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GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*) - definitive test

i2L Research Ltd
Capital Business Park
Wentloog
Cardiff CF3 2PX
UK

Dr Jitka Zelová

11th December 2018

- Chemicals Regulation Directorate Certificated
- GLP (Good Laboratory Practice) Compliant

Compliance statement

The study was conducted in compliance with the Czech GLP compliance programme (Act No. 350/2011 Coll. and Decree No. 163/2012 Coll. as amended), which follows the Organisation for Economic Co-Operation and Development (OECD) Principles of Good Laboratory Practice (revised, 1997).

This report represents a true and accurate record of all data obtained.

Signed..... Date 11. 12. 2018

Jitka Zelová
Study Director

Signed..... Date 11. 12. 2018

Dr Pavel Foltan
GLP Facility Management

All raw data and a copy of the final report will be archived at i2LResearch Ltd for a period of ten years. At the end of this period all data relating to this report will either be retained by i2LResearch Ltd for a further disclosed period of time at an extra cost, destroyed at the sponsor`s request or passed on to the sponsor at the sponsor`s expense.

Report circulated to: Organic Laboratories, Inc. (1 copy)

*i2L*Research Ltd (1 copy)

Quality assurance statement

Study code: 18/523
Study title: GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*) – definitive test

Study director: Jitka Zelová

The conduct of this study was audited on the dates given below. The report has been audited to ensure that it accurately describes the methods used and that the reported results accurately reflect the raw data of the study.

Date of audit	Date of QA report to Study Director	Date of QA Report to Management	Phase audited
07.11.2018	07.11.2018	07.11.2018	Identification of critical phase of the study
19.11.2018	19.11.2018	19.11.2018	Test item application on thorax of immobilized bees, distribution of the bees to the test chambers
28.11.2018	29.11.2018	29.11.2018	Study folder audit

Quality Assurance:

Signed…… Date…11.12.18……

Pavlína Wiedenová
Quality assurer

Contents

Study Information

GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*) – definitive test

Testing organisation:	*i2L*Research Ltd Capital Business Park Wentloog Cardiff CF3 2PX UK
Testing facility:	*i2L*Research LTD Lipová 1789/9 České Budějovice 37005 Czech Republic
Sponsor:	Organic Laboratories, Inc. 5520 Glades Cut Off Road Fort Pierce, FL 34981 USA
Study Director:	Dr Jitka Zelová Email: jitka@i2lresearch.com Tel: +420603739011
Primary personnel:	Jana Nácarová
Study start date:	31.10.2018
Experimental start date:	19.11.2018
Experimental end date:	21.11.2018
Study end date:	11.12.2018
Certification:	GLP certificate no. 60464 /ENV/15

Test chemical:

Test Product	Active ingredient	Lot Number	Physical description	Storage conditions	Expiry date	i2LResearch Ltd code number
Organocide Bee Safe 3-in-1 Garden Spray	5% Sesame Oil	3238AK07EZ	yellowish liquid in white bottle with label	Ambient room temperature	13.11.2020*	18111302

**i2L* Research default expiry date (2 years from receipt).

Positive control:

Test Product	Active ingredient	Lot Number	Physical description	Storage conditions	Expiry date	i2LResearch Ltd code number
Danadim Progress	Dimethoate (400g/L EC)	10210326A	Turquoise colour liquid in white 5L bottle	Ambient room temperature	31.08.2019	1166

Summary

A laboratory study (contact toxicity definitive test) was conducted to assess acute contact toxicity of Organocide Bee Safe 3-in-1 Garden Spray (Sesame oil 5%) to honey bees (*Apis mellifera*). A single dose was applied to the dorsal side of the thorax of each bee via micropipette. 25 bees were used for each dosage level, for the control and for the toxic standard. The test item was tested at 5 rates: 6.4, 16, 40, 100 and 250 µg a.i./bee at application volume 5 µL per bee. A negative control (distilled water) at the same application volume and toxic standard (Dimethoate) at a rate of 0.3 µg a.i./bee was also included.

Dose-response curve for honey bee mortality after 48 h acute contact was not determined at significance level $p(F) \leq 0.05$. However, at the significance level $p(F) \geq 0.10$ contact toxicity or any other adverse effect was found and LD50 was determined to be 201.7 µg a.i./bee.

