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Labor für biologische und chemische Analytik GmbH

FINAL REPORT

Imidacloprid SL 200

STUDY TITLE

Acute toxicity of Imidacloprid SL 200 to the honeybee *Apis mellifera* L. under laboratory conditions

Guidelines:

OECD 213 (1998), OECD 214 (1998)

Author

[redacted]

STUDY COMPLETED ON

November 5, 2001

PERFORMING LABORATORY

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LABORATORY PROJECT IDENTIFICATION

BioChem project number: 01 10 48 048

SPONSOR

Bayer AG
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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

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SPONSOR

Study Title:	Acute toxicity of Imidacloprid SL 200 to the honeybee *Apis mellifera* L. under laboratory conditions
Guidelines:	OECD 213 (1998), OECD 214 (1998)
Test item:	Imidacloprid SL 200 (TOX No.: 05752-00)
Sponsor's Representative:	07/11/2007 [redacted]

LIST OF AMENDMENTS TO STUDY PLAN

Amendment No.	Date	Concerning	Reason for alteration
none			

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SUMMARY

Report: [redacted]
Acute toxicity of Imidacloprid SL 200 to the honeybee *Apis mellifera* L. under laboratory conditions
BioChem agrar, unpublished report No.: 01 10 48 048; 2001/11/05

Guideline(s): OECD 213 (1998), OECD 214 (1998)

GLP: yes (certified laboratory)

Material and methods:

Test species: *Apis mellifera carnica* L.

Test system: oral toxicity and contact toxicity test of Imidacloprid SL 200 on honeybees

Treatments: control, test item and toxic standard (Dimethoate EC 400)

Test item treatment levels: the test item was applied at the following doses:
oral toxicity test: 0.0064, 0.0128, 0.0256, 0.0512 and 0.1025 µg a.i./bee
contact toxicity test: 0.0029, 0.0057, 0.0114, 0.0229, 0.0457 and 0.0914 µg a.i./bee

Toxic standard: Dimethoate EC 400 was applied at the following doses:
oral toxicity test: 0.074, 0.089, 0.104, 0.126, 0.149 µg a.i./bee
contact toxicity test: 0.012, 0.023, 0.046, 0.093, 0.186 µg a.i./bee

Dates of work: August 28 –. September 10, 2001

The insecticide Imidacloprid SL 200 (purity: 200.9 g/l; specification: Development No.: 3000249869, TOX No.: 05752-00, Formulation No.: 03833/0818 (0753) was tested under laboratory conditions on the honeybee *A. mellifera* after oral and contact exposure. Endpoints were mortality and behaviour of the bees compared to control up to 96 h after application. Mortality values were used to provide a regression line and calculate the median lethal dose value (LD_{50}) expressed in µg of active ingredient or product per bee.

Findings:

Table: Oral and contact toxicity LD_{50} values of bees treated with Imidacloprid SL 200

Test item		Imidacloprid SL 200					
Test object		Honeybee *Apis mellifera L.*					
Exposure		oral / contact					
Treatment		**LD_{50}**					
Test item	time	oral toxicity test			contact toxicity test		
		µg a.i./bee	slope b	µg product/bee	µg a.i./bee	slope b	µg product/bee
Imidacloprid SL 200	**24 h**	n.d.		n.d.	n.d.		n.d.
	95 %-cl lower						
	upper						
	48 h	**0.066**	1.72	**0.361**	**0.056**	2.32	**0.306**
	95 %-cl lower	0.045		0.246	0.042		0.230
	upper	0.098		0.536	0.074		0.404
	72 h	**0.056**	1.89	**0.306**	**0.048**	2.03	**0.262**
	95 %-cl lower	0.040		0.219	0.036		0.197
	upper	0.077		0.421	0.065		0.355
	96 h	**0.053**	1.84	**0.290**	**0.045**	2.09	**0.246**
	95 %-cl lower	0.038		0.208	0.034		0.186
	upper	0.074		0.404	0.060		0.328

cl: confidence limits n.d.: not defined

Table (continued): Oral and contact toxicity LD_{50} values of bees treated with Imidacloprid SL 200

Treatment		LD_{50}					
Reference item	time	oral toxicity test			contact toxicity test		
Dimethoate EC 400		µg a.i./bee	slope b	µg product/bee	µg a.i./bee	slope b	µg product/bee
	24 h	**0.133**	8.85	**0.358**	**0.113**	2.22	**0.304**
	95 %-cl lower	0.123		0.331	0.084		0.226
	upper	0.144		0.388	0.152		0.409
	48 h	**0.129**	8.17	**0.347**	**0.102**	2.37	**0.275**
	95 %-cl lower	0.120		0.323	0.078		0.210
	upper	0.139		0.374	0.134		0.361

cl: confidence limits

No statistically significant effects on survival were observed at doses of 0.0064 and 0.0128 µg a.i. per bee in the oral toxicity test (0 and 13.3 % mortality, respectively) during 48 hours. Statistically significant effects on survival were observed at doses of 0.0256, 0.0512 and 0.1025 µg a.i. per bee in the oral toxicity test (23.3, 36.7 and 66.7 % mortality, respectively) during 48 hours. The calculated LD_{50} (48 h) is 0.066 µg a.i. per bee in the oral toxicity test (equivalent to 0.361 µg product/bee based on analysed content of a.i.).

In the contact toxicity test no statistically significant effects on survival were observed at doses of 0.0029, 0.0057, 0.0114 and 0.0229 µg a.i. per bee (0, 0, 10 and 13.3 % mortality, respectively) during 48 hours. Statistically significant effects on survival were observed only at doses of 0.0457 and 0.0914 µg a.i. per bee in the contact toxicity test (33.3 and 75.0 % mortality, respectively) during 48 hours. Therefore the calculated LD_{50} (48 h) is 0.056 µg a.i. per bee in the contact toxicity test (equivalent to 0.306 µg product/bee based on analysed content of a.i.).

