STUDY REPORT

Name and address of sponsor:	Bayer AG Landwirtschaftszentrum Monheim Institut fur Okobiologie 51368 Leverkusen Germany
Sponsor's Representative	[redacted]
Name and address of test facility:	Environmental R&D Team Environmental Biology Group Central Science Laboratory Sand Hutton York N Yorks YO41 1LZ Tel 01904 462515, Fax 01904 462240
Study Director	[redacted]
Study dates:	
Initiation	27 April 2000
Experiment start	8 May 2000
Experiment end	11 May 2000
Study Completion	9 June 2000

Study Number:HT0400b

Substance A:
Acute Oral Toxicity to Honey Bees
(Apis mellifera).

1. Summary

Tests were carried out to determine the acute oral toxicity of Substance A to adult honey bees (*Apis mellifera* L.). The protocol followed the EPPO guidelines (1992) and OECD guideline 213 Honeybees, Acute Oral Toxicity Test (September 1998). All doses and toxicity data for the test substance refer to Substance A as the active ingredient.

Three batches of bees, in groups of 10 bees, were offered the equivalent doses of 73.6, 24.6, 8.2, 2.8, 0.94 ng /bee Substance A in 50% w/v aqueous sucrose solution, the test substance having first been dissolved in acetone. At the highest treatment level the mean dose consumed was 45 ng /bee Substance A, 39% less than the actual dose offered. This lowered intake may be due to repellency or to the large numbers of bees observed as knocked down at 4 hrs, the bees were on their feet but immobile and therefore unable to feed.

Mortality was assessed at 4 hours after dosing. Glass test feeders were then removed and further assessments made at 24 and 48 hours after removal of the glass test feeders. Results indicated that the 24-hour and 48-hour oral LD_{50} of Substance A is greater than 45 ng /bee. Significant sub-lethal effects (50-100% knockdown) were observed at 4 hrs in the highest two doses but only 10% knockdown was observed in the highest dose at 24 hrs.

2. Introduction

This study was carried out on behalf of Bayer AG to establish the acute oral toxicity of Substance A to adult worker honey bees in the laboratory.

The honey bee was chosen as the test organism, being representative of the pollinating insects which may be at risk if flowering crops are sprayed with pesticides. The effects of oral exposure are assessed in aqueous sucrose solution, first dispersing the test substance in acetone and then dispersing in 50% w/v aqueous sucrose.

The protocol followed the European Plant Protection Organisation guidelines (1992) and methods are in accordance OECD guideline 213 Honeybees, Acute Oral Toxicity Test (September 1998). All doses and toxicity data for the test substance refer to Substance A as the active ingredient.

3. Materials and methods

3.1 Test substance

Substance A supplied by Bayer AG was used in this study. Each sample of the material tested in this study was uniquely labelled with Test Substance number NBU83. The test substance was stored, as recommended by the sponsor, at 4°C in the dark.

Substance A, a white solid, was dispersed in acetone and then 50% aqueous sucrose for dosing for a maximum of two hours before use.

Although there were no data on the stability of the test substance in solution the test substance was assumed to be stable under the conditions of this study.

3.2 Test bees

Test bees for all the tests in this study were adult worker bees (*Apis mellifera* L.) taken from a single colony (colony Thorpe 1) owned and maintained by the Central Science Laboratory's National Bee Unit for use in such tests.

3.3 General Method

Worker bees were collected from the hive by using a small amount of smoke, gently shaking them from the combs and transferring them (40-50 per cage) into cylindrical mesh cages.

In the laboratory the mesh cages were placed into the incubator (25 $\pm$ 1°C, 65 $\pm$ 5% relative humidity) for 1.5 to 2 hours before being used for the test. Immediately prior to treatment each mesh cage of bees was anaesthetised with carbon dioxide gas by placing the cage into a 2 litre beaker filled with CO_2.

A range of dose rates and a control were used, with three replicates of 10 bees per dose rate. During the test period the bees were kept in the dark (except during observations) in an incubator at 25 ± 1°C and 65 ± 5% relative humidity. Mortality and sub-lethal effects were assessed at 4 hours after offering doses. The glass test feeders, containing any unconsumed portions of the doses, were then removed. Fresh 50% w/v aqueous sucrose was then supplied in feeders within the cages and further assessments made at 24 and 48 hours after removal of the glass test feeders.

Sub-lethal effects were assessed according to pre-determined categories:
Knocked down (i.e. alive but immobile)
Stumbling (i.e. moving but in a poorly co-ordinated manner)

In this report "solution" is used to include material which may be suspended or dispersed. Homogeneity of such "solutions" of the Substance A was checked visually immediately before use. Solutions of the test doses were mixed well immediately prior to use and were homogenous for the purpose of administration.

Doses for the oral toxicity test (Table 1) were prepared from a stock solution of 1390 µg/ml Substance A in acetone. The highest dose was prepared by diluting 0.263 ml stock to10 ml with 50% w/v aqueous sucrose and then preparing serial dilutions of this. Each group of 10 bees were offered 0.2 ml in a glass feeder. Doses were offered within 2 hours of preparation.

Control bees were given 50% w/v aqueous sucrose solution containing 0.263 ml acetone (the same level as in the highest dose).

Table 1. Oral test dose levels

Dose group	Substance A ng /bee
1	73.6
2	24.6
3	8.2
4	2.8
5	0.94
Control	0.263 ml acetone made up to10 ml with 50% w/v aqueous sucrose

The bees were anaesthetised with carbon dioxide immediately before dosing and were gently tipped out onto filter paper and counted into the petri dish cage (drones were discarded). Each group of 10 bees was offered 0.2 ml of a given concentration (or controls as above), the dose being measured into a small, pre-weighed, glass feeder within the cage using a variable volume Gilson pipette. This was equivalent to 20 µl/bee.

