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INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY
BRANCH PSZCZYNA

REPORT

PALIOGEN VIOLET 5011

Daphnia magna acute immobilization test

According to OECD Guideline No 202 (2004)

STUDY CODE: W/68/11

Study director:	[redacted]
Test facility:	Institute of Industrial Organic Chemistry Branch Pszczyna Department of Ecotoxicology [redacted]
Sponsor:	BASF SE [redacted]
BASF Project No.	50E00223/11X287
Version:	Final
Study completion date:	May 2012

Head of the Branch

[redacted]

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DECLARATION OF STUDY DIRECTOR

The toxicity assessment of Paliogen Violet 5011 for *Daphnia magna* was carried out according to order from November 28, 2011 from BASF SE, [redacted].

The study coded W/68/11 was performed according to the OECD Guidelines for Testing of Chemicals No 202 (2006) [1]. The study was in compliance with the principles of Good Laboratory Practice, OECD 1997 [4]. The study was performed in compliance with the study plan. The preliminary tests were performed non-GLP.

The study was performed in the Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, which holds a Statement of GLP Compliance Registration Number 6/2011/DPL issued on June 13, 2011 by the Polish Bureau for Chemical Substances, valid from June 7, 2011 (Appendix 3) [2].

I hereby declare that the work was performed under my supervision and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. I declare that the report contains an authentic description of all results obtained and observations made during the study. I bear the responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

Study director: [redacted]

Institute of Industrial Organic Chemistry
Branch Pszczyna

[redacted]

signature

PERFORMERS, AUTORS

Performing the biological part: Date, signature

[redacted] 09.05.12
[redacted] 09.05.12
[redacted] 09.05.12
[redacted] 09.05.12
[redacted] 09.05.12
[redacted] 09.05.12

Performing the chemical analysis:

[redacted]
[redacted] 09.05.12
[redacted] 09.05.12

Head of the Department of Ecotoxicology:

[redacted] 9.5.12

REPORT OF QUALITY ASSURANCE UNIT

The study plan, course of the study and final report of *Daphnia magna* acute immobilization test of Paliogen Violet 5011 (study code: W/68/11) [SOP/W/4], were controlled by the person responsible for quality assurance unit:

Scope of control	Date of control	Date of protocol submission to Head of testing facility
Study plan	30.03.2012	30.03.2012
Experiment course	04.04.2012	04.04.2012
Draft report	16-17.04.2012	17.04.2012
Final report	09.05.2012	09.05.2012

The control was conducted according to the Good Laboratory Practice [2, 4], [SOP/PJ/1].
The person responsible for quality assurance hereby confirms that the study was performed in compliance with the requirements of GLP (except for the preliminary test) and as outlined in the study plan. The final report reflects the study course and contains all generated data.

[redacted]

09.05.2012
date

[redacted]

ARCHIVING

The following documents will be archived at ten years in the Archive of Documentation and Test Samples at the Institute of Industrial Organic Chemistry, Branch Pszczyna, [redacted]

- Order,
- Study plan – true copy,
- Raw data (test sheets, results sheets – originals, temperature records, correspondence with sponsor),
- Documentation of analytical measurements,
- Quality assurance control protocols – original,
- Final report – true copy.

After the archiving period all materials will be transferred to the archives of the sponsor or, with prior notification of the sponsor, destroyed.

The archival standard of the test item will be stored in the Department of The Test Material (PAA) at the Institute of Industrial Organic Chemistry, Branch Pszczyna, [redacted], until its expiry date (November 18, 2020) [SOP/PB/1].

Archives and conditions of archiving of documentation and archival standard of the test item are regulated according to GLP principles and organization of quality system in the Institute of Industrial Organic Chemistry, Branch Pszczyna.

DISTRIBUTION:

Study plan:

Original: BASF SE, [redacted]

True copy: Institute of Industrial Organic Chemistry Branch Pszczyna, [redacted]

Final report:

Original: BASF SE, [redacted]

True copy: Institute of Industrial Organic Chemistry Branch Pszczyna, [redacted]

Raw data:

Original: Institute of Industrial Organic Chemistry Branch Pszczyna, [redacted]

True copy: BASF SE, [redacted]

Electronic version of final report: BASF SE, [redacted]

Institute of Industrial Organic Chemistry Branch Pszczyna, [redacted]

SUMMARY

In a static acute toxicity study, young *Daphnia magna* individuals (< 24 h old) were exposed to the test item for 48 hours. Eight replicates of the test concentration and control were tested with five daphnids per replicate (100 mL test volume). A nominal concentration of 100.0 mg/L of PALIOGEN VIOLET 5011 was applied as a limit test in eight replicates (Limit Test).

Daphnids were observed for immobilization after 24 and 48 hours of exposure. The test organisms were considered immobile if they showed no ability to swim within 15 seconds after swirling the test vessel. In the concentration 100 mg/L and in the control no immobilization was observed until test termination.

The content of test item in the test solutions was determined at test initiation (t_0) and at test termination (t_{48}).

In solutions collected at test initiation the determined concentration of test item was 6.5 µg/L. In samples at test termination the determined concentration of test item was 7.8 µg/L.

The results confirm the low solubility of the test item in water according to the information from Sponsor. The test was conducted at the water solubility limit under test conditions. The maximum solubility value under test conditions may differ from the standard water solubility value because of the composition of the test medium.

Since the recovery of PALIOGEN VIOLET 5011 at test termination is 120% of initial concentration, the EC_x values were determined based on nominal and measured concentration of the test item.

Material and methods

Test item:	PALIOGEN VIOLET 5011, Batch P 100012, [redacted].
Test species:	*Daphnia magna* Straus (< 24 h old at test initiation), not first brood progeny, range of parent daphnid age: 21 – 25 days, neonates collected from laboratory culture in the Institute of Industrial Organic Chemistry.
Test design:	Static system (48 hours), 8 replicates per treatment with 5 daphnids each.
Test concentrations:	Control; 100 mg/L.
Test conditions:	Temperature: 20.5 – 21.2°C °C, pH of control: 7.91 – 7.95, oxygen concentration: 7.5 – 8.8 mg/L, day: night regime 16 : 8 h, no feeding, no aeration
Analytics	The content of the test item was determined by spectrophotometric method.
Endpoints:	EC_{50}, EC_0, EC_{100}, LOEC and NOEC

Conclusion:

EC_{50}(48 h) value is above 100 mg/L - based on nominal concentration of PALIOGEN VIOLET 5011 (loading). The EC_0(48 h) value is equal or above 100 mg/L and EC_{100}(48 h) is above 100 mg/L - based on nominal concentration of PALIOGEN VIOLET 5011 (loading). The LOEC/48 h is above 100 mg/L

and the NOEC/48 h is equal or above 100 mg/L – based on nominal concentrations of test item (loading).

The EC_{50}(48 h) value is above 0.0065 mg/L. The EC_0(48 h) value is equal or above 0.0065 mg/L and EC_{100}(48 h) is above 0.0065 mg/L. The LOEC/48 h is above 0.0065 mg/L and the NOEC/48 h is equal or above 0.0065 mg/L – based on measured concentrations of test item. No toxicity was observed up to the limit of solubility of the test substance under test conditions.

1. AIM OF THE STUDY

The aim of the study was the determination of the concentration of the test item causing immobilization of 50% of *Daphnia magna* Straus population, i.e. EC_{50} and also EC_0 and EC_{100} values after 24 and 48 hours. The LOEC and NOEC values should be estimated, if possible.