Aim

A laboratory study (contact toxicity definitive test) was required to assess acute contact toxicity of Organocide Bee Safe 3-in-1 Garden Spray (Sesame oil 5%) to honey bees (*Apis mellifera*). The goal of the definitive test was to determine the dose-response curve for honey bee mortality after a 48 h acute contact, and to establish the median lethal dose (LD50) (and its 95 percent confidence limits), as well as the slope of the dose-response curve and its 95% confidence limits. The methodology followed EPA (2012) test guideline. The product was tested at 5 rates provided by the sponsor, the highest rate was undiluted Organocide Bee Safe 3-in-1 Garden Spray (Sesame oil 5%). The experiment was conducted at *i2L*Research Central Europe, a branch office of *i2L*Research Ltd, in Ceske Budejovice, Czech Republic.

Test systems

The test was conducted using young adult worker honey bees (*Apis mellifera*) that were of a similar age and feeding status.

Bees were obtained from a commercial apiary in Ceske Budejovice, Czech Republic. All control and treatment bees used in a test originated from the same apparently disease-free colony. Bees were not treated with chemical substances, such as antibiotics or anti-varroa. Bees used in the test were in apparent good health. During holding and testing, bees were shielded from excessive activity or other disturbance and they were kept in conditions conforming to proper cultural practices. Bees were handled only as much as necessary to conform to test procedures. On the day of test initiation, bees were randomly collected from the hive, and placed into a holding cage. Dead or moribund bees were rejected and replaced by healthy bees before starting the test. Approximately 50% (w/w) solution of sucrose/water (500 grams/litre) were provided ad libitum throughout the holding period.

Test item

The test item (Organocide Bee Safe 3-in-1 Garden Spray) was provided by the sponsor. The test item was diluted in deionised water shortly prior to application and the solutions were thoroughly agitated to ensure their homogeneity. The test item was tested at 5 rates: 6.4, 16, 40, 100* and 250 µg a.i./bee at an application volume of 5 µL per bee. A negative control (deionised water) was also included, at the same application volume. In addition, a toxic

* Corresponds to a limit test dose according to OECD Test No. 214 (1998)

standard (Dimethoate 400g/L) at one rate (0.3 µg a.i./bee) was used to verify the sensitivity of the bees and the precision of the test procedure.

Test cages

The testing cages were wire-mesh frustum cones with 220 mm top diameter, 180 mm bottom diameter and 240 mm height covered by a netting (volume approximately 8 L). Groups of 25 bees per cage were used.

Figure 1. Test cage.

Experimental design

To initiate the test, bees in the holding cage were immobilized by cold, and distributed into treatment groups of 25 bees. A single dose was applied to the dorsal side of the thorax of each bee via micropipette. 25 bees were used for each dosage level and for each control (deionised water and toxic standard). After application, bees were placed into test cages (Figure 1). The test cages were wire-mesh frustum cones with 220 mm top diameter, 180 mm bottom diameter and 240 mm height covered by a netting (volume approximately 8 L). During the test period, the 50% (w/v) solution of sucrose/water was provided *ad libitum*.

Bees were observed for mortality and any other adverse effect at 4, 24, and 48 hours after the application. All signs of intoxication, other abnormal behaviour (denoted as "affected"), and mortality were recorded at each interval.
Dead bees were not removed from the test chambers until the test was terminated.

During the test, temperature and relative air humidity were recorded by a data logger and maintained at 23 - 26°C, with relative humidity between 54% and 71% (see Appendix III and V). The bees were kept in an incubator in the dark except for during insect introduction and observations.

Results and Conclusions

Raw data are provided in Appendix 1.

For the test to be valid according to the EPA (2012) test guidelines, mortality in the control treatment should not exceed 20% over a 48 h test period. The actual mortality was 0%. The mortality of the toxic standard (dimethoate) at 0.3 µg a.i./bee must be ≥ 50 %. In the present test the mortality of dimethoate was 100% (Table 1). The test fulfils both criteria and is thus considered valid.