Before bees died in the test item treatments apathy and immobility were observed.

The test period was prolonged up to 96 h because progressive mortality of the bees was observed at some doses between 24 and 48 hours in both the oral and contact toxicity tests. The prolongation of the study resulted in a statistically significantly increased mortality in the oral and the contact toxicity tests for the test item doses including and above 0.0256 and 0.0229 µg a.i./bee after 96 h. The calculated LD_{50} (96 h) are 0.053 and 0.045µg a.i. per bee in the oral and the contact toxicity tests (equivalent to 0.290 and 0.246 µg product/bee, respectively, based on analysed content of a.i.).

The LD_{50} of the reference item Dimethoate was 0.133 µg a.i./bee in the oral toxicity test after 24 hours. This value was also within the preferred range of 0.10-0.35 µg a.i./bee cited in the OECD Guideline 213. The LD_{50} of the reference item was 0.113 µg a.i./bee in the contact toxicity test after 24 hours. This value corresponds to the expected range for the oral 24h - LD_{50} (0.10-0.30 µg a.i./bee) published in the OECD Guideline 214.

In the reference treatments apathy, discoordinated movements and immobility were observed before bees died.

The study was performed in compliance with the GLP principles.
The validity criterion - mortality in the control ≤ 10 % - was accomplished (being 0 % in the oral and in the contact toxicity tests after 48 hours).
The LD_{50} –24 h values for the toxic standard of 0.1-0.35 µg a.i./bee (oral) and 0.1-0.30 µg a.i./bee (contact) were accomplished (being 0.133 µg a.i./bee and 0.113 µg a.i./bee in the oral and the contact toxicity test, respectively).

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1. General information

1.1. Objective

The purpose of this study was to determine the acute toxicity of Imidacloprid SL 200 to 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 (1998).
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) and the recommendations of the SETAC-Europe (1995).

1.2. Project staff

Study Director: [redacted]

Personnel: [redacted]

1.3. Time schedule

Study initiation date:	11.06.01
Signature of the study plan by the Monitor:	12.06.01
Experimental start date:	28.08.01
Experimental termination date:	10.09.01
Study completion date:	05.11.01

1.4. Test guidelines

OECD Guideline No. 213 (1998)
OECD Guideline No. 214 (1998)

1.5. Archiving

Study plan, final report, raw data and QA inspection reports will be kept in the GLP archive of BioChem agrar GmbH in compliance with the GLP principles for 15 years. A sample of the test item will be also stored there for 5 years.
Disposal of any data or the sample of the test item requires the sponsor's consent.

2. Performance of the test

2.1. Test item

Common name:	Imidacloprid SL 200 (= Confidor SL 200 Forte)
Formulation:	SL 200
Development No.:	3000249869
Formulation No.:	03833/0818(0753)
Proposed use:	insecticide
Active ingredient(s):	NTN 33893 (Imidacloprid)
CAS-No.:	138261-41-3
TOX No.:	05752-00
Analysed content of active ingredient(s):	200.9 g/l
Analytical findings from:	May 31, 2001
Approved until:	December 1, 2001
Appearance of the test item:	yellow liquid
Density:	1.098 g/cm^3
Storage conditions:	room temperature
Safety precautions:	designation according to "EU Classification": R 92, R 916; R43; S24, S 28 B, S 37 consideration of the safety measures in general use with handling of plant protection products
Dose applied in the test:	<u>oral toxicity test</u>: 0.0064, 0.0128, 0.0256, 0.0512 and 0.1025 µg a.i./bee <u>contact toxicity test</u>: 0.0029, 0.0057, 0.0114, 0.0229, 0.0457 and 0.0914 µg a.i/bee
Applied/exposed volume:	<u>oral toxicity test</u>: - 200 µl sucrose solution/10 bees = 20 µl/bee <u>contact toxicity test</u>: - 1 µl/bee (acetone)
Further details:	"Approval of Preparation Sample" of 01.06.2001 "Sicherheitsdatenblatt" (no. 416227/02) of 06.06.00

2.2. Reference item (toxic standard)

Product name:	Dimethoate EC 400
Batch No.:	99-1
Approved until:	October 2002
Formulation:	EC 400
Active ingredient/content:	Dimethoate 417.5 g/l (according to certificate PCP06027)
Density (20 °C):	1.077 g/cm^3
Classification:	„Xn" - Harmful; R 10, 21/22; S 2, 13, 20/21, 28, 46
Dose applied in the test:	<u>oral toxicity test</u>: 0.074, 0.089, 0.104, 0.126 and 0.149 µg a.i./bee <u>contact toxicity test</u>: 0.012, 0.023, 0.046, 0.093, 0.186 µg a.i./bee

2.3. Control

The oral and contact control groups were treated with 50 % (w/v) sucrose solution, water and acetone, respectively.

2.4. Test system

Test organism: honeybee - *Apis mellifera carnica* L.
(worker bees of a colony in good healthy condition)

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

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.
Ten 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 had walked into 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. 1-2 hours (corresponding to the starvation period in the oral 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/dose:	3
Number of honeybees/ dose:	30
Food:	50 % (w/v) aqueous sucrose solution
Feeding:	continuously during the test, using a glass feeding tube which was placed on the floor in the test cage

Climatic conditions (test room)

Temperature:	23.5-25 °C (according to study plan: (25±2) °C)
Relative humidity:	64-74 % (according to study plan: around 50-70 %)
Recording:	continuously by a data logger manufacturer: Testo GmbH & Co., type: testostor 175-2
Illumination:	constant darkness throughout the test (diffuse artificial light (about 100 lx) only during handling and assessments)
Test duration:	96 hours