2. TERMS

The preliminary tests started:	01.02.2012
The preliminary tests ended:	21.03.2012
The study started:	30.03.2012
The experimental starting date:	31.03.2012
The experimental completion date:	06.04.2012
Draft report:	17.04.2012
The study ended:	09.05.2012

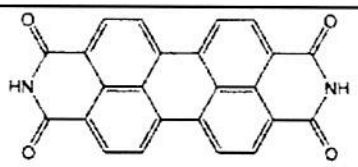
3. MATERIALS AND METHODS

3.1. Test item

The test item PALIOGEN VIOLET 5011 is a powder of dark violet colour and specific odour[1]. The sample of test item is labelled with Name: Paliogen Violet, Batch: P 100012, Expiry date: 18.11.2020, [redacted]. The sample of test item in AN amount of 50 g in a plastic container was provided by the Sponsor on December 20, 2011 with the material safety data sheet. According to information provided by the Sponsor the test item contains Anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone (CAS name), CAS No. 81-33-4. The sponsor did provide a Characterization of the test substance. According to Sponsor's information the solubility of the detected substance is 10 µg/L. The sample is stored at room temperature under dry conditions without exposure to light in a tightly sealed container [SOP/W/3, SOP/PB/1].
Data relating to the identity, purity and stability of test item are the responsibility of the Sponsor.

Paliogen Violet 5011:
Chemical name: Anthra[2,1,9-def; 6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone
CAS No.: 81-33-4
Total formula: $C_{24}H_{10}N_2O_4$
Molecular mass: 390.4 g/mol
Structural formula:

[1] Colour and the odour according to data in MSDS, were not determined according to GLP



3.2. Test organism

The test organism, *Daphnia magna* Straus, originates from the standard laboratory culture maintained in the Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, [redacted] Only organisms up to 24 h old (not first brood progeny) and in good physiological condition were used in the experiments. The sensitivity of the culture was monitored on regular basis with the reference substance potassium dichromate [SOP/W/72].

3.2.1. Culturing of *Daphnia magna*

Daphnia magna was cultured in glass beakers of 150 mL volume (one parent per vessel) at room temperature 18 – 22° C with the light : dark ratio of 16 h : 8 h (electronic time programmer, KANLUX, Poland). The culture was maintained in Elendt M7 medium [SOP/W/63, SOP/W/67]. The daphnids were fed with *Pseudokirchneriella subcapitata* algae suspension originating from the culture at the [redacted] Group B vitamins and micronutrients necessary for proper growth were supplied with lyophilized *Spirulina* sp. suspension.

Daphnia magna reproduce parthenogenetically, therefore in each beaker young daphnids were produced. The young (less than 24 h old) *Daphnia magna*, progeny of 21 – 25 days old parent, were used in the tests.

3.2.2. Culturing medium

The Elendt M7 medium recommended by OECD Guideline No 202 (2004) was used for culturing and as the diluent/solvent for the test item. The medium was prepared on the basis of deionized water (filtering system SolPure7 [SOP/W/71]) by adding stock solutions of reagent-grade chemicals [SOP/W/18]. The stock solutions were renewed on regular basis for *Daphnia* sp. culturing. The composition of Elendt M7 medium and concentration of each ingredient are presented in Appendix 1.

3.3. Performance of acute immobilization test

The acute immobilization test with *Daphnia magna* was performed according to the OECD Guideline No 202 (2004) [1] and SOP/W/21.

3.4. Test conditions

3.4.1. The preliminary tests (non-GLP)

The first toxicity evaluation of the test substance to *Daphnia magna* was performed in a preliminary static test. The test was performed in Elendt M7 medium used for culturing and as diluent for preparing the test concentrations. The medium was aerated prior to test initiation. The test was conducted in glass beakers of 150 mL capacity covered with transparent lids in order to minimize evaporation and to prevent accidental contamination. In the preliminary test the volume of the test substance solution and the control in every replicate was 50 mL. Five individuals of *Daphnia magna* were used in each replicate. In accordance with the OECD Guideline (202) which requires at least 2 mL [1] 10 mL of test suspension were applied per daphnid. The test concentration and control was tested in eight replicates.

Three preliminary tests were performed all using the same concentrations: 0.1; 1.0; 10.0 and 100 mg/L (Table 1-3). The three test differed in the way of preparing concentrations of test item.

In the first test the highest test concentration 100 mg/L was prepared by weighing the test item into a glass flask and mixing it with the test medium in an ultrasonic bath at a temperature of 40-50 °C (for 48 h with sonication for 30 min) on a mechanic stirrer. Clear and transparent basic solutions were obtained after filtration [SOP/W/37]. The solution was diluted with test medium to prepare the remaining test concentrations.

In the second test the concentration 100 mg/L was stirred over 72 h at 40 °C (in the incubator), then conditioned to 20 °C over 24 h (stirring) and the suspension was filtered over a 0.45 µm membrane disc. This filtrate was used to prepare the other concentrations: 10.0; 1.0 and 0.1 mg/L.

In the third test 100 mg and 1.0 mg were weighed separately. Each of two weighted amounts was mixed with 1 L of Elendt medium and then the procedure was similar as in the second test. After conditioning the concentration 100 mg/L was diluted in order to prepare a concentration of 10 mg/L and the concentration 1.0 mg/L was diluted in order to prepare a concentration of 0.10 mg/L. Next the control and all concentrations were filtrated over a 0.20 µm membrane disc. The filter was saturated with the test solution.

In the preliminary tests the concentrations of the test item were chemically determined in the highest concentration 100 mg/L and in the concentration 10 mg/L in the third test. The chemical analyses of the 100 mg/L concentration of the second preliminary non-GLP test revealed a concentration above 1 mg/L, which indicated, that the filtration was not successful. Particles of the test substance were observed in the test solution. In conclusion the effects in the highest concentration were most likely caused by unsolved test substance.

During the test observations of toxic effects were performed and recorded 24 and 48 h after test initiation.

During the test daphnids were not fed and the temperature of solutions was constantly recorded (thermologger HI 141, Hanna Instruments, USA [SOP/W/51]). The pH and dissolved oxygen content were measured at test initiation before division into replicates and at test termination in pooled

replicates (pH-Oxi-meter inoLAB Level 3, WTW, Germany) [SOP/W/36]. The test was conducted with day : night regime of 16 : 8 h (electronic time programmer, KANLUX, Poland) provided by fluorescent lamps.

3.4.2. The definitive test

In the definitive test for *Daphnia magna* the test item was used in one concentration of 100 mg/L in a static limit test. The time of exposure was 48 hours.

The test was performed in Elendt M7 medium used for culturing and as diluent for preparing the test concentrations. The medium was aerated prior to test initiation. The test was conducted in glass beakers of 150 mL capacity covered with transparent lids in order to minimize evaporation and to prevent accidental contamination. In the definitive test the volume of test concentration and control in every replicate was 100 mL. Five individuals of *Daphnia magna* were used in each replicate. In the test a volume of 20 mL of test suspension per daphnid was used in accordance with the OECD Guideline (202) which requires at least 2 mL [1]. The test concentration and control was tested in eight replicates.