After 4 hours the glass feeders were removed and weighed and the sucrose feeders filled with approximately 3 ml 50% w/v aqueous sucrose so that bees had continuous access to sucrose for the remainder of the study. The dose consumed was determined by comparison of the weight of the dose remaining in the glass feeders with the weight of a known volume of the test solutions.

4. Results

Substance A readily dissolved in acetone at 1390 µg/ ml to give a clear, colourless solution and remained in solution when further diluted in 50% w/v aqueous sucrose.

The mortality results for the oral test are listed in Table 2 for Substance A. Sub-lethal effects recorded are shown in Table 3. At 4 hours in the two highest doses 50-100% of bees were observed as knocked down, the bees were on their feet but immobile, but by 24 hrs only 10% of bees were knocked down at the highest dose.

The mean maximum dose consumed was 45 ng/ bee, 39% less than the actual dose offered compared with only 1% remaining in control doses. This lowered intake may be due to repellency or to the large numbers of bees observed as knocked down at 4 hrs and therefore unable to feed.

The 24-hour and 48-hour LD_{50} for Substance A are given in Table 4.

With the Substance A the 24-hour and 48-hour LD_{50} levels are > 45 ng /bee Substance A. Control mortality was 0% at 24 and 48 hours. The 48-hour results were similar to those at 24 hours, indicating there were no delayed effects.

TABLE 2. Results of oral dosing tests with Substance A

Dose (ng /bee)	Actual dose (ng /bee)	Cage No.	Number dead (n=10) 4 hours	24 hours	48 hours
73.6	52	87	0	0	0
73.6	41	88	0	2	2
73.6	43	89	0	0	2
24.6	20	90	0	0	0
24.6	22	91	0	0	0
24.6	22	92	0	1	1
8.2	7.8	93	0	3	3
8.2	8.1	94	0	0	0
8.2	7.9	95	0	0	0
2.8	2.8	96	0	0	0
2.8	2.8	97	0	1	1
2.8	2.8	98	0	1	1
0.94	0.94	99	0	0	0
0.94	0.94	100	0	0	0
0.94	0.94	101	0	0	0
0	0	102	0	0	0
0	0	103	0	0	0
0	0	104	0	0	0

TABLE 3. Sub-lethal effects observed in oral dosing tests with Substance A.

Dose	Actual dose	Cage	Number knockdown (K) or stumbling (S) (n=10)		
(ng /bee)	(ng /bee)	No.	4 hours	24 hours	48 hours
73.6	52	87	1S, 9K	1K	1K
73.6	41	88	8K	1K	0
73.6	43	89	10K	1K	0
24.6	20	90	5K	0	0
24.6	22	91	9K	0	0
24.6	22	92	10K	0	0
8.2	7.8	93	2K	0	0
8.2	8.1	94	0	0	0
8.2	7.9	95	0	0	0
2.8	2.8	96	0	0	0
2.8	2.8	97	0	0	0
2.8	2.8	98	0	0	0
0.94	0.94	99	0	0	0
0.94	0.94	100	0	0	0
0.94	0.94	101	0	0	0
0	0	102	0	0	0
0	0	103	0	0	0
0	0	104	0	0	0

Table 4. Oral toxicity of Substance A

Time	LD_{50} (ng Substance A /bee)	95% Confidence Interval for LD_{50}	Estimate of slope of response line	NOEL ng/ bee
24 hours	>45*	-	-	22
48 hours	>45*	-	-	22

* mean maximum dose consumed

6. Discussion and conclusions

Reduced intake of Substance A was observed at the highest dose (73.6 ng/bee) with 39% of the dose remaining compared with less than 1% remaining in the control doses. This reduced intake may be due to repellency or the large numbers of bees observed as knocked down at 4 hrs, on their feet but immobile and therefore unable to feed. The mean intake at the maximum dose was 45 ng /bee Substance A.

The 24-hour and 48-hour oral LD_{50} of Substance A based on actual uptake is greater than 45 ng/bee Substance A.

7. References

1992 European and Mediterranean Plant Protection Organisation
"Guideline on test methods for evaluating the side-effects of plant protection products on honey bees"
EPPO Bulletin 22, 203-215

September 1998 OECD
"OECD Guidelines for the testing of chemicals Guideline 213. Honeybees, acute oral toxicity test."

Distribution

Sponsor
Study Director [redacted]

APPENDIX I

ACTUAL TIMETABLE FOR THE STUDY

ACUTE ORAL HONEYBEE TOXICITY TEST HT0400b

Day	Time (hours)	Date & Actual Time	Activity
-3	pm	08/05/00	Dispense test substance
0	-2½	11/05/00	Start bee collection
	-2	11/05/00	Stock prepared and dilutions started
	-2	11/05/00	Bees in incubator
	-1	11/05/00 09:28	Dilutions finished
	0	11/05/00 11:15	Orals dosed
	+4	11/05/00 15:13	Assess orals, remove feeders and weigh, feed
	+28	11/05/00 15:11	Assess orals
	+52	11/05/00 15:18	Assess orals

STUDY REPORT AMENDMENT 1

Name and address of sponsor:	Bayer AG Landwirtschaftszentrum Monheim Institut fur Okobiologie 51368 Leverkusen Germany
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(*Apis mellifera*).

HT0400b

Clarification
The bees used were fully developed nurse bees which had not just emerged, i.e. they did not still have hairs on their thorax and had fully extended wings. As guidance they were between 2 days and 2-3 weeks of age.

Amendment to Report No. HT0400b

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:	Central Science Laboratory, York, United Kingdom
Date of delivery as substance A:	19.4.2000
Delivered amount:	1.15 g
Order no.:	2670

Leverkusen, 21.6.00

[redacted]