2.5. Experimental procedure

2.5.1. Preparation of the test solutions

Table 1: Test concentrations used in the test

treatment group	Item applied	concentration (% w/v)	dose (µg product /bee)	dose (µg a.i./bee)*
control (oral)	50 % (w/v) sucrose solution	-	-	-
control (contact)	water	-	-	-
control (contact)	acetone	-	-	-
reference item	Dimethoate EC 400	oral toxicity test (20 µl sucrose solution/bee)		
		0.002	0.40	0.149
		0.0017	0.34	0.126
		0.0014	0.28	0.104
		0.0012	0.24	0.089
		0.0010	0.20	0.074
		contact toxicity test (1 µl acetone/bee)		
		0.05	0.5	0.186
		0.025	0.25	0.093
		0.0125	0.125	0.046
		0.00625	0.0625	0.023
		0.00313	0.0313	0.012
test item	Imidacloprid SL 200	oral toxicity test (20 µl sucrose solution /bee)		
		0.0028	0.56	0.1025
		0.0014	0.28	0.0512
		0.0007	0.14	0.0256
		0.00035	0.07	0.0128
		0.00018	0.036	0.0064
		contact toxicity test (1 µl acetone/bee)		
		0.05	0.5	0.0914
		0.025	0.25	0.0457
		0.0125	0.125	0.0229
		0.00625	0.0625	0.0114
		0.00313	0.0313	0.0057
		0.00156	0.0156	0.0029

* For calculation unrounded values were used.
The 50 % (w/v) sucrose solution was prepared with deionised water, just prior to application.

The exactly weighed out amounts of the items were dispersed in sucrose solution or in acetone, immediately before application (see appendix 1).

2.5.2. Course of the trial

Worker bees of the honeybee *Apis mellifera* L. were exposed to different doses of the test and the reference item. The treated bees were kept under controlled climatic conditions and assessed for toxic effects for up to 96 hours.
Two different routes of exposure were used permitting the evaluation of both toxic feeding effects and contact effects of the test and the reference item. 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 µg of the test item per bee.

At the beginning of the test, 10 healthy worker bees 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 starved for about 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 approx. 2 hours after collecting bees from the hive.
The preparation of the test solution was performed according to point 2.5.1.
After 4 hours in the oral toxicity test and immediately after application in the contact toxicity test, untreated 50 % (w/v) aqueous sucrose solution was provided as food in both 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 4, 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 either the test item or the reference item. The control treatments were fed with 50 % aqueous sucrose solution.
Before the sucrose solution was filled in the feeding tubes they were weighed. In the test, groups of 10 bees were provided with 200 µl (= 0.235 g) of the test solution which was presented to the bees in a glass ampoule (half-open on its 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 item (i.e. about 20 µl/bee). Maximally 4 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 % (w/v) aqueous sucrose solution.

Contact toxicity test

The test item and reference items were dissolved in acetone.
Bees shortly anaesthetised with CO_2 were treated individually by topical application of the test solution with an Eppendorf Micropipette. 1 µl of the test item or reference solution was applied to the dorsal thorax of each bee.
In the control treatment groups 1 µl pure water and 1 µl of untreated acetone, respectively, was applied in the same manner.
After application, the treated bees were returned to the test cages which were supplied with a feeding tube containing 50 % (w/v) aqueous sucrose solution.

Order of application and cleaning procedure
The order of feeding or topical application was the following:
- control
- test item (from the lowest to the highest concentration)
- reference item (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 Mucasol®, 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 test cage (without anaesthesia) and acclimatisation for approximately 1-2 hours in both tests to the test room conditions
- 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), food was provided during application (application=feeding, i.e. first after 2 hours after collecting bees from the hive)
- In the contact toxicity tests no food was provided before application because the bees were treated immediately after collecting bees from the hive (the food was provided immediately after application)
- 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 % (w/v) sucrose solution into the contact treatment test cages
- 4 hours after the oral application, feeding tubes reweighed and replaced with tubes containing untreated 50 % (w/v) sucrose solution
- Observation of the bees throughout the experiment (including 4, 24, 72 and 96 hours) and feeding as required
- Record and listing of the findings
- Final assessment 96 h after application

2.5.3. Assessment of the effects

Time and frequency of assessments:
· 4, 24, 48, 72 and 96 hours after application

Evaluation parameters:
· mortality: number of dead bees
· behaviour: number of bees/replicate/time of assessment: healthy, affected (paralysis, lateral position, lying on the back), abnormal amount and colour of excretion all in comparison with control bees

Validity criteria:
· mortality in the control (48 h): ≤ 10 %
· LD_{50}-24 h of toxic standard (dimethoate):
oral toxicity test: 0.10-0.35 µg a.i./bee
contact toxicity test: 0.10-0.30 µg a.i./bee

2.6. Calculation and statistics

The corrected mortality according to ABBOTT (1925) 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 group
t	=	number of surviving bees in the treated group

Statistical analysis
For statistical calculation of the mortality results the DUNNETT-test was used. The accepted significance level was $p \leq 0.05$ (one-sided smaller). The LD_{50} for the mortality was calculated by Probit analysis according to the maximum likelihood method (FINNEY 1971). The goodness-of-fit of the model was evaluated by Pearson's X^2 test. The calculation of statistical significance and the LD_{50} was performed with the computer programs EASY ASSAY (Multiple Testing and Critical Values) by RATTE (1998).

3. Results

The findings are summarized in the tables 4 and 5 and the detailed set of results is presented in the appendices.
0 % mortality were observed in the oral and the contact control treatments during 48 hours, respectively. Thus, the test accomplished the validity criterion (mortality in the control ≤ 10 %). The LD_{50} –24h of the reference item Dimethoate EC 400 was 0.133 µg a.i./bee in the oral toxicity test and 0.113 µg a.i./bee in the contact toxicity test.
These determined values correspond to the specified range for the oral and contact 24h - LD_{50} of 0.10-0.35 µg a.i./bee and 0.10-0.30 µg a.i./bee, respectively, published in the OECD Guidelines 213/214 and showed that the test system was sensitive.