During the test daphnids were not fed and the temperature of the solutions was constantly recorded in an additional test vessel with test medium adjancent to the test vessels. The recorded temperature was 20.5 – 21.2°C during the exposure period (Figure 1) (thermologger HI 141, Hanna Instruments, USA [SOP/W/51]). The pH and dissolved oxygen content were measured at test initiation before division into replicates and at test termination in pooled replicates (Table 5) (pH-Oxi-meter inoLAB Level 3, WTW, Germany) [SOP/W/36]. The test was conducted with a day : night regime of 16 : 8 h (electronic time programmer, KANLUX, Poland) provided by fluorescent lamps.

3.4.3. Preparing concentrations of test item

In order to determine the concentrations of test item in the definitive test, the preliminary range finding tests were performed.

In the definitive test the test item was used in one concentration of 100.0 mg/L of PALIOGEN VIOLET 5011 as a limit test.

100.3 mg of test item was weighed [SOP/W/7] into a glass flask and mixed with Elendt medium up to 1L. The stock solution was mixed thoroughly in an incubator at temperature of 40 °C for 3 days with stirring resulting in a homogeneous, intensive grey mixture with a concentration of 100 mg/L. The stock solution was conditioned at a temperature of 20°C with continuous stirring [SOP/W/57]. Next the control and the test concentration were filtrated over a 0.20 µm membrane disc. After the filtration a clear and transparent solution was observed in the concentration 100.0 mg/L. The filter was previously saturated with the test mixture. From each test concentration one sample for chemical analysis was collected (200 mL).

The pH and dissolved oxygen measurements were made and the saturated test soution was divided onto eight replicates. The test medium for the control group was processed in the same way.

Subsequently, five daphnids were introduced into each vessels.

3.5. Definitions

Immobilization - the test organisms were considered immobile if they showed no ability to swim within 15 seconds after swirling the test vessel.

EC_{50} - median concentration causing immobilization, i.e. the inability to swim, of 50% daphnids after 24 and 48 hours of exposure,

EC_0 - the highest concentration causing no immobilization

EC_{100} - the lowest concentration causing 100% of immobilization.

NOEC - No Observed Effect Concentration is the highest test concentration without significant effect as compared with the control.

LOEC - the Lowest Observed Effect Concentration is the lowest test concentration with significant mortality as compared with the control.

3.6. The reference test

The test with reference substance – potassium dichromate (produced by Sigma-Aldrich) was performed at a temperature of 22.2 – 23.3°C, oxygen concentration 8.4 – 8.7 mg/L, Elendt M7 medium) [SOP/W/72]. The test was conducted from March 21 - 23, 2012.

The reference substance was used in five concentrations: 0.32; 0.56; 1.00; 1.80; 3.20 mg/L. Every concentration and control was tested in four replicates. The results of the test are given in Appendix 2 (Tables A and B). The EC_{50}(24 h) value was 0.84 mg/L and EC_{50}(48 h) value was 0.47 mg/L, both within the range given in literature [5], [SOP/W/68].

3.7. Analytical measurements

The aim of the analytical measurements of the study was to verify the concentrations of Paliogen Violet 5011 in the test system. The samples of fresh water solutions at test initiation and samples of 48 hour old water solutions at test termination were analysed in the test concentration and in the control.

The analytical measurements of Paliogen Violet 5011 were performed by spectrophotometric method [SPR/C/205].

3.7.1. Conditions of analyses performed for Paliogen Violet 5011

Reagents and solvents:

- sulfuric acid, concentrated, pure p.a.,
- deionized water,
- Paliogen Violet 5011, standard, BASF,

- standard solution 1 mg/mL of of Paliogen Violet 5011 in concentrated sulfuric acid and stock solutions 100.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL of Paliogen Violet 5011 in concentrated sulfuric acid.

Apparatus:

- laboratory glassware,
- analytical balance,
- rotary vacuum evaporator with water bath,
- UV – Visible spectrophotometer Cary Scan 50 (Varian, USA).

The following spectrophotometer parameters was used:

Wavelength	595 nm
Quartzcuvettes	1 cm

3.7.2. Sample preparation for chromatography analysis

The sample of a volume from 20 to 100 mL (i.e. control sample, test sample, sample fortified with standard) was taken and evaporated to dryness using vacuum rotary evaporator. The dry residue was dissolved in an appropriate volume of concentrated sulfuric acid and analyzed spectrophotometrically.

3.7.3. Method validation

Linearity of response for the analysis method, its specificity, precision, recovery of Paliogen Violet 5011, limit of quantification and detection were assessed in the process of analytical method validation.

Linearity

Working solutions containing 10.0, 5.0, 2.0, 1.0, 0.5, 0.1 and 0.05 µg/mL of Paliogen Violet 5011 were analyzed spectrophotometrically and the absorbance was recorded. The standard curve (absorbance versus quantity of the standard) was linear with a regression coefficient of 0.99749. The range of linearity of the analytical graph is from 0.05 µg/mL to 10.0 µg/mL. The standard curve is presented in Figure 2.

Specificity

The analytical method specificity was estimated based on the analysis of absorbance obtained for control samples of water and fortification samples. Considering the results of analysis no signal of detected substances was overlapping with the matrix signal of control samples under experimental conditions. Therefore the criterion for the specificity of the method is fulfilled.

Precision

The precision is determined as the repeatability (RSD – relative standard deviation [%]). The repeatability for Paliogen Violet 5011 is presented in Table 4.

Extraction recovery level

In order to study the recovery level, the solution of the detected substance was added to non-treated samples of water and then analyzed by the method described. The results are presented in Table 4.

Limit of Quantification and Detection

The Limit of Quantification was estimated as the lowest concentration of the detected substances, at which an acceptable mean recovery is obtained (normally 70 – 110% with a relative standard deviation of preferably ≤ 20%). The Limit of Detection was estimated as the lowest concentration of the detected substances that the analytical procedure can reliably differentiate from background noise. The Limit of Quantification (LoQ) and the Limit of Detection (LoD) for Paliogen Violet 5011 in water are both 0.001 mg/L.

4. RESULTS

4.1. The preliminary tests (non-GLP)

At the end of the first preliminary test the immobilization was 5% in the nominal concentration 100 mg/L (Table 1). At the end of the second preliminary test the immobilization was 0% in the nominal concentration 100 mg/L (Table 2).
At the end of the third preliminary test the immobilization was 45% in the nominal concentration 100 mg/L. The results of immobilization are given in Table 3.
The chemical analyses of the 100 mg/L concentration of the third preliminary non-GLP test revealed a concentration above 1 mg/L, which indicated, that the filtration was not successful. Particles of the test substance were observed in the test solution. In conclusion the effects in the highest concentration were most likely caused by unsolved test substance.

4.2. Definitive test

In the control and the test concentration 100 mg/L no immobilization of Daphnia magna was observed after 24 and 48 h. The results are presented in Table 6.

4.2.1. Results of analytical measurements

The content of the PALIOGEN VIOLET 5011 was chemically analysed in samples of the test concentration collected at test initiation and termination. The results are given in Table 7.
In the sample collected at test initiation the concentration of the PALIOGEN VIOLET 5011 was 6.5 µg/L. The results confirm the low solubility of the test item in water according to the information from Sponsor.

The determined concentration of the PALIOGEN VIOLET 5011 in the sample collected at test termination from the test concentration – 100 mg/L was 7.8 µg/L. The test item was stable under test conditions.

Therefore, the results should be based on nominal and measured concentration of the test item.

