the positive control substance (Dimethoate 40%)
Identification	Each cage used for testing was labelled uniquely.
Randomisation	Allocation of the bumblebees to any particular treatment 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

(used in the LD_{50} / ED_{50} test of 08-06-1999)

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

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.
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.

- 0.5033 gram imidacloprid techn was dissolved in 3.9873 gram acetone (101 µg / 1 µl). This resulted in a white suspension.
- 0.3228 gram imidacloprid techn was dissolved in 3.9672 gram acetone (65 µg / 1 µl). This resulted in a white suspension.
- 0.1537 gram imidacloprid techn was dissolved in 3.9972 gram acetone (31 µg / 1 µl). This resulted in a clear solution.
- 0.1633 gram imidacloprid techn was dissolved in 15.9249 gram acetone (8 µg / 1 µl). This resulted in a clear solution.
- 4.007 gram imidacloprid techn. solution (8 µg / 1 µl) + 3.9697 gram acetone resulted in an Imidacloprid techn. concentration of 4 µg / 1 µl, a clear solution,
- 0.8122 gram imidacloprid techn. solution (4 µg / 1 µl) + 2.3976 gram acetone resulted in an Imidacloprid techn. concentration of 1 µg / 1 µl, a clear solution. This was an intermediate step in the dilution of imidacloprid 4 µg / 1 µl into imidacloprid 0.1 µg / 1 µl.
- 0.8120 gram imidacloprid techn. solution (1 µg / 1 µl) + 7.183 gram acetone resulted in an Imidacloprid techn. concentration of 0.1 µg / 1 µl, a clear solution.

Dimethoate:

The specific range of the LD_{50} in 24 hours of Dimethoate is 4 to 10 µg a.i. / bumblebee.
A concentration range of approximately 4, 7.5 and 10 µg dimethoate a.i./ bumblebee = 10, 19 and 25 µg Dimethoate formulation was tested.

- 0.0528 gram Dimethoate was dissolved in 3.9559 gram acetone. This resulted in the concentration of 11 µg Dimethoate / 1 µl acetone.
- 0.1015 gram Dimethoate was dissolved in 3.9659 gram acetone. This resulted in the concentration of 20 µg Dimethoate / 1 µl acetone.
- 0.1389 gram Dimethoate was dissolved in 3.9750 gram acetone. This resulted in the concentration of 27 µg Dimethoate / 1 µl acetone.

TREATMENT

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.
For the administration of the test substances, the bumblebees were anaesthetised with CO_2 again and 1 µl test solution was pipetted on the ventral part of the thorax between the base of the 2nd and 3rd pair of legs. Per concentration 30 bumblebees were treated.
After treatment the bumblebees, treated with the same concentration, were housed together and fed sucrose solution 50% ad libitum. The sucrose-solution was changed daily.
A range finding test preceded the definitive test. In this test, six concentrations of approximately 0.001, 0.01, 0.05, 0.1, 1, 10 and 100 µg / bumblebee, were tested. Effect within 24 hours was observed after treatment with concentrations of 0.1 µg and more / bumblebee. Within 24 hours only in the group, treated with 100 µg / bumblebee, mortality was recorded. The mortality rate increased in time and after 72 hours, mortality was recorded in the groups, treated with 1 µg and more / bumblebee. Due to the significant difference between the effective and lethal dose it was, in consultation with the sponsor, decided to test the range of approximately 8 µg, 4 µg and 0.1 µg imidacloprid techn. per 1 µl acetone to determine a effective dose and approximately 101 µg, 65 µg and 31 µg imidacloprid techn. per 1 µl acetone to determine a lethal dose.
The test was carried out once.

OBSERVATIONS

Behaviour abnormalities, e.g. paralysis, uncoordinated movements or any locomotor disabilities as well as mortality were recorded 24 hours, 48 hours and 72 hours after administration of the test solutions.