Exposure to Imidacloprid SL 200 resulted in no statistically significantly increased mortality at doses of 0.0064 and 0.0128 µg a.i. per bee in the oral toxicity test (0 and 13.3 %, respectively) during 48 hours.
Statistically significant effects on survival were observed at doses of 0.0256, 0.0512 and 0.1025 µg a.i. per bee in the oral toxicity test (23.3, 36.7 and 66.7 % mortality, respectively) during 48 hours. The calculated LD_{50} (48 h) is 0.066 µg a.i. per bee in the oral toxicity test (equivalent to 0.361 µg product/bee based on analysed content of a.i.).
In the oral toxicity test the contaminated sucrose solutions were completely consumed at each dose level within 3 hours of dose administration. The results are presented in terms of the actual measured consumed dose (see appendix 3).

In the contact toxicity test no statistically significant effects on survival were observed at doses of 0.0029, 0.0057, 0.0114 and 0.0229 µg a.i. per bee (0, 0, 10 and 13.3 % mortality, respectively) during 48 hours. Statistically significant effects on survival were observed only at doses of 0.0457 and 0.0914 µg a.i. per bee in the contact toxicity test (33.3 and 75.0 % mortality, respectively) during 48 hours. Therefore the calculated LD_{50} (48 h) is 0.056 µg a.i. per bee in the contact toxicity test (equivalent to 0.306 µg product/bee based on analysed content of a.i.).

The test period was prolonged up to 96 h because progressive mortality of the bees was observed at some doses between 24 and 48 hours in both the oral and contact toxicity test.
0 % mortality was observed in the oral toxicity control treatments during 96 hours. Thus, the test accomplished the validity criterion (mortality in the control ≤ 10 %) up to 96 hours.

No statistically significantly mortality was observed at doses 0.0064 and 0.0128 µg a.i./bee after oral exposure to Imidacloprid SL 200 (3.3 and 16.7 %, respectively) during 96 hours.
The increased mortality was statistically significant compared to control only for the doses of 0.0256, 0.0512 and 0.1025 µg a.i./bee (26.7, 43.3 and 73.3 % mortality) during 96 hours.
Therefore, the calculated LD_{50} (96 h) is 0.053 µg a.i. per bee in the oral toxicity test (equivalent to 0.290 µg product/bee based on analysed content of a.i.).

In the contact toxicity test 0 % mortality was observed in the control treatments during 96 hours. Thus, the test accomplished the validity criterion (mortality in the control ≤ 10 %) until 96 hours after exposure. At the last assessment at 96 hours after contact exposure the mortality increased up to 0, 6.7, 10.0, 26.7, 33.3 and 86.7 % at doses of 0.0029, 0.057, 0.0114, 0.0229, 0.0457 and 0.0914 µg a.i. per bee, respectively. Therefore, the calculated LD_{50} (96 h) is 0.045 µg a.i. per bee in the contact toxicity test (equivalent to 0.246 µg product/bee based on analysed content of a.i.).

Behaviour of bees

After contact exposure most of the bees showed uncoordinated movement during walking or were inactive at doses of 0.0114, 0.0229, 0.0457 and 0.0914 µg a.i./bee between 24 and 48 hours. The activity of bees was reduced most at the two highest applied doses of 0.0457 and 0.0914 µg a.i./bee after contact exposure between 24 and 48 hours. At 72 hours following contact exposure all healthy bees were active in all test item treatment groups.
The most bees demonstrating this behaviour were observed between 48 and 72 hours.

In the oral toxicity test bees showed the same behaviour as observed after contact exposure to the test item. Bees exposed to the test item showed uncoordinated movement during walking or were inactive at the 24, 48 and 72 hours assessments at doses of 1.025 and 0.0512 µg a.i./bee.
After 72 and 96 hours of oral exposure bees exposed at doses of 1.025 and 0.0512 µg a.i./bee had recovered and were walking or feeding.

With regard to the test item Imidacloprid SL 200 LD_{50} values were calculated and are presented in table 2.

Table 2: Oral and contact toxicity LD_{50} values of bees treated with Imidacloprid SL 200

Treatment		**LD_{50}**					
Test item	time	oral toxicity test			contact toxicity test		
Imidacloprid SL 200		µg a.i./bee	slope b	µg product/bee	µg a.i./bee	slope b	µg product/bee
	24 h 95 %-cl lower upper	n.d.		n.d.	n.d.		n.d.
	48 h 95 %-cl lower upper	**0.066** 0.045 0.098	1.72	**0.361** 0.246 0.536	**0.056** 0.042 0.074	2.319	**0.306** 0.230 0.404
	72 h 95 %-cl lower upper	**0.056** 0.04 0.077	1.89	**0.306** 0.219 0.421	**0.048** 0.036 0.065	2.030	**0.262** 0.197 0.355
	96 h 95 %-cl lower upper	**0.053** 0.038 0.074	1.84	**0.290** 0.208 0.404	**0.045** 0.034 0.060	2.092	**0.246** 0.186 0.328

cl: confidence limits
n.d.: not defined

In order to determine the sensitivity of the test system the oral and the contact toxicity of Dimethoate were determined in a study run concurrently with the test item toxicity tests.
With regard to the reference item Dimethoate EC 400 the LD_{50} values were calculated and are presented in table 3.

Table 3: Oral and contact toxicity LD_{50} values of bees treated with Dimethoate EC 400

Treatment		LD_{50}					
Reference item	time	oral toxicity test			contact toxicity test		
		µg a.i./bee	slope b	µg product/bee	µg a.i./bee	slope b	µg product/bee
Dimethoate EC 400	**24 h**	**0.133**	8.85	**0.358**	**0.113**	2.22	**0.304**
	95 %-cl lower	0.123		0.331	0.084		0.226
	upper	0.144		0.388	0.152		0.409
	48 h	**0.129**	8.17	**0.347**	**0.102**	2.37	**0.275**
	95 %-cl lower	0.120		0.323	0.078		0.210
	upper	0.139		0.374	0.134		0.361

cl: confidence limits

In the reference treatments apathy, discoordinated movements and immobility were observed before bees died.