4.3. Endpoint values

The EC_{50}(48 h) value is above 100 mg/L. The EC_0(48 h) value is equal or above 100 mg/L and EC_{100}(48 h) is above 100 mg/L. The LOEC/48 h is above 100 mg/L and the NOEC/48 h is equal or above 100 mg/L – based on nominal concentrations of test item (loading) (Table 8)

The EC_{50}(48 h) value is above 0.0065 mg/L. The EC_0(48 h) value is equal or above 0.0065 mg/L and EC_{100}(48 h) is above 0.0065 mg/L. The LOEC/48 h is above 0.0065 mg/L and the NOEC/48 h is equal or above 0.0065 mg/L – based on measured concentrations of test item (loading) (Table 9).

No toxicity was observed up to the water solubility limit of the test substance under test conditions.

5. THE VALIDITY CRITERIA

The validity criteria were met according to OECD Guideline No 202:

- Immobilization of *Daphnia magna* in control was 0 % (allowed not more than 10 %).
- Oxygen concentration in the test vessels was between 7.5 – 8.8 mg/L (allowed not less than 3 mg/L).

6. DEVIATIONS FROM STUDY PLAN

No deviations occured during the definitive test.

In the Study plan the deadline for final report was April 2012. However, due to obligation to acquire Sponsor's acceptance of the draft report, the deadline was postponed.

7. REFERENCES

[1] OECD Guideline for Testing of Chemicals No 202 (2004): "*Daphnia* sp., Acute Immobilization Test".

[2] The regulation of the minister of Heath of 28th May 2010 concerning the criteria, that must be met by institutions conducting tests of chemical substances and preparations, and the verification of compliance with these criteria (Dz.U. Nr 109, poz. 722)

[3] ToxRat Professional 2.10 – Software for Statistical Evaluation of Biotests in Ecotoxicology, ToxRat Solutions GmbH, Alsdorf, Germany.

[4] Directive 2004/10/EC on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

[5] Directive 67/548 EC, Appendix No. V, Part C: C.2.

8. STANDARD OPERATING PROCEDURES USED IN THE STUDY

SOP/W/3	Proceeding with test item
SOP/W/4	Studies coding
SOP/W/7	The analytical balance – instructions manual
SOP/W/18	Preparing and labeling of chemical reagents
SOP/W/21	Daphnia, acute immobilization test
SOP/W/35	The documentation and archiving of test records and notes
SOP/W/36	The pH-oxi meter inoLAB pH/Oxi Level 3 – instructions manual
SOP/W/37	NALGENE filtration system – instructions manual
SOP/W/51	Temperature recorder HI 141 – instructions manual
SOP/W/57	Mechanic shaker – instructions manual
SOP/W/63	Standardized Elendt M7 medium – preparation method
SOP/W/65	Algae and cyanobacteria, laboratory cultivation
SOP/W/67	Daphnia magna laboratory culture
SOP/W/68	ToxRat Professional programme – manual
SOP/W/71	Water deionization system SolPure7 – instructions manual
SOP/W/72	Reference tests
SOP/C/205	The determination method of Paliogen Violet 5011 in water
SOP/PB/1	Receiving, distribution, storage, registry and elimination of test item
SOP/PB/2	Archiving of documentation and test samples
SOP/PJ/1	Control of planning, conducting and preparing of study report

Table 1. Immobilization of *Daphnia magna*, preliminary test I (non-GLP)

Nominal concentration of test item [mg/L]	Number of tested organisms	Number of immobilized								Total immobilized [%]	
		24 h				48 h					
		Replicates									
		A	B	C	D	A	B	C	D	24 h	48 h
Control	20	0	0	0	0	0	0	0	0	0	0
0.1	20	0	0	0	0	0	0	0	0	0	0
1.0	20	0	0	0	0	0	0	0	0	0	0
10.0	20	0	0	0	0	0	0	0	0	0	0
100.0	20	0	0	0	0	0	1	0	0	0	5

The test was performed on 01 – 03.02.2012

Table 2. Immobilization of *Daphnia magna*, preliminary test II (non-GLP)

Nominal concentration of test item [mg/L]	Number of tested organisms	Number of immobilized								Total immobilized [%]	
		24 h				48 h					
		Replicates									
		A	B	C	D	A	B	C	D	24 h	48 h
Control	20	0	0	0	0	0	0	0	0	0	0
0.1	20	0	0	0	0	0	0	0	0	0	0
1.0	20	0	0	0	0	0	0	0	0	0	0
10.0	20	0	0	0	0	0	0	0	0	0	0
100.0	20	0	0	0	0	0	0	0	0	0	0

The test exposure 25.02.2012 – 27.02.2012

Table 3. Immobilization of *Daphnia magna*, preliminary test III

Nominal concentration of test item [mg/L]	Number of tested organisms	Number of immobilized								Total percentage of immobilized [%]	
		24 h				48 h					
		Replicates									
		A	B	C	D	A	B	C	D	24 h	48 h
Control	20	0	0	0	0	0	0	0	0	0	0
0.1	20	0	0	0	0	0	0	0	0	0	0
1.0	20	0	0	0	0	0	0	0	1	0	5
10.0	20	0	0	0	0	0	0	1	0	0	5
100.0	20	0	0	0	1	3	1	3	2	5	45

The test exposure 19.03.2012 – 21.03.2012.

Table 4. Recovery level of Paliogen Violet 5011 in fortified samples (n = 5)

Nominal concentration [mg/L]	Determined concentration of Paliogen Violet 5011 in replicates [mg/L]					Average [mg/L]	Recovery [%]	SD [mg/L]	RSD [%]
	1	2	3	4	5				
Control	0.0000	0.0000	--	--	--	0.0000	--	0.0000	--
0.001	0.0009	0.0009	0.0010	0.0010	0.0009	0.0010	96.00	0.0000	4.17
0.100	0.0985	0.0988	0.0979	0.0981	0.0989	0.0984	98.44	0.0004	0.44

LoQ = 0.001 mg/L
LoD = 0.001 mg/L

Table 5. pH values and oxygen concentration, definitive test

Nominal concentration of test item [mg/L]	pH values # at test initiation	oxygen concentration [mg/L] # at test initiation	pH values * at test termination	oxygen concentration [mg/L] * at test termination
Control	7.91	7.7	7.95	8.6
100	8.04	7.5	7.94	8.8

- pH and O_2 measured in samples before division into replicates.
* - pH and O_2 measured in samples of pooled replicates.

Table 6. Immobilization of *Daphnia magna*,definitive test

Nominal concentration of test item [mg/L]	Number of tested organisms	Number of immobilized								Total immobilized [%]	
		24 h				48 h					
		Replicates									
		A	B	C	D	A	B	C	D	24 h	48 h
Control	20	0	0	0	0	0	0	0	0	0	0
Control	20	0	0	0	0	0	0	0	0	0	0
100.0	20	0	0	0	0	0	0	0	0	0	0
100.0	20	0	0	0	0	0	0	0	0	0	0

The test exposure 04.04 – 06.04.2012

Table 7. Concentration and stability of test item - definitive test

Nominal concentration of test item [mg/L]	Mean concentration (n=3) of test item measured in samples collected [mg/L]			
	at test initiation		at test termination	
	fresh solutions	% of nominal concentration	48 h old solutions	% of initial concentration
Control	<LoD	--	<LoD	--
100	0.0065	0.0065	0.0078	120

Table 8. Endpoint values based on nominal concentrations of test item (as loadings)

Endpoint values	EC_x, LOEC and NOEC values [mg/L] at time of exposure	
	24 h	48 h
EC_0	≥ 100	≥ 100
EC_{50}	> 100	> 100
EC_{100}	> 100	> 100
LOEC	> 100	> 100
NOEC	≥ 100	≥ 100