The evaluation of toxic effect of the tested item was performed in program ToxRat 3.3.0. For details on the bee mortality in each treatment after 48 h see Table 2. Mortality proportion data were arcsine-transformed prior to analysis. Mortality data were analyzed by probit analysis (normal sigmoid, linear regression with max. likelihood fit) with confidence limits after Fieller. Parameters and results of the probit analysis are summarized in Table 3.
A dose-response curve of the Organocide Bee Safe 3-in-1 Garden Spray for honey bee mortality after 48 h acute contact was not determined at significance level p(F) ≤ 0.05. Nonetheless, at the significance level p(F) ≥ 0.10 significant dose/response was found and LD50 was calculated by the software to be 201.7 µg a.i./bee (Table 4). To get more reliable results, one rate above 250 µg a.i./bee would have to be tested, however this was not possible as this highest rate was alreay conducted using undiluted test item.

Table 1. Overview of Control and Standard Toxic (Dimethoate) mortality in the honey bee at 48 h.

Treatm.[µg a.i./bee]	Total Introduced	Survived	Dead	% Mortality
Control	25	25	0	0.0
0.3	25	0	25	100.0

Table 2. Overview of the honey bee mortality after 48 h acute contact with Organocide Bee Safe 3-in-1 Garden Spray. *Corresponds to a limit test dose according to OECD Test No. 214 (1998) **Application rate on label.

Treatm.[µg a.i./bee]	Total Introduced	Survived	Dead	% Mortality
Control	25	25	0	0.0
6.4**	25	25	0	0.0
16	25	24	1	4.0
40	25	23	2	8.0
100*	25	24	1	4.0
250	25	7	18	72.0

Table 3. Parameters of the probit analysis of the toxicity of Organocide Bee Safe 3-in-1 Garden Spray to honey bees with mortality at 48 h and results of the regression analysis

Parameter	Value
Computation runs:	8.00000
Slope b:	2.21998
Intercept a:	-5.11647
Variance of b:	0.22728
Goodness of Fit	
Chi2:	11.72025
Degrees of freedom:	3.00000
p(Chi2):	0.00800
Log LD50:	2.30474
SE Log LD50:	0.09006
g-Criterion:	1.82473
F:	5.55000
p(F) (df: 1;3):	0.10000

Table 4. Critical Dose of Organocide Bee Safe 3-in-1 Garden Spray for honey bee mortality after 48 h acute contact. Confidential intervals could not be determinded due to mathematical reasons. n.d. - not determined.

Critical Doses [µg a.i./bee]		0-48 h
Mortality		
	LD50	201.715
95%-CL	lower	n.d.
	upper	n.d.

It can be concluded that contact toxicity or any other adverse effect of Organocide Bee Safe 3-in-1 Garden Spray to honey bees was determined at the greater significance level ($p(F) \geq 0.10$) and LD50 was determined to be 201.7 µg a.i./bee.

References:

EPA (2012) Ecological Effects Test Guidelines: OCSPP 850.3020: Honey Bee Acute Contact Toxicity Test. United States Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention (7101), EPA 712-C-019

OECD Test No. 214 (1998) Honeybees, Acute Contact Toxicity Test. OECD Guidelines for the Testing of Chemicals, Section 2

Appendix I Raw data

Table 1A. Number of dead/affected/unaffected bees in control, toxic standard (Tox. Std.) and five rates of test item at 4, 24 and 48 hours after application.

Time from application (hrs)	Treatment [µg a.i./bee]	Dead	Affected	Unaffected
4	Control	0	0	25
4	250	7	12	6
4	100	1	0	24
4	40	2	0	23
4	16	1	0	24
4	6.4	0	0	25
4	Tox. Std.	11	11	3
24	Control	0	0	25
24	250	14	3	8
24	100	1	0	24
24	40	2	0	23
24	16	1	0	24
24	6.4	0	0	25
24	Tox. Std.	25	0	0
48	Control	0	0	25
48	250	18	0	7
48	100	1	0	24
48	40	2	0	23
48	16	1	0	24
48	6.4	0	0	25
48	Tox. Std.	25	0	0

Appendix II Study protocol

Study Code: 18/523	Sponsor: Organic Laboratories, Inc.
Proposal approval date: N/A	Proposal approval method: email
Test system(s): *Apis mellifera*	Study Director: Dr Jitka Zelova
Title of Study: GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*) - definitive test	
Test substances Test item(s): 1 product Control: Negative control and solvent (or vehicle) control	Study schedule: Study start date: October 2018 Study completion date: January 2019 Experimental start date: November 2018 Experimental completion date: December 2018
Distribution of Study protocol/Final report:	Organic Laboratories, Inc. (1 copy) i2LResearch Ltd (1 copy) Quality Assurance (1 copy)

Study director:..... Date:.....