Table 4: Mortality of bees in the oral toxicity test with Imidacloprid SL 200 and Dimethoate (group feeding)

acute oral toxicity (mortality %)							
		Imidacloprid SL 200					
time after application		concentration of test solution (%)					
		control	0.00018	0.00035	0.0007	0.0014	0.0028
		dose (µg product/bee) *)					
		control	0.036	0.07	0.14	0.28	0.56
		dose (µg a.i./bee) *)					
		control	0.0064	0.0128	0.0256	0.0512	0.1025
4 h	m	0.0	0.0	0.0	0.0	0.0	0.0
	cm		0.0	0.0	0.0	0.0	0.0
24 h	m	0.0	0.0	0.0	10.0	23.3	36.7
	cm		0.0	0.0	10.0	23.3	36.7
48 h	m	0.0	0.0	13.3	23.3	36.7	66.7
	cm		0.0	13.3	23.3	36.7	66.7
72 h	m	0.0	0.0	13.3	26.7	40.0	73.3
	cm		0.0	13.3	26.7	40.0	73.3
96 h	m	0.0	3.3	16.7	26.7	43.3	73.3
	cm		3.3	16.7	26.7	43.3	73.3
		reference item Dimethoate EC 400					
time after application		concentration of test solution (%)					
		control	0.001	0.0012	0.0014	0.0017	0.002
		dose (µg product/bee)					
		control	0.20	0.24	0.28	0.34	0.40
		dose (µg a.i./bee)					
		control	0.074	0.089	0.104	0.126	0.149
4 h	m	0.0	0.0	0.0	0.0	0.0	0.0
	cm		0.0	0.0	0.0	0.0	0.0
24 h	m	0.0	0.0	10.0	16.7	26.7	76.7
	cm		0.0	10.0	16.7	26.7	76.7
48 h	m	0.0	0.0	13.3	20.0	36.7	76.7
	cm		0.0	13.3	20.0	36.7	76.7
72 h	m	0.0	3.3	16.7	26.7	36.7	80.0
	cm		3.3	16.7	26.7	36.7	80.0
96 h	m	0.0	3.3	20.0	26.7	40.0	80.0
	cm		3.3	20.0	26.7	40.0	80.0

m: mortality
cm: corrected mortality (according to ABBOTT 1925)

*) calculated from the quantity of test solution remaining after 4 hours of dose administration (see appendix 3);
provided 200 µl per 10 bees
consumed volume of sucrose solution per single bee: 20 µl

Table 5: Mortality of bees in the contact toxicity test with test item Imidacloprid SL 200 and Dimethoate (topical application)

acute contact toxicity (mortality %)							
time after application		**Imidacloprid SL 200**					
		concentration of test solution (%)					
		control (acetone)	0.00156	0.00313	0.00625	0.0125	0.025	0.05
		dose (µg product/bee)						
		control (acetone)	0.0156	0.0313	0.0625	0.125	0.25	0.5
		dose (µg a.i./bee)						
		control (acetone)	0.0029	0.0057	0.0114	0.0229	0.0457	0.0914
4 h	m	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	cm		0.0	0.0	0.0	0.0	0.0	0.0
24 h	m	0.0	0.0	0.0	10.0	0.0	16.7	23.3
	cm		0.0	0.0	10.0	0.0	16.7	23.3
48 h	m	0.0	0.0	0.0	10.0	13.3	33.3	76.7
	cm		0.0	0.0	10.0	13.3	33.3	76.7
72 h	m	0.0	0.0	6.7	10.0	23.3	33.3	83.3
	cm		0.0	6.7	10.0	23.3	33.3	83.3
96 h	m	0.0	0.0	6.7	10.0	26.7	33.3	86.7
	cm		0.0	6.7	10.0	26.7	33.3	86.7
time after application		**reference item Dimethoate EC 400**					
		concentration of test solution (%)					
		control	0.00313	0.00625	0.0125	0.025	0.05
		dose (µg product/bee)					
		control	0.0313	0.0625	0.125	0.25	0.5
		dose (µg a.i./bee)					
		control	0.012	0.023	0.046	0.093	0.186
4 h	m	0.0	0.0	0.0	0.0	0.0	6.7
	cm		0.0	0.0	0.0	0.0	6.7
24 h	m	0.0	0.0	6.7	13.3	53.3	63.3
	cm		0.0	6.7	13.3	53.3	63.3
48 h	m	0.0	0.0	6.7	13.3	60.0	66.7
	cm		0.0	6.7	13.3	60.0	66.7
72 h	m	0.0	0.0	6.7	13.3	66.7	76.7
	cm		0.0	6.7	13.3	66.7	76.7
96 h	m	0.0	3.3	6.7	16.7	70.0	80.0
	cm		3.3	6.7	16.7	70.0	80.0

m: mortality
cm: corrected mortality (according to ABBOTT 1925)

4. Conclusions

Oral exposure to Imidacloprid SL 200 resulted in statistically significant effects on honeybee survival at doses of 0.0256, 0.0512 and 0.1025 µg a.i. per bee. The 48-hour LD_{50} value was 0.066 µg a.i./bee in the oral toxicity test (equivalent to 0.361 µg product/bee).
In the contact toxicity test bees exposed to the test item doses of 0.0457 and 0.0914 µg a.i./bee showed statistically significant effects on survival. The 48-hour LD_{50} values was 0.056 µg a.i./bee in the contact toxicity test (equivalent to 0.306 µg product/bee).
The observed behaviour of the test item exposed bees was different to those in the control, with a high number of bees observed as inactive up to 72 hours after exposure, depending on the dose applied.