Table 9. Endpoint values based on measured concentrations of test item

Endpoint values	EC_x, LOEC and NOEC values [mg/L] after 48 h
EC_0	≥ 0.0065
EC_{50}	> 0.0065
EC_{100}	> 0.0065
LOEC	> 0.0065
NOEC	≥ 0.0065

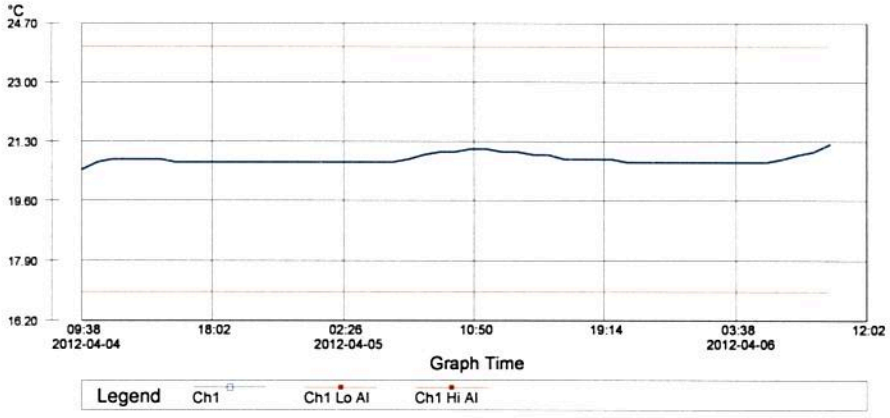


Figure 1. Temperature - definitive test

Paliogen Violet 5011, *Daphnia magna*, Acute immobilization test
Study code: W/68/11

Calibration eqn	Abs = 0.23371*Conc +0.00661
Correlation Coefficient	0.99749

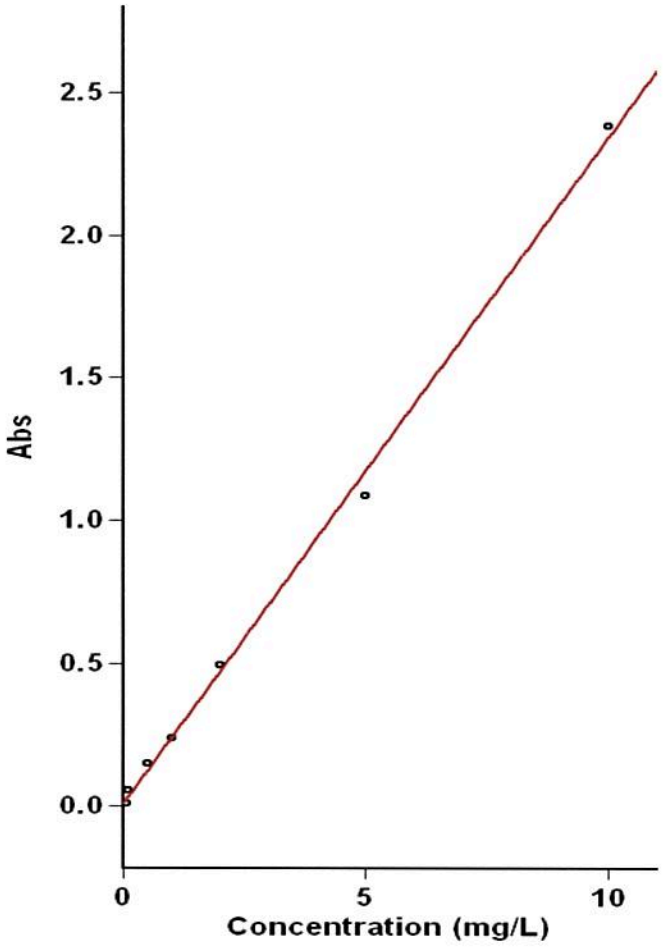


Figure 2. Calibration curve of Paliogen Violet 5011

Appendix 1.Composition of the Elendt M7 medium

Substance	Concentration in Elendt M7 medium [mg/L]
Micronutrients	
H_3BO_3	0.715
$MnCl_4 \cdot 4H_2O$	0.090
LiCl	0.076
RbCl	0.018
$SrCl_2 \cdot 6\ H_2O$	0.038
NaBr	0.004
$Na_2MoO_4 \cdot 2H_2O$	0.016
$CuCl_2 \cdot 2H_2O$	$4.19 \cdot 10^{-3}$
$ZnCl_2$	0.013
$CoCl_2 \cdot 6H_2O$	0.01
KJ	$3.25 \cdot 10^{-3}$
Na_2SeO_3	$2.19 \cdot 10^{-3}$
NH_4VO_3	$5.75 \cdot 10^{-4}$
$Na_2EDTA \cdot 2H_2O$	0.625
$FeSO_4 \cdot 7H_2O$	0.249
Macronutrients	
$CaCl_2 \cdot 2H_2O$	293.8
$MgSO_4 \cdot 7H_2O$	123.3
KCl	5.8
$NaHCO_3$	64.8
Na_2SiO_3	10.0
$NaNO_3$	0.274
KH_2PO_4	0.143
K_2HPO_4	0.184
Vitamins	
thiamine hydrochloride	0.075
cyanocobalamin	0.001
biotin	$7.5 \cdot 10^{-4}$

Appendix 2. The reference test

Table A. Immobilization of *Daphnia magna*, reference test

Nominal concentration of test item [mg/L]	Number of tested organisms	Number of immobilized								Total immobilized [%]	
		24 h				48 h					
		Replicates									
		A	B	C	D	A	B	C	D	24 h	48 h
Control	20	0	0	0	0	0	0	0	0	0	0
0.32	20	0	0	0	0	1	0	1	0	0	10
0.56	20	0	0	1	1	4	4	2	4	10	70
1.0	20	3	4	4	3	5	5	5	5	70	100
1.8	20	5	5	5	5	5	5	5	5	100	100
3.2	20	5	5	5	5	5	5	5	5	100	100

The test was performed on 23 – 25.01.2012

Table B. Endpoint values based on nominal reference substance concentrations, reference test

The ECx values	EC_x, LOEC and NOEC values [mg/L] at time of exposure	
	24 h	48 h
EC_0	0.32	< 0.32
EC_{50}	0.84 (0.72-0.97)	0.47 (0.41-0.55)
EC_{100}	1.8	1.00
LOEC	0.56	0.32
NOEC	0.32	< 0.32

(...) – confidence interval

Appendix 3. Copy of Statement of GLP compliance

STATEMENT OF GLP COMPLIANCE

Registration number: 6/2011/DPL

Assessment of conformity with GLP according to the Directive 2004/9/EC of the European Parliament and of the Council

On the basis of the inspection which was held on 6th – 7th June 2011 and in accordance with the criteria specified in the order of the Inspector for Chemical Substances and Preparations of 1st April 2011 concerning the rules on the inspection and verification of compliance with the principles of Good Laboratory Practice, as well as in accordance with Directive 2004/9/EC and the relevant OECD regulations, the Inspector for Chemical Substances hereby confirms that

Department of Ecotoxicology
Institute of Industrial Organic Chemistry
Branch Pszczyna
[redacted]

complies with the OECD and the EU principles of Good Laboratory Practices in the fields of:

- **environmental toxicity studies on aquatic and terrestrial organisms;**
- **studies on behaviour in water, soil and air; bioaccumulation;**
- **residue studies.**

The certificate is valid from 7th June 2011. The next inspection of compliance with the principles of Good Laboratory Practice is to be held according to the provisions laid down in paragraph 6 (3)(2) of the regulation of the Minister of Health of 28th May 2010 concerning the criteria, that must be met by institutions conducting tests of chemical substances and preparations, and the verification of compliance with these criteria (Dz.U. Nr 109, poz. 722).