Management:..... Date:.....

Sponsor:..... Date:.....

Quality Assurance..... Date:.....

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Product testing and development for the pest control industry

GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*) - definitive test

Testing organization

i2LResearch Ltd
Capital Business Park
Wentloog
Cardiff, CF3 2PX
UK

Testing facility

i2LResearch Ltd
Lipova 1789/9
Ceske Budejovice, 37005
Czech Republic

Sponsor

Organic Laboratories, Inc.
5520 Glades Cut Off Road
Fort Pierce, FL 34981
USA

Submitted by: **Dr Pavel Foltan**

Study Director: **Dr Jitka Zelova**
Jitka@i2lresearch.com

Quality Assurance: **Pavlina Wiedenova**

Aim

A GLP laboratory study (contact toxicity definitive test) is required to assess acute contact toxicity of Sesame oil (5% w/w) to honey bees (*Apis mellifera*). The goal of the definitive test is to determine the dose-response curve for honey bee mortality after 48 h acute contact, and if possible to establish the median lethal dose (LD50) (and its 95 percent (95%) confidence limits), as well as the slope of the dose response curve, its associated standard error and 95% confidence limits. The methodology follows EPA (2012) test guidelines, the product will be tested at 5 rates agreed with the sponsor (range finding test will not be conducted).
Experiments will be conducted at i2LResearch Ltd, in Ceske Budejovice, Czech Republic.

GLP compliance

The study will be in compliance with the Czech GLP compliance programme (Title III of Act No. 350/2011 Coll. and Decree No. 163/2012 Coll. as amended), which is in accordance with the Organisation for Economic Co-Operation and Development (OECD) Principles of Good Laboratory Practice (revised, 1997).

Test systems

The test will be conducted using young adult worker honey bees (*Apis mellifera*) that are of a similar age and feeding status.
Bees will be obtained from a commercial apiary. All control and treatment bees used in a test will be from the same source and race. Collection in early spring or late autumn should be avoided, as the bees have a changed physiology during this time. If tests have to be conducted during these times, bees can be emerged in an incubator and reared for one week with "bee bread" (pollen collected from the comb) and a sucrose solution. Bees used in the test should be in apparent good health. Only bees from apparently disease-free colonies will be used, and they will be kept in conditions conforming to proper cultural practices. Bees treated with chemical substances, such as antibiotics, anti-varroa, etc., will not be not be used. During holding and testing, bees will be shielded from excessive activity or other disturbance. Bees should be handled only as much as is necessary to conform to test procedures. Solution of sucrose/water (approximately 500 grams/litre) or a honey comb will be provided ad libitum throughout the holding and test periods.
On the day of test initiation or the evening before, bees will be randomly collected from the incubator or directly from the hive, immobilized with cold temperatures and placed into

holding cage. Dead or moribund bees should be rejected and replaced by healthy bees before starting the test.

Test Items.

The test items and its certificate of analysis will be provided by the sponsor. Two concurrent controls will be included in the test: a negative control and a solvent (or vehicle) control if applicable. The particular solvent (water is preferred) will be agreed with the sponsor.

Test item will be diluted in the solvent shortly prior to application and the solution will be thoroughly agitated to ensure its homogeneity. The test item will be tested at 5 rates diluted from the product concentrate with dilution step 2.5 (applied at the maximal allowed volume of 5 µL/bee):

a) 5 % sesame oil in water (~ 250 µg a.i./bee)
b) 2 % sesame oil in water (~ 100 µg a.i./bee*)
c) 0.8 % sesame oil in water (~ 40 µg a.i./bee)
d) 0.32 % sesame oil in water (~ 16 µg a.i./bee)
e) 0.128% sesame oil in water (~ 6.4 µg a.i./bee)

*Corresponds to a limit test dose according to OECD Test No. 214 (1998).

Experimental design

To initiate the test, bees in the holding cages will be again immobilized, and distributed into treatment groups of at least 25 bees. A single dose of 5µL will applied to the dorsal side of the thorax of each bee via a microapplicator. A minimum of 25 bees will be used for each dosage level and for each control. After treatment, the bees will placed into test chambers constructed from wire and mesh (volume approximately 8,000 cm^3), separately for each treatment.