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

1. Preparation of sucrose solution (= control)

- Weighed: 250.0 g of sucrose
- Dissolving in deionized water to a total volume of 500 ml yielding the 50 % (w/v) sucrose solution;
- Applied volume 200 µl 50 % (w/v) aqueous sucrose solution (equivalent to 235 µg) per 10 bees (group feeding)

2. Preparation of test item solutions (based on analysed content of a.i.)

- Weighed: 0.0280 g Imidacloprid SL 200
- Made up to 1000 ml with 50 % (w/v) sucrose solution, which gave 1000 ml 0.0028 % (w/v) stock solution AT (equivalent to 0.028 µg product/µl, to 0.56 µg product/bee and to 0.1025 µg a.i./bee)

Preparation of dilutions:

volume	name of solution	+ 50 % (w/v) sucrose solution to total volume of solution	concentration (% w/v)	dose (µg product/µl)	dose (µg product/bee) (20 µl solution/bee)	dose (µg a.i./bee) (20 µl solution/bee)
5 ml	AT	to 10 ml BT	0.0014	0.0140	0.28	0.0512
5 ml	BT	to 10 ml CT	0.0007	0.007	0.14	0.0256
5 ml	CT	to 10 ml DT	0.00035	0.0035	0.07	0.0128
5 ml	DT	to 10 ml ET	0.00018	0.0018	0.036	0.0064

The solutions AT to ET were used for dosing.

3. Preparation of reference item solutions (based on nominal content of a.i.)

- Weighed: 0.200 g Dimethoate EC 400
- Made up to 10 ml with 50 % (w/v) sucrose solution, which gave 10 ml 2 % (w/v) stock solution AR
- Preparation of dilutions:

volume	name of solution	+ 50 % (w/v) sucrose solution to total volume of solution	concentration (% w/v)	dose (µg product/µl)	dose (µg product/bee) (20 µl solution/bee)	dose (µg a.i./bee) (20 µl solution/bee)
1 ml	AR	to 100 ml BR	0.02	0.2		
5.0 ml	BR	to 50 ml CR	0.002	0.02	0.40	0.149
8.4 ml	CR	to 10 ml DR	0.0017	0.017	0.34	0.126
7.1 ml	CR	to 10 ml ER	0.0014	0.014	0.28	0.104
5.9 ml	CR	to 10 ml FR	0.0012	0.012	0.24	0.089
5.0 ml	CR	to 10 ml GR	0.0010	0.010	0.20	0.074

The solutions CR to GR were used for dosing.

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

1. Acetone (=control)

- Applied volume: 1 µl untreated acetone

2. Preparation of test item solutions (based on analysed content of a.i.)

- Weighed: 0.0500 g Imidacloprid SL 200
- Made up to 100 ml with acetone, which gave a 0.05 % (w/v) stock solution AT (equivalent to 0.5 µg product/µl and 0.0914 µg a.i./bee)
-

volume of solution	name	+ acetone	concentration (% w/v)	dose (µg product/µl)	dose (µg product/bee) (1µl solution/bee)	dose (µg a.i./bee) (1 µl solution/bee)
5 ml	AT	to 10 ml BT	0.025	0.25	0.25	0.0457
5 ml	BT	to 10 ml CT	0.0125	0.125	0.125	0.0229
5 ml	CT	to 10 ml DT	0.00625	0.0625	0.0625	0.0114
5 ml	DT	to 10 ml ET	0.00313	0.0313	0.0313	0.0057
5 ml	ET	to 10 ml FT	0.00156	0.0156	0.0156	0.0029

The solutions AT to ET were used for dosing.

3. Preparation of reference item solutions (based on nominal content of a.i.)

- Weighed: 0.100 g Dimethoate EC 400
- Made up to 100 ml with acetone, which gave a 0.1 % (w/v) stock solution AR
- Preparation of dilutions:

volume of solution	name	+ acetone	concentration (% w/v)	dose (µg product/µl)	dose (µg product/bee) (1 µl solution/bee)	dose (µg a.i./bee) (1 µl solution/bee)
5 ml	AR	to 10 ml BR	0.05	0.5	0.5	0.186
5 ml	BR	to 10 ml CR	0.025	0.25	0.25	0.093
5 ml	CR	to 10 ml DR	0.0125	0.125	0.125	0.046
5 ml	DR	to 10 ml ER	0.00625	0.0625	0.0625	0.023
5 ml	ER	to 10 ml FR	0.00313	0.0313	0.0313	0.012

The solutions BR to FR were used for dosing.

Appendix 3: **Table of results – oral toxicity test – consumed amount of test solution**

treatment	replicate	concentration of test solution (%)	consumed test solution per 10 bees (g)*	a.i. per bee (µg)**
control (sucrose solution)	1	.	0.235	
	2		0.235	
	3		0.235	
	mean		0.235	
test item **Imidacloprid SL 200**	1	0.0028	0.235	0.1025
	2		0.235	0.1025
	3		0.235	0.1025
	mean		0.235	0.1025
	1	0.0014	0.235	0.0512
	2		0.235	0.0512
	3		0.235	0.0512
	mean		0.235	0.0512
	1	0.0007	0.235	0.0256
	2		0.235	0.0256
	3		0.235	0.0256
	mean		0.235	0.0256
	1	0.00035	0.235	0.0128
	2		0.235	0.0128
	3		0.235	0.0128
	mean		0.235	0.0128
	1	0.00018	0.235	0.0064
	2		0.235	0.0064
	3		0.235	0.0064
	mean		0.235	0.0064
reference item **Dimethoate EC 400**	1	0.002	0.235	0.149
	2		0.235	0.149
	3		0.235	0.149
	mean		0.235	0.149
	1	0.0017	0.235	0.126
	2		0.235	0.126
	3		0.235	0.126
	mean		0.235	0.126
	1	0.0014	0.235	0.104
	2		0.235	0.104
	3		0.235	0.104
	mean		0.235	0.104
	1	0.0012	0.235	0.089
	2		0.235	0.089
	3		0.235	0.089
	mean		0.235	0.089
	1	0.001	0.235	0.074
	2		0.235	0.074
	3		0.235	0.074
	mean		0.235	0.074

* all test solutions administered to each replicate as 0.235 g (200 µl/replicate, i.e. about 20 µl/bee)
** calculated from the quantity of test solution remaining after 3 hours of dose administration, in the case of this refers to µg test item/bee rather than µg a.i. per bee.

Appendix 4: **Table of results – oral toxicity test – test item**

Surviving bees after oral exposure of the test item Imidacloprid SL 200							
Assessment time	replicate	control	µg a.i./bee				
			0.0064	0.0128	0.0256	0.0512	0.1025
4h	1	10.0	10.0	10.0	10.0	10.0	10.0
	2	10.0	10.0	10.0	10.0	10.0	10.0
	3	10.0	10.0	10.0	10.0	10.0	10.0
	sum	30.0	30.0	30.0	30.0	30.0	30.0
	mean	10.0	10.0	10.0	10.0	10.0	10.0
	m	0.0	0.0	0.0	0.0	0.0	0.0
	cm		0.0	0.0	0.0	0.0	0.0
24h	1	10.0	10.0	10.0	10.0	7.0	6.0
	2	10.0	10.0	10.0	9.0	8.0	7.0
	3	10.0	10.0	10.0	8.0	8.0	6.0
	sum	30.0	30.0	30.0	27.0	23.0	19.0
	mean	10.0	10.0	10.0	9.0	7.7	6.3
	m	0.0	0.0	0.0	10.0	23.3	36.7
	cm		0.0	0.0	10.0	23.3	36.7
48h	1	10.0	10.0	8.0	9.0	7.0	4.0
	2	10.0	10.0	8.0	8.0	5.0	2.0
	3	10.0	10.0	10.0	6.0	7.0	4.0
	sum	30.0	30.0	26.0	23.0	19.0	10.0
	mean	10.0	10.0	8.7	7.7	6.3	3.3
	m	0.0	0.0	13.3	23.3	36.7	66.7
	cm		0.0	13.3	23.3	36.7	66.7
72h	1	10.0	10.0	8.0	9.0	7.0	3.0
	2	10.0	10.0	8.0	7.0	5.0	2.0
	3	10.0	10.0	10.0	6.0	6.0	3.0
	sum	30.0	30.0	26.0	22.0	18.0	8.0
	mean	10.0	10.0	8.7	7.3	6.0	2.7
	m	0.0	0.0	13.3	26.7	40.0	73.3
	cm		0.0	13.3	26.7	40.0	73.3
96h	1	10.0	9.0	7.0	9.0	7.0	3.0
	2	10.0	10.0	8.0	7.0	4.0	2.0
	3	10.0	10.0	10.0	6.0	6.0	3.0
	sum	30.0	29.0	25.0	22.0	17.0	8.0
	mean	10.0	9.7	8.3	7.3	5.7	2.7
	m	0.0	3.3	16.7	26.7	43.3	73.3
	cm		3.3	16.7	26.7	43.3	73.3

m: mortality
cm: corrected mortality (according to ABBOTT 1925)

Appendix 5: **Table of results – oral toxicity test – reference item**

Surviving bees after oral exposure of the reference item Dimethoate EC 400							
Assessment time	replicate	control	µg a.i./ bee				
			0.074	0.089	0.104	0.126	0.149
4h	1	10.0	10.0	10.0	10.0	10.0	10.0
	2	10.0	10.0	10.0	10.0	10.0	10.0
	3	10.0	10.0	10.0	10.0	10.0	10.0
	sum	30.0	30.0	30.0	30.0	30.0	30.0
	mean	10.0	10.0	10.0	10.0	10.0	10.0
	m	0.0	0.0	0.0	0.0	0.0	0.0
	cm		0.0	0.0	0.0	0.0	0.0
24h	1	10.0	10.0	9.0	8.0	8.0	3.0
	2	10.0	10.0	9.0	9.0	7.0	2.0
	3	10.0	10.0	9.0	8.0	7.0	2.0
	sum	30.0	30.0	27.0	25.0	22.0	7.0
	mean	10.0	10.0	9.0	8.3	7.3	2.3
	m	0.0	0.0	10.0	16.7	26.7	76.7
	cm		0.0	10.0	16.7	26.7	76.7
48h	1	10.0	10.0	9.0	8.0	7.0	3.0
	2	10.0	10.0	8.0	8.0	6.0	2.0
	3	10.0	10.0	9.0	8.0	6.0	2.0
	sum	30.0	30.0	26.0	24.0	19.0	7.0
	mean	10.0	10.0	8.7	8.0	6.3	2.3
	m	0.0	0.0	13.3	20.0	36.7	76.7
	cm		0.0	13.3	20.0	36.7	76.7
72h	1	10.0	10.0	9.0	8.0	7.0	2.0
	2	10.0	10.0	8.0	7.0	6.0	2.0
	3	10.0	9.0	8.0	7.0	6.0	2.0
	sum	30.0	29.0	25.0	22.0	19.0	6.0
	mean	10.0	9.7	8.3	7.3	6.3	2.0
	m	0.0	3.3	16.7	26.7	36.7	80.0
	cm		3.3	16.7	26.7	36.7	80.0
96h	1	10.0	10.0	9.0	8.0	6.0	2.0
	2	10.0	10.0	7.0	7.0	6.0	2.0
	3	10.0	9.0	8.0	7.0	6.0	2.0
	sum	30.0	29.0	24.0	22.0	18.0	6.0
	mean	10.0	9.7	8.0	7.3	6.0	2.0
	m	0.0	3.3	20.0	26.7	40.0	80.0
	cm		3.3	20.0	26.7	40.0	80.0

m: mortality
cm: corrected mortality (according to ABBOTT 1925)

Appendix 6: **Table of results - contact toxicity test – test item**

Surviving bees after contact exposure of the test item Imidacloprid SL 200								
Assessment time	replicate	control (acetone)	µg a.i./bee 0.0029	0.0057	0.0114	0.0229	0.0457	0.0914
4h	1	10.0	10.0	10.0	10.0	10.0	10.0	10.0
	2	10.0	10.0	10.0	10.0	10.0	10.0	10.0
	3	10.0	10.0	10.0	10.0	10.0	10.0	10.0
	sum	30.0	30.0	30.0	30.0	30.0	30.0	30.0
	mean	10.0	10.0	10.0	10.0	10.0	10.0	10.0
	mort	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	cm		0.0	0.0	0.0	0.0	0.0	0.0
24h	1	10.0	10.0	10.0	10.0	10.0	8.0	7.0
	2	10.0	10.0	10.0	8.0	10.0	9.0	9.0
	3	10.0	10.0	10.0	9.0	10.0	8.0	7.0
	sum	30.0	30.0	30.0	27.0	30.0	25.0	23.0
	mean	10.0	10.0	10.0	9.0	10.0	8.3	7.7
	m	0.0	0.0	0.0	10.0	0.0	16.7	23.3
	cm		0.0	0.0	10.0	0.0	16.7	23.3
48h	1	10.0	10.0	10.0	10.0	10.0	7.0	3.0
	2	10.0	10.0	10.0	8.0	8.0	7.0	1.0
	3	10.0	10.0	10.0	9.0	8.0	6.0	3.0
	sum	30.0	30.0	30.0	27.0	26.0	20.0	7.0
	mean	10.0	10.0	10.0	9.0	8.7	6.7	2.3
	m	0.0	0.0	0.0	10.0	13.3	33.3	76.7
	cm		0.0	0.0	10.0	13.3	33.3	76.7
72h	1	10.0	10.0	9.0	10.0	7.0	7.0	2.0
	2	10.0	10.0	10.0	8.0	8.0	7.0	1.0
	3	10.0	10.0	9.0	9.0	8.0	6.0	2.0
	sum	30.0	30.0	28.0	27.0	23.0	20.0	5.0
	mean	10.0	10.0	9.3	9.0	7.7	6.7	1.7
	m	0.0	0.0	6.7	10.0	23.3	33.3	83.3
	cm		0.0	6.7	10.0	23.3	33.3	83.3
96h	1	10.0	10.0	9.0	10.0	7.0	7.0	1.0
	2	10.0	10.0	10.0	8.0	7.0	7.0	1.0
	3	10.0	10.0	9.0	9.0	8.0	6.0	2.0
	sum	30.0	30.0	28.0	27.0	22.0	20.0	4.0
	mean	10.0	10.0	9.3	9.0	7.3	6.7	1.3
	m	0.0	0.0	6.7	10.0	26.7	33.3	86.7
	cm		0.0	6.7	10.0	26.7	33.3	86.7

m: mortality
cm: corrected mortality (according to ABBOTT 1925)

Appendix 7: **Table of results - contact toxicity test – reference item**

Surviving bees after contact exposure of the reference item Dimethoate EC 400							
Assessment time	replicate	control (acetone)	µg a.i./bee 0.0012	0.0253	0.046	0.093	0.186
4h	1	10.0	10.0	10.0	10.0	10.0	10.0
	2	10.0	10.0	10.0	10.0	10.0	9.0
	3	10.0	10.0	10.0	10.0	10.0	9.0
	sum	30.0	30.0	30.0	30.0	30.0	28.0
	mean	10.0	10.0	10.0	10.0	10.0	9.3
	m	0.0	0.0	0.0	0.0	0.0	6.7
	cm		0.0	0.0	0.0	0.0	6.7
24h	1	10.0	10.0	10.0	9.0	5.0	4.0
	2	10.0	10.0	9.0	8.0	4.0	3.0
	3	10.0	10.0	9.0	9.0	5.0	4.0
	sum	30.0	30.0	28.0	26.0	14.0	11.0
	mean	10.0	10.0	9.3	8.7	4.7	3.7
	m	0.0	0.0	6.7	13.3	53.3	63.3
	cm		0.0	6.7	13.3	53.3	63.3
48h	1	10.0	10.0	10.0	9.0	4.0	4.0
	2	10.0	10.0	9.0	8.0	4.0	3.0
	3	10.0	10.0	9.0	9.0	4.0	3.0
	sum	30.0	30.0	28.0	26.0	12.0	10.0
	mean	10.0	10.0	9.3	8.7	4.0	3.3
	m	0.0	0.0	6.7	13.3	60.0	66.7
	cm		0.0	6.7	13.3	60.0	66.7
72h	1	10.0	10.0	10.0	9.0	3.0	3.0
	2	10.0	10.0	9.0	8.0	4.0	2.0
	3	10.0	10.0	9.0	9.0	3.0	2.0
	sum	30.0	30.0	28.0	26.0	10.0	7.0
	mean	10.0	10.0	9.3	8.7	3.3	2.3
	m	0.0	0.0	6.7	13.3	66.7	76.7
	cm		0.0	6.7	13.3	66.7	76.7
96h	1	10.0	10.0	10.0	9.0	3.0	2.0
	2	10.0	9.0	9.0	7.0	4.0	2.0
	3	10.0	10.0	9.0	9.0	2.0	2.0
	sum	30.0	29.0	28.0	25.0	9.0	6.0
	mean	10.0	9.7	9.3	8.3	3.0	2.0
	m	0.0	3.3	6.7	16.7	70.0	80.0
	cm		3.3	6.7	16.7	70.0	80.0

m: mortality
cm: corrected mortality (according to ABBOTT 1925)

Appendix 8: Deviations to study plan (not amended)

Deviation No.	Concerning	Reason for deviation
none		

Appendix 9: GLP Certificate

[redacted]