Lodz, 13th June 2011

GLP Inspectors

[redacted]

Inspector for Chemical Substances

[redacted]

Appendix 4. Copy of Study plan

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY
BRANCH PSZCZYNA

STUDY PLAN

PALIOGEN VIOLET 5011

Daphnia magna, acute immobilisation test

According to OECD No 202 (2004)

STUDY CODE: W/68/11

Study director: [redacted]

Test facility: Institute of Industrial Organic Chemistry
Branch Pszczyna
Department of Ecotoxicology
[redacted]

Sponsor: BASF SE
[redacted]

BASF Project No. 50E0223/11X287

Institute of Industrial Organic Chemistry
Branch Pszczyna
[redacted]

This is an true copy of
the original document
from page 1 to page 16
[redacted]
Date 30.03.12

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
DEPARTMENT OF ECOTOXICOLOGY

Paliogen Violet 5011, *Daphnia magna*, acute immobilization test
Study code: W/68/11

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Paliogen Violet 5011, *Daphnia magna*, acute immobilization test
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The following information and signatures are necessary to be in compliance with Good Laboratory Practice Principles and requirements of the quality control system at the Institute of Industrial Organic Chemistry Branch Pszczyna, Department of Ecotoxicology.

SPONSOR

BASF SE,

[redacted]

Note:

written confirmation of purity or active substances content with information on specific activity, use, molecular mass, production and expiry dates, if applicable, should be provided with true copy of the study plan at the latest.

The duty of the sponsor is to provide material safety data sheet (MSDS), if available, and all other information concerning safe and proper handling, storage and transport of the test item.

The duty of the sponsor is to inform about any known or potential risks and hazards on contact with the test item.

The Sponsor agrees to accept all unused remains of test item.

Study plan approval:

Sponsor/Representative of the sponsor:

Stamp: [redacted] 30 Mar 2012 [redacted]

date signature

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
DEPARTMENT OF ECOTOXICOLOGY

Paliogen Violet 5011, *Daphnia magna*, acute immobilization test
Study code: W/68/11

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
DEPARTMENT OF ECOTOXICOLOGY

Paliogen Violet 5011; *Pseudokirchneriella subcapitata* SAG.61.81 Growth inhibition test
Study code: W/67/11

TEST FACILITY

Institute of Industrial Organic Chemistry Branch Pszczyna

Department of Ecotoxicology

[redacted]

Study plan acceptance:

Study director:

[redacted]

Institute of Industrial Organic Chemistry
Branch Pszczyna

[redacted]

30.03.12
date

[redacted]

Chemical analysis

30.03.12
date

Head of Departament of
Ecotoxicology:

30.3.12
date

STATEMENT OF QUALITY CONTROL ABOUT ACCORDANCE WITH GOOD LABORATORY PRACTICE PRINCIPLES (GLP)

Hereby we state that the study plan is in compliance with the OECD Guidelines for Testing of Chemicals No 201 (2006) 'Freshwater Alga and Cyanobacteria, Growth Inhibition Test' [1], SOP/W/20 'Alga and cyanobacteria, growth inhibition test' and in compliance with GLP principles [4, 5]. [SOP/PJ/1]

[redacted]

30.03.2012
date

[redacted]

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DISTRIBUTION

Original: BASF SE, [redacted]

True copy: Institute of Industrial Organic Chemistry Branch Pszczyna, [redacted]

Acquainted with the study plan *(to be filled in by IPO in True copy of signed study plan only)*:

[redacted]

Internal distribution of photocopies *(to be filled in by IPO in True copy of plan only)*:

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
DEPARTMENT OF ECOTOXICOLOGY

Paliogen Violet 5011, *Daphnia magna*, acute immobilization test
Study code: W/68/11

1. BASIS FOR PERFORMING THE STUDY

The toxicity assessment of Paliogen Violet 5011 for *Daphnia magna* will be carried out according to the order from BASF SE, [redacted] dated November 28, 2011, BASF SE.

2. INTRODUCTION

The toxicity evaluation to *Daphnia magna* will be performed according to OECD Guideline No 201 '*Daphnia* sp., Acute immobilization test' (2004) [1] and according to SOP/W/21 '*Daphnia*, acute immobilization test'.

The study was coded: W/68/11 [SOP/W/4]

The study will be performed in compliance with the principles of Good Laboratory Practice OECD 1997 except non-GLP preliminary tests [5]. The Institute of Industrial Organic Chemistry Branch Pszczyna, Department of Ecotoxicology owns a GLP Certificate (Statement of GLP Compliance No 6/2011/DPL, issued on June, 13 2011 by Polish Bureau for Chemical Substances, valid from June 7, 2011 – Appendix 1 to study plan) [4].

3. AIM OF THE STUDY

The aim of the study is the determination of acute toxicity of the test item for *Daphnia magna* and the determination of the EC_{50}, EC_0 and EC_{100} values after 48 h, as well as LOEC and NOEC (if possible). Test organisms of the species *Daphnia magna* will be exposed in a static test to various concentrations of the test item under defined conditions.

The NOEC and LOEC values will be estimated, if possible.

The test is valid if validity criteria according to OECD Guideline No 202 are met.

4. TERMS

Start of the study:	March 2012
Proposed starting date of definitive study:	April 2012
Proposed termination date of definitive study:	April 2012
Planned the end of the study :	April 2012

5. MATERIAL AND METHODS

5.1. Test species

The test organism, *Daphnia magna* Straus, originates from the standard laboratory culture maintained in Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, [redacted] Only organisms up to 24 h old (not first brood progeny) and in good physiological condition will be used in the experiments. The sensitivity of the culture is monitored on regular basis with the reference substance potassium dichromate [SOP/W/72].

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Paliogen Violet 5011, *Daphnia magna*, acute immobilization test
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5.2. Test item

The test item Paliogen Violet 5011 is a powder of dark violet colour and specific odour[1]. The sample of test item is labelled with Name: Paliogen Violet, Batch: P 100012, Expiry date: 18.11.2020, [redacted] The sample of test item in amount of 50 g in a plastic container was provided by the Sponsor on December 20, 2011 with the material safety data sheet. According to information provided by the Sponsor the test item contains Anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone (CAS name), CAS No. 81-33-4. The sponsor did not provide a Certificate of Analysis. According to Sponsor's information the solubility of the detected substance is 10 µg/L. The sample is stored at room temperature under dry conditions without exposure to light in tightly sealed container [SOP/W/3, SOP/PB/1].
Data relating to the identity, purity and stability of test item are the responsibility of the Sponsor.

5.3. The preliminary test (non-GPL)

In order to provide details in the study plan on the range of the test concentrations and the test design [2, 3], the preliminary tests were performed before initiation of the study.

5.3.1. Test design

The first preliminary test was performed on Daphnia magna during 48 h with the test concentrations: 0.1, 1.0, 10.0 and 100.0 mg/L. The highest test concentration 100 mg/L was prepared by weighing the test item into a glass flask and mixing it with the test medium in an ultrasonic bath at a temperature of 40-50 °C (for 48 h and with sonication for the first 30 min) on a mechanic stirrer. The clear and transparent basic solutions were obtained after filtration. The test concentration was diluted with test medium to prepare the remaining test concentrations.

The second preliminary test was performed in the following way:
A concentration 100 mg/L was stirred over 72 h at 40 °C (in the incubator), then conditioned to 20 °C over 24 h (stirring) and the suspension was filtered over a 0.45 µm membrane disc. This filtrate was used to prepare the other concentrations: 10.0; 1.0 and 0.1 mg/L.

The third preliminary test was performed in the following way:
100 mg and 1.0 mg were weighed separately. Each of two weighted amounts was mixed with 1 L of Elendt medium and then the procedure was similar as in the fourth test. After conditioning the concentration 100 mg/L was diluted in order to prepare a concentration of 10 mg/L and the concentration 1.0 mg/L was diluted in order to prepare a concentration of 0.10 mg/L. Next the control and all concentrations were filtrated over a 0.20 µm membrane disc. The filter was saturated with the test solution.

[1] Colour and the odour according to data in MSDS, were not determined according to GLP

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In the preliminary tests glass beakers of 150 mL capacity were used containing 50 mL of test concentration. The test vessels were covered with a transparent plastic lid in order to minimize evaporation and to prevent accidental contamination. The test concentrations and control were tested in four replicates. In each test vessel five organisms of Daphnia magna were exposed to the test item for 48 h. The preliminary tests were of static design.

5.3.2. Test medium

Elendt medium recommended by OECD Guideline No 202 (2004) was used as diluent/solvent of the test item. The same medium is used for culturing.

5.3.2.1. Elendt medium preparation

The Elendt medium [SOP/W/63] was prepared based on deionised water [SOP/W/71] by adding appropriate amounts of stock solutions of reagent grade chemicals.
The stock solutions are renewed on regular basis for *Daphnia* sp. culturing and stored in a refrigerator.
The composition of stock solutions is presented in Table 1.
In order to prepare 10 L of Elendt M7 medium each stock solution was diluted with deionised water by adding 500 mL of combined microelements stock solution, appropriate amounts of each macroelements stock solution and 1.0 mL of combined vitamins stock solution and filled up to 10 L with deionised water.

Table 1. Composition of Elendt M7 medium

Combined microelements stock solution			Volume of each ingredient in combined stock solution [mL/2 L]
Ingredient		Concentration [g/100 mL]	
1	H_3BO_3	2.860	1.0
	$MnCl_2 \cdot 4H_2O$	0.721	0.5
	LiCl	0.612	0.5
	RbCl	0.142	0.5
	$SrCl_2 \cdot 6H_2O$	0.304	0.5
	NaBr	0.032	0.5
	$Na_2MoO_4 \cdot 2H_2O$	0.123	0.5
	$CuCl_2 \cdot 2H_2O$	0.0335	0.5
	KI	0.0065	2.0
	$CoCl_2 \cdot 6H_2O$	0.020	2.0
	$ZnCl_2$	0.026	2.0
	Na_2SeO_3	0.0044	2.0
	NH_4VO_3	0.005	2.0
2	$FeCl_3 \cdot 6 H_2O$	0.1991	20.0
	$Na_2EDTA \cdot 2 H_2O$	0.500	

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Table 1 continued. Composition of Elendt M7 medium

Macroelements stock solutions			Volume of each ingredient in Elendt M7 medium [mL/10 L]
Ingredient		Concentration [g/100 mL]	
3	$NaNO_3$	0.274	1.0
4	$MgSO_4 \cdot 7\ H_2O$	24.660	5.0
5	$CaCl_2 \cdot 2\ H_2O$	29.380	10.0
6	KCl	5.800	1.0
7	$NaHCO_3$	6.480	10.0
8	K_2HPO_4	0.143	1.0
9	KH_2PO_4	0.184	1.0

Combined vitamins stock solution		
Ingredient		Concentration [mg/100 mL]
10	thiamine hydrochloride	75.00
	cyanocobalamine	1.00
	biotine	0.75

5.3.3. Test conditions

The test was conducted in day-night light regime (16 h day – 8 h night) with artificial fluorescent light source employed. Test organisms were not fed during the test.

5.3.4. Measurements and observations

During the preliminary test observations of toxic effects were conducted and recorded after 24 and 48 h from test initiation. Daphnids were observed for immobilization (inability to swim after gentle agitation of test vessel). Observations were recorded on test result sheets. The pH values and dissolved oxygen concentration of each test concentration and control were measured [SOP/W/36] before division into replicates and at test termination in pooled replicates.
control.The results of immobilization are given in Table 2.

Table 2. Inhibition – the first preliminary non-GPL test

Nominal concentration of test item [mg/L]	% of immobilization	
	24 h	48 h
Control	--	--
0.1	0	0
1.0	0	0
10.0	0	0
100.0	0	5

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At the end of the second preliminary test the immobilization was 0% in the nominal concentration 100 mg/L. The results of immobilization are given in Table 3.

Table 3. Inhibition – the second preliminary non-GPL test

Nominal concentration of test item [mg/L]	% of immobilization	
	24 h	48 h
Control	--	--
0.1	0	0
1.0	0	0
10.0	0	0
100.0	0	0

At the end of the third preliminary test the immobilization was 45% in the nominal concentration 100 mg/L. The results of immobilization are given in Table 4.

Table 4. Inhibition – the second preliminary non-GPL test

Nominal concentration of test item [mg/L]	% of immobilization	
	24 h	48 h
Control	--	--
0.1	0	0
1.0	0	0
10.0	0	0
100.0	5	45

5.3.5. Chemical analysis

In the preliminary tests the concentrations of the test item were chemically determined only in the highest concentration. The chemical analyses of the 100 mg/L concentration of the second preliminary non-GLP test revealed a concentration above 1 mg/L, which indicated, that the filtration was not successful. Particles of the test substance were observed in the test solution. In conclusion the effects in the highest concentration were most likely caused by unsolved test substance.

5.4. The definitive test

Based on the results of the preliminary tests, the definitive test will be performed according to the following procedure.

5.4.1. Test design

The test will be conducted as a limit test in static design. The test will be performed with 100.0 mg/L. The test concentration will be prepared by weighing the test item into a flask and mixing it with the test

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medium for 72 h at temperature of 30-40° C in order to maximum dissolving (mechanic shaker, WL-2000, JW Electronic) [SOP/W/57]. Then the concentration will be conditioned at room temperature stirring continuously.

After 24 h the control and the test concentration will be filtrated through conditioned nitrocellulose membrane (Filter type HAWG, 0.20 µm pores) [6], [SOP/W/37]. The test solution is very thoroughly checked for unsolved parts of the test substance before test organisms are introduced.

Test glass vessels of 150 mL capacity will be used containing 100 mL of the test concentration. Test vessels will be covered with a transparent plastic lid in order to minimize evaporation and to prevent from accidental contamination. Eight replicates with five organisms will be used for the concentration and the control. Total time of exposure will be 48 h.

5.4.2. Test medium

Elendt medium (M 7) recommended by OECD Guideline No 202 (2004) was used as diluent/solvent of the test item. The same medium is used for culturing (see point 5.3.2.1)

5.4.3. Test conditions

The conditions of the definitive test will be in accordance with the requirements of OECD 202 [1], [SOP/W/21].