Bees will be observed for mortality and any other adverse effects at approximately 4, 24, 48, and if applicable (If mortality increases by more than 10% between 24 and 48 h, the test duration should be extended), 96 hours after dosing. All signs of intoxication, other abnormal behaviour, and mortality will be recorded throughout the test period.

Dead bees will not be removed from the test chambers until the test is terminated.

Temperature will be maintained between 25 and 35 °C, with relative humidity between 50% and 80%. Air temperature and humidity will be recorded during the course of the trial. If the

ambient temperature or humidity falls outside of the required parameters for a short period of time, such as when assessments are made, this will not be considered to be a protocol deviation.

Test bees should be maintained in the dark except of during dosing and observations.

Validity criteria

For the test to be considered valid, mortality in the control treatment/s should not exceed 20%.

Statistical analyses

Statistical procedures will be employed to calculate the 48-h LD_{50} (standard error and 95% confidence limits) based upon mortality. Results will be presented in a tabular format. Analyses will be performed by the Study Director and will depend on the outcome of the testing at the discretion of the Study Director. Statistical analyses performed will be fully documented in the report.

Protocol amendments and deviations

Any protocol amendments and/or deviations will be documented, fully justified and maintained with the protocol. All protocol amendments will be approved by the study director and sent to the sponsor.

Archiving records

The original raw data, final report and any amendments will be archived at *i2L*Research for a period of ten years. Following this ten year period the study file will either be destroyed at the sponsor's request, kept for a further period at an extra cost, or returned to the sponsor at the sponsor's expense. The final report, including any protocol amendments or deviations, will be forwarded to the Sponsor. Any unused test substances will be either be returned to the sponsor or disposed of with the sponsor's consent.

References:

EPA (2012) Ecological Effects Test Guidelines: OCSPP 850.3020: Honey Bee Acute Contact Toxicity Test. United States Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention (7101), EPA 712-C-019

OECD Test No. 214 (1998) Honeybees, Acute Contact Toxicity Test. OECD Guidelines for the Testing of Chemicals, Section 2

Appendix III Amendments and Deviations

Protocol deviation no. 1

Study code: 18/523

Title: GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*) - definitive test

Description of deviation	Temperature in the incubator was maintained between 23 – 26°C instead of 25-35°C as stated in the protocol.
Reason for deviation	More suitable temperature range for honey bees in the winter season (bee-keeper recommendation).
Impact on study	No impact.

Study Director ..... Date.....

QA ..... Date.....

Appendix IV Calculation of concentration

Calculation of concentration range

Study code: 18/523

Test item: Organocide Bee Safe 3-in-1 Garden Spray
content of active ingredient (sesame oil): 5%
density: 0.95 g/ml (MSDS)
Solvent: distilled water

Application volume: 5 µL per bee (25 bees per treatment)

- 5 dose range required, dilution step 2.5:
  T1: 250 µg a.i./bee (5 % sesame oil in water)
  T2: 100 µg a.i./bee (2 % sesame oil in water)
  T3: 40 µg a.i./bee (0.8 % sesame oil in water)
  T4: 16 µg a.i./bee (0.32 % sesame oil in water)
  T5: 6.4 µg a.i./bee (0.128 % sesame oil in water)

  T1:
  the test item is 5 % sesame oil (a.i.) containing 250 µg a.i./bee in 5 µL - to get T1 the test item will not be dilluted

  T2 – T5:
  4 g of T1 (5% sesame oil) will be filled up to 10 g by distilled water to reach T2. The same dilution step will be applied to get T3 – T5.

- Toxic reference: **Danadim** (400g dimethoate/L) at rate 0.3 µg a.s./bee, application volume 1µL per bee

  7.5 mg product $((10mL/0.001mL_{app.\ volume}*0.0003mg_{\ a.s/bee})/0.4)$ fill up to **10 mL** of distilled water.