RESULTS

DATA / TABLES

Table 1
Mortality and effect during the observation period of the bumblebees treated with 101 µg imidacloprid techn. / 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:33	12	40%	30	100%
10-06-1999	50:43	24	80%	28	93%
11-06-1999	73:33	26	87%	30	100%

Table 2
Mortality and effect during the observation period of the bumblebees treated with 65 µg imidacloprid techn ./ 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:33	8	27%	30	100%
10-06-1999	50:54	19	63%	30	100%
11-06-1999	73:42	25	83%	30	100%

Table 3
Mortality and effect during the observation period of the bumblebees treated with 31 µg imidacloprid techn./ 1µl acetone (LD_{50} / ED_{50} test 08-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:43	14	47%	29	97%
10-06-1999	51:03	24	80%	29	97%
11-06-1999	73:49	25	83%	30	100%

Table 4
Mortality and effect during the observation period of the bumblebees treated with 8 µg imidacloprid techn. / 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:39	17	57%	29	97%
10-06-1999	51:10	25	83%	30	100%
11-06-1999	73:54	29	97%	30	100%

Table 5
Mortality and effect during the observation period of the bumblebees treated with 4 µg imidacloprid techn. / 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:40	13	43%	28	93%
10-06-1999	51:17	23	77%	28	93%
11-06-1999	74:00	25	83%	29	97%

Table 6
Mortality and effect during the observation period of the bumblebees treated with 0.1 µg imidacloprid techn. / 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:36	0	0%	1	3%
10-06-1999	51:25	7	23%	12	40%
11-06-1999	74:07	14	47%	18	60%

Table 7
Mortality and effect during the observation period of the bumblebees treated with 27 µg Dimethoate / 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:38	21	70%	21	70%
10-06-1999	51:31	24	80%	24	80%
11-06-1999	74:13	24	80%	24	80%

Table 8
Mortality and effect during the observation period of the bumblebees treated with 20 µg Dimethoate / 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:39	18	60%	18	60%
10-06-1999	51:37	24	80%	24	80%
11-06-1999	74:18	24	80%	24	80%

Table 9
Mortality and effect during the observation period of the bumblebees treated with 11 µg Dimethoate / 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:36	10	33%	10	33%
10-06-1999	51:42	14	47%	14	47%
11-06-1999	74:22	14	47%	14	47%

Table 10
Mortality and effect during the observation period of the bumblebees treated with 1µl acetone

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
09-06-1999	25:39	0	0%	0	0%
10-06-1999	51:44	0	0%	0	0%
11-06-1999	74:29	0	0%	0	0%

MORTALITY AND OTHER EFFECTS

The most significant effect is the "frozen behaviour" at which the bumblebees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus. Beside this spasms and paralysis were observed as well. The effect continued during the observation period without causing an immediate mortality. Mortality increased during the observation period. Bumblebees that were affected may have died from starvation.

STATISTICAL ANALYSIS

As there is no mortality in the negative control, the data are not corrected for control mortality.
No statistical analysis was carried out.

EVALUATION OF THE RESULTS

All concentrations tested 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. Besides that, spasms and paralysis were observed. These effects lasted at least during the observation period of 72 hours.

There was no correlation between the amount of imidacloprid techn. and the number of dead and affected bumblebees, in the range from 4 µg imidacloprid techn. to 101 µg imidacloprid techn. Within 72 hours these treatments resulted in 90% to 100% dead or affected bumblebees.
Imidacloprid techn. administered to bumblebees in the amount of 0.1 µg resulted in 47% mortality and 60% effect.
Concentrations imidacloprid techn. < 0.05 µg / 1 µl, administered per bumblebee, did not cause effect and mortality (result range finding test).
Mortality continued during the observation period.
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 data provide no basis for an accurate LD_{50} and ED_{50} but it is obvious that the exposure of 0.1 µg imidacloprid techn. or more per bumblebees does seriously affect bumblebees.

VALIDY OF THE TEST

The LD_{50} in 24 hours of Dimethoate, determined in this test was 17 µg Dimethoate / bumblebee (= 7 µg a.i. / bumblebee). The acceptable range of the LD_{50} in 24 hours of Dimethoate is 4 to 10 µg a.i. / bumblebee. The mortality in the negative control did 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, treated with imidacloprid techn. can be considered as caused by imidacloprid techn.

APPENDIX

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.

Data of the range finding test

Table 1
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees treated with 104 ng / 1µl acetone (range finding test 02-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	24:55	0	0%	4	13%
04-06-1999	49:05	0	0%	0	0%
05-06-1999	77:46	1	3%	4	13%

Table 2
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees treated with 52 ng / 1µl acetone (range finding test 02-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	24:58	0	0%	0	0%
04-06-1999	49:10	0	0%	0	0%
05-06-1999	77:50	0	0%	0	0%

Table 3
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees treated with 9 ng / 1µl acetone (range finding test 02-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	25:03	0	0%	0	0%
04-06-1999	49:14	0	0%	0	0%
05-06-1999	77:54	0	0%	0	0%

Table 4
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees treated with 0.9 ng / 1µl acetone (range finding test 02-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	25:07	0	0%	0	0%
04-06-1999	49:18	0	0%	0	0%
05-06-1999	77:58	0	0%	0	0%

Table 5
Dimethoate.
Mortality and effect during the observation period of the bumblebees treated with 24 µg / 1µl acetone (range finding test 02-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	24:48	26	87%	26	87%
04-06-1999	48:57	28	93%	28	93%
05-06-1999	77:32	29	97%	29	97%

Table 6
Dimethoate.
Mortality and effect during the observation period of the bumblebees treated with 19 µg / 1µl acetone (range finding test 02-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	24:31	20	67%	20	67%
04-06-1999	48:56	22	73%	22	73%
05-06-1999	77:35	24	80%	24	80%

Table 7
Dimethoate.
Mortality and effect during the observation period of the bumblebees treated with 10 µg / 1µl acetone (range finding test 02-06-1999)

date	observation period (houts)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	24:41	4	13%	4	13%
04-06-1999	49:03	4	13%	4	13%
05-06-1999	77:41	9	30%	9	30%

Table 8
Acetone
Mortality and effect during the observation period of the bumblebees treated with 1µl acetone (range finding test 02-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
03-06-1999	25:00	0	0%	0	0%
04-06-1999	49:30	0	0%	0	0%
05-06-1999	78:03	0	0%	0	0%

Table 9
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees treated with 99 µg / 1µl acetone (range finding test 03-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
04-06-1999	26:25	3	10%	30	100%
05-06-1999	53:18	9	30%	30	100%
06-06-1999	77:16	18	60%	30	100%

Table 10
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees treated with 10 µg / 1µl acetone (range finding test 03-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
04-06-1999	26:38	0	0%	22	73%
05-06-1999	53:27	5	17%	26	87%
06-06-1999	77:22	10	33%	30	100%

Table 11
imidacloprid techn.
Mortality and effect during the observation period of the bumblebees treated with 1 µg / 1µl acetone (range finding test 03-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
04-06-1999	26:43	0	0%	17	56%
05-06-1999	53:26	6	20%	26	87%
06-06-1999	77:24	10	33%	26	87%

Table 12
Dimethoate.
Mortality and effect during the observation period of the bumblebees treated with 24 µg / 1µl acetone (range finding test 03-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
04-06-1999	24:49	19	63%	19	63%
05-06-1999	47:29	21	70%	21	70%
06-06-1999	77:27	23	77%	23	77%

Table 13
Dimethoate.
Mortality and effect during the observation period of the bumblebees treated with 19 µg / 1µl acetone (range finding test 03-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
04-06-1999	24:54	12	40%	12	40%
05-06-1999	47:30	19	63%	19	63%
06-06-1999	77:30	19	63%	19	63%

Table 14
Dimethoate.
Mortality and effect during the observation period of the bumblebees treated with 11 µg / 1µl acetone (range finding test 03-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
04-06-1999	25:00	9	30%	9	30%
05-06-1999	47:33	9	30%	9	30%
06-06-1999	77:33	9	30%	9	30%

Table 15
Acetone
Mortality and effect during the observation period of the bumblebees treated with 1µl acetone (range finding test 03-06-1999)

date	observation period (hours)	mortality (n bumblebees)	percentage	effect (n bumblebees)	percentage
04-06-1999	25:05	0	0%	0	0%
05-06-1999	47:38	0	0%	0	0%
06-06-1999	77:37	0	0%	0	0%