5.4.4. Measurements and observations

During the test observations of toxic effects will be performed and recorded after 24 and 48 h from test initiation. Daphnids will be observed for immobilization (inability to swim after gentle agitation of test vessel). The pH values and dissolved oxygen concentration of test concentration and control will be measured [SOP/W/36] at the initiation of the test before division into replicates and at the end of the test. Temperature will be measured continuously using electronic device [SOP/W/51] with a sensor submerged in additional test vessel with 100 mL test medium. Observations for unsolved test substance will be made daily.

5.4.5. Chemical analysis

The concentration of PALIOGEN VIOLET 5011 will be chemically determined. The samples of all fresh solutions at test initiation and 48 hour old solutions at test termination will be analyzed in all test concentrations [SOP/W/83].

The content of test item will be determined by validated spectrophotometric method [SOP/C/205]. The validation process will be described in the report.

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5.5. Analysis of the results

Results generated will be discussed and presented in tables. Based on the definitive test results the analysis of data will be performed in order to calculate EC_x value with 95 % confidence interval (if applicable) as well as to determine LOEC and NOEC values. ToxRat Professional 2.10 statistical computer software will be used. The report of statistical calculations will be archived in raw data and added to the report [7], [SOP/W/68].

6. FINAL REPORT

The original of the final report will be submitted to the sponsor, as well as electronic version. Additional copies will be provided at an additional cost.
The true copies of raw data with report will be submitted to the sponsor.
The final report will contain the following information:
- Signatures of staff responsible for the experiment performance,
- Statement of Quality Control Unit regarding the performance of the study,
- Archiving,
- Objectives and test method description,
- Dates of start and end of the study,
- Dates of start and end of preliminary and definitive tests,
- Experimental starting and completion date,
- Description of the test item,
- Description of the test species,
- Description of test design,
- Test conditions,
- Results generated per replicate,
- Results of the most recent test with the reference substance,
- Reference to the statistical methods used,
- Analytical data with description of analytical procedure,
- Deviations from the study plan,
- GLP compliance statement
- Study plan.

7. ARCHIVING

The following documents concerning the experiment labeled with the study code W/68/11 will be archived (at least 10 years) in Archive of Documentation and Test Samples [SOP/W/35, SOP/PB/2]:
- Raw data,
- Quality assurance control protocols,
- Correspondence with sponsor,
- Study plan (true copy),

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- Final report (true copy).

True copies of the raw data will be sent to the sponsor.

After archiving period all documents will be transferred to the archives of sponsor.

The standard of the test item labelled with the study code will be stored in the Department of The Test Material at the Institute of Industrial Organic Chemistry, Branch Pszczyna, [redacted] till its expiry date e.g. 18.11.2020 [SOP/PB/1].

8. STUDY PLAN CHANGES AND STUDY PLAN DEVIATIONS

The study director, upon approval of the sponsor or sponsor's representative, may make changes to this study plan. All proposed changes will take the form of a written amendment to study plan describing the changes and the reason for the change. All amendments will be signed and dated by the study director, the sponsor and verified by Quality Control in the range of GLP. The signed amendments to study plan will be archived with the study plan.

The information on deviation from the study plan/guideline will be described in the final report.

9. QUALITY ASSURANCE CONTROL

The aim is to assure, that the study will be performed in compliance with Principles of Good Laboratory Practice (GLP) [4, 5], [SOP/PJ/1]

The person responsible for quality assurance performs the following internal controls:

Before test start

- Study plan,
- Standard operational procedures.

During the study

- Compliance of the study course with study plan and SOPs,
- Accuracy of the study records,

Final report

- Exact description of test methods, procedures and observations,
- Accuracy of raw data reflection in the report,
- All changes in relation to approved study plan.

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DEPARTMENT OF ECOTOXICOLOGY

Paliogen Violet 5011, *Daphnia magna*, acute immobilization test
Study code: W/68/11

10. REFERENCES

[1] OECD Guideline for Testing of Chemicals No 202 (2004): '*Daphnia* sp., Acute Immobilization Test'.

[2] The application of the GLP Principles to short-term studies, Number 7, OECD, September 1999, ENV/JM/MONO(99)23.

[3] OECD series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, OECD, January 1998, ENV/MC/CHEM(98)17.

[4] Polish legislation: The regulation of the minister of Heath of 28th May 2010 concerning the criteria, that must be met by institutions conducting tests of chemical substances and preparations, and the verification of compliance with these criteria (Dz.U. Nr 109, poz. 722).

[5] Directive 2004/10/EC on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

[6] OECD Series on Testing and Assessment No 23 "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures", ENV/JM/MONO(2000)6, December 15, 2000.

[7] ToxRat Professional 2.10 – Software for Statistical Evaluation of Biotests in Ecotoxicology, ToxRat Solutions GmbH, Alsdorf, Germany

11. STANDARD OPERATIONAL PROCEDURES USED IN THE STUDY

SOP/W/3	Proceeding with the test item
SOP/W/4	Studies coding
SOP/W/7	Analytical balance – instruction manual
SOP/W/18	Preparing and labelling of chemical reagents
SOP/W/21	Daphnia, acute immobilization test
SOP/W/35	The documentation and archiving of test records and notes
SOP/W/36	The pH-oxi meter inoLAB pH/Oxi Level 3 – instructions manual
SOP/W/37	NALGENE filtration system – instructions manual
SOP/W/39	Luxmeter L-50 – instruction manual
SOP/W/51	Temperature logger HI 141 – instruction manual
SOP/W/57	Mechanic shaker – instructions manual
SOP/W/63	Standardized Elendt M7 medium
SOP/W/67	Daphnia magna laboratory culture
SOP/W/68	ToxRat Professional – instruction manual
SOP/W/71	Water deionization system SolPure7 – instructions manual
SOP/W/72	Reference tests
SOP/W/75	Incubator with built-in shaker – instructions manual
SOP/W/83	Collecting the samples
SOP/C/205	Analytical method for determination of Paliogen Violet 5011 in water.
SOP/PB/1	Receiving, distribution, storage, registry and elimination of test item

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SOP/PB/2	Archiving of documentation and test samples
SOP/PJ/1	Control of planning, conducting and preparing of study

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STATEMENT OF GLP COMPLIANCE

Registration number: 6/2011/DPL

Assessment of conformity with GLP according to the Directive 2004/9/EC of the European Parliament and of the Council

On the basis of the inspection which was held on 6th – 7th June 2011 and in accordance with the criteria specified in the order of the Inspector for Chemical Substances and Preparations of 1st April 2011 concerning the rules on the inspection and verification of compliance with the principles of Good Laboratory Practice, as well as in accordance with Directive 2004/9/EC and the relevant OECD regulations, the Inspector for Chemical Substances hereby confirms that

Department of Ecotoxicology
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[redacted]

complies with the OECD and the EU principles of Good Laboratory Practices in the fields of:

- **environmental toxicity studies on aquatic and terrestrial organisms;**
- **studies on behaviour in water, soil and air; bioaccumulation;**
- **residue studies.**

The certificate is valid from 7th June 2011. The next inspection of compliance with the principles of Good Laboratory Practice is to be held according to the provisions laid down in paragraph 6 (3)(2) of the regulation of the Minister of Health of 28th May 2010 concerning the criteria, that must be met by institutions conducting tests of chemical substances and preparations, and the verification of compliance with these criteria (Dz.U. Nr 109, poz. 722).

Lodz, 13th June 2011

GLP Inspectors

[redacted]

Inspector for Chemical Substances

[redacted]