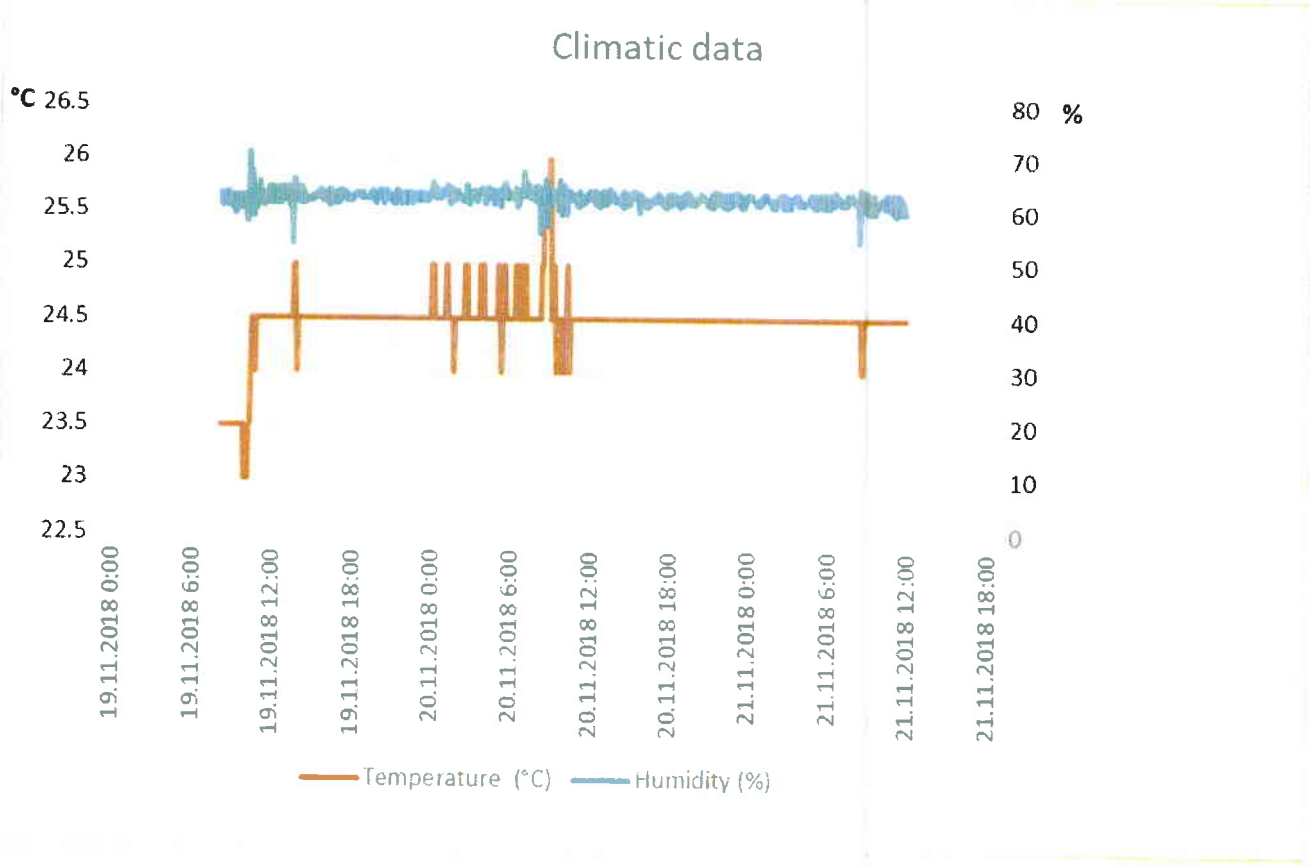
- Negative control: distilled water

Calculated by……..Date……

Checked by……....Date……

Study director……Date……

Appendix V Climatic data



Appendix VI GLP certification

Ministry of the Environment of the Czech Republic

August 28, 2015
Our ref.:60464 /ENV/15

MINISTRY OF THE ENVIRONMENT ON THE BASIS OF VERIFYING THE COMPLIANCE WITH THE PRINCIPLES ON GOOD LABORATORY PRACTICE IS ISSUING in accordance with Act. No. 350/2011 Coll.

to

I2L RESEARCH LTD.,
Lipová 1789/9
370 05 České Budějovice
Identification No: 27845281

CERTIFICATE

on the compliance with Principles on Good Laboratory Practice for

4. Ecotoxicology tests on aquatic and terrestrial orgasnisms
9. The efficacy testing of insecticide, biocides and repellent products

Signature:

Stamp: