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INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY
BRANCH PSZCZYNA

REPORT

Paliogen Violet 5011

Lemna gibba L. CPCC 310 GROWTH INHIBITION TEST

According to OECD Guideline No 221 (2006)

STUDY CODE: W/120/12

Study Director:	[redacted]
Test facility:	Institute of Industrial Organic Chemistry Branch Pszczyna Department of Ecotoxicology [redacted]
Sponsor:	BASF SE [redacted]
Version:	Final
BASF project No.	99E0223/11X541
BASF Order No.	1089966059
Study completion date:	October 2012

Head of the Branch

[redacted]

10.10.2012 [redacted]

date signature

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DECLARATION OF THE STUDY DIRECTOR

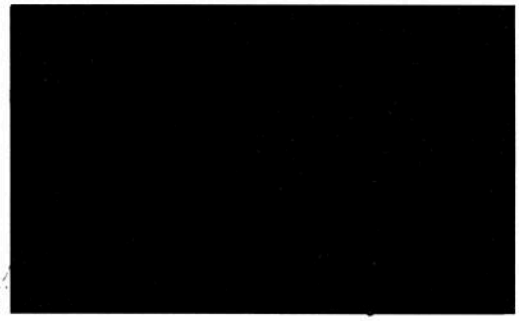
Formal basis for performing the study Paliogen Violet 5011 *Lemna gibba* L. CPCC 310 growth inhibition test, was the order from BASF SE [redacted] dated July 24, 2012.

The study coded W/120/12 was performed according to the OECD Guidelines for Testing of Chemicals No 221 (2006) [1] and SOP/W/47. The study was in compliance with the principles of Good Laboratory Practice, OECD 1997 [2]. The study was performed in compliance with the study plan (Appendix 5). The preliminary test was made non-GLP.

The study was performed in Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, which holds Statement of GLP Compliance Registration Number 6/2011/DPL issued on June 13, 2011 by the Polish Bureau for Chemical Substances [3], (Appendix 5).

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. No circumstances have been left unreported which might have a potential bearing on the validity and reproducibility of this 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:



10.
date

AUTHORS AND PERFORMERS

Performing the biological part: Date, signature

[redacted] 10.10.12
[redacted] 10.10.12
[redacted] 10.10.12
[redacted] 10.10.2012
[redacted] 10.10.2012
[redacted] 10.10.12

Performing the chemical analysis:

[redacted] 10.12.12
[redacted] 10.10.12
[redacted] 10.10.2012

Head of the Department of Ecotoxicology:

[redacted] 10.10.2012 [redacted]

REPORT OF THE QUALITY ASSURANCE UNIT

The study plan, course of the study and final report of the toxicity evaluation of Paliogen Violet 5011 for duckweed (*Lemna gibba*) (study code: W/120/12) [SOP/W/4], were controlled by the person responsible for quality assurance:

Scope of control	Date of control	Date of protocol submission to Head of testing facility
Study plan	24.08.2012	24.08.2012
Experiment course	07.09.2012	07.09.2012
	12.09.2012	13.09.2012
Draft report	26 - 27.09.2012	27.09.2012
Final report	10.10.2012	10.10.2012

The control was conducted according to the Good Laboratory Practice [2, 3], [SOP/PJ/1].
The report is in compliance with GLP criteria. The person responsible for quality assurance hereby confirms that the study was performed in compliance with the study plan and the requirements of GLP (except for the preliminary test). The final report reflects the study course (methods, procedures and observations) and presents results completely described raw date obtained during the study.

10.10.20 [redacted]
date

ARCHIVING

The following documents will be archived at ten years from the end of the study in the Archive of Documentation and Test Samples at the Institute of Industrial Organic Chemistry, Branch Pszczyna, [redacted] [SOP/W/35, SOP/PB/2]:

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

After the archiving period all materials will be destroyed with prior notification of the sponsor.

The archival standard of the test item will be stored in the Department of The Test Material at the Institute of Industrial Organic Chemistry, Branch Pszczyna, [redacted], [redacted], i.e. till 11.2020 [SOP/PB/1].

Archives and conditions of archive of documentation and a 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, Archives [redacted]

Final report:

Original: BASF SE, [redacted]

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

Raw data:

Original: BASF SE, [redacted]

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

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

Institute of Industrial Organic Chemistry Branch Pszczyna, Archives, [redacted]

SUMMARY

The growth of the aquatic plant *Lemna gibba* was investigated in a 7 days semi-static toxicity laboratory study with renewal of test solutions on days 2 and 5. The initial frond number in each replicate was 9, i.e. three colonies of three fronds each. Three replicates per concentration and 6 replicates for the control were tested.

The following concentrations of the test item Paliogen Violet 5011 were applied: 1.0; 3.2; 10; 32 and 100 mg/L (based on loading). The test concentrations were prepared separately (differential loadings) and undisolved parts were removed before insertion of test organisms.

The number of fronds distinctly visible in each test vessel was recorded as well as changes in plant development which were observed at each renewal (i.e. day 2 and day 5) and after 7 days of exposure. The dry weight of test organisms was measured at test termination.

Morphological effects after 7 days of exposure as compared to the normal development of fronds in the control were recorded. In none of the tested concentrations from 1.0 to 100 mg/L the colonies were different in comparison to the control group.

The test item contains Anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone.

The concentrations of the test item in the test concentrations were chemically determined by validated methods. The results confirm the low solubility of the test item in water (10 µg/L at 20 °C according to the information from sponsor). In fresh solutions the concentration of test item was between 0.0067 – 0.0070 mg/L in all tested concentrations and the measured concentrations were not correlated with the loading used for the preparation of the test solution. In conclusion the measured values represent the solubility limit in the test medium under test conditions. In 48 and 72 hour old solutions the concentration of test item was between 0.0067 – 0.0071 mg/L, which demonstrates that the test concentrations were kept stable over the study period.

Since the measurements in test vessels demonstrated the presence and stability of the test item in solution, mean measured concentrations were calculated.

The EC_x values were determined based on the loading of the test item. The EC_x values were also determined based on time-weighted mean of measured concentrations of the test item in test vessels with *Lemna gibba*.

Material and methods

Test item:	Paliogen Violet 5011, Batch: P 100012, Test substance No. 11/0223-1, Expiry date: November 18, 2020. contains Anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone
Test species:	Freshwater aquatic plant *Lemna gibba* L. specification CPCC 310, stock G3 from [redacted].
Test design:	Semi-static system (7 days), three replicates per each test concentration and six for control with *Lemna gibba*.
Test concentrations:	Control; 1.0; 3.2; 10.0; 32 and 100 mg/L of test item in 20XAAP medium (concentrations based on loading of the test item).
Test conditions:	20X AAP nutrient solution, pH of control: 7.54 – 9.28, light intensity: 8.26 – 9.60 klx, constant illumination, glass vessels with 100 mL test volume; initial frond number 9,

i.e. 3 plants per 3 fronds; temperature measured in an additional test vessel was in the range of 24.2 – 24.7°C.

Analytics: The contents of active substance in test concentrations were determined by spectrophotometric measurements.

Statistics: Probit method calculations and analysis: Shapiro-Wilk's Test on Normal Distribution, Levene's Test on Variance Homogeneity (with Residuals), Williams Multiple Sequential t-test Procedure

Endpoints: E_rC_{50}, E_yC_{50}, E_rC_{20}, E_yC_{20}, E_rC_{10}, E_yC_{10}, LOEC and NOEC based on frond number and dry weight.

Conclusion

The results after 7 days based on theoretical concentrations (loadings) of the test item:

In a semi-static *Lemna gibba* growth inhibition test the EC_{50} values were estimated based on frond number and dry weight.

From frond number

The E_rC_{50} is above the highest test concentration i.e. 100 mg/L and E_yC_{50} is above the highest test concentration i.e. 100 mg/L. The values E_rC_{20} and E_rC_{10} are above 100 mg/L. The value E_yC_{20} is above 100 mg/L and E_yC_{10} is above 100 mg/L.

The LOEC values based on growth rate and yield are above 100 mg/L and the NOEC values are above or equal to 100 mg/L.

From dry weight

The E_rC_{50} and E_yC_{50} are above the highest test concentration i.e. 100 mg/L The values E_rC_{20} and E_rC_{10} are above 100 mg/L.

The value E_yC_{20} is above 100 mg/L and E_yC_{10} is 100 mg/L.

The LOEC value based on growth rate is above 100 mg/L and the NOEC value is above or equal to 100 mg/L.

The LOEC value based on yield from dry weight is higher than 100 mg/L and NOEC value is above than 100 mg/L or equal 100 mg/L.

The results after 7 days based on time-weighted means of the measured concentrations:

From frond number

The E_rC_{50} is above 0.0070 mg/L responding the test concentration 100 mg/L and the E_yC_{50} is above 0.0070 mg/L. The values E_rC_{20} and E_rC_{10} are above 0.0070 mg/L. The value E_yC_{20} is above 0.0070 mg/L and E_yC_{10} is above 0.0070 mg/L.

The LOEC values based on growth rate and yield are above 0.0070 mg/L and the NOEC values are above or equal to 0.0070 mg/L.

From dry weight

The values E_rC_{50}, E_rC_{20}, E_rC_{10} and E_yC_{50}, E_yC_{20}, E_yC_{10} are above 0.0070 mg/L.

The LOEC value based on growth rate from dry weight is above 0.007 mg/L and NOEC value is above or equal to 0.007 mg/L.

The LOEC value based on yield is above 0.0070 mg/L and the NOEC value is above or equal to 0.0070 mg/L.

The test substance had no effect on the test organisms up to the saturation concentration in the test medium under the test conditions.

1. AIM OF THE STUDY

An ecotoxicological study of Paliogen Violet 5011 for freshwater aquatic plant *Lemna gibba* was performed. The aim of the study was the evaluation of toxicity for duckweed and determination of EC_{50}, EC_{20} and EC_{10} values as well as LOEC and NOEC values based on the average specific growth rate and yield.

2. TERMS

The preliminary test started (time of exposure):	13.08.2012
The preliminary test ended:	20.08.2012
The study started:	24.08.2012
The experimental starting date	01.09.2012
The experimental completion date	12.09.2012
The definitive test started (time of exposure)	05.09.2012
The definitive test ended:	12.09.2012
Draft report:	26.09.2012
The study ended:	10.10.2012

3. MATERIAL 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 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 characterization of the test substance. According to sponsor's information the solubility of

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

the detected substance is 10 µg/L. The sample was stored at room temperature in dry conditions without exposure to light in a tightly sealed container [SOP/W/3, SOP/PB/1].
Proceeding with the test item was according to procedures of Department of the Test Material (PAA) [SOP/PB/1] and obligatory procedure at the Laboratory of Aquatic Toxicology [SOP/W/3].
Data relating to the identity, purity and stability of test item are the responsibility of the sponsor.

3.2. Test organism

The freshwater aquatic plant *Lemna gibba* L. originates form the standard laboratory culture cultivated in the Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, Laboratory of Aquatic Toxicology according to OECD 221 recommendations [1], [SOP/W/66]. Duckweed *Lemna gibba* CPCC 310 on agar bevels was received from the [redacted].

3.2.1. Culturing of Lemna

Duckweed was transferred from agar bevels to the fresh medium (for *Lemna gibba* 20XAAP medium is recommended, Appendix 3) in 600 mL glass vessels with transparent covers and incubated at 22 - 26°C with constant illumination (pre-culture) [1], [SOP/W/66]. The duckweed culture was inoculated to fresh medium once a week.
A pre-culture was started seven days before the experiment. Only organisms in good physiological condition without any discoloration were used for inoculation of the test concentrations and the control.

3.2.2. Culturing medium

The 20X AAP medium recommended by OECD Guideline No 221 was the culturing medium for test organism and the diluent/solvent for the test item. The same medium was used in duckweed culturing. The 20X AAP medium was the source of nutrients for plant growth. The 20XAAP medium was prepared on the basis of deionised water (filtering system SolPure7 [SOP/W/71]) by adding stock solutions of reagent-grade chemicals [SOP/W/18, SOP/W/66]. After 24 h of aeration the pH of test medium was measured and adjusted with 0.1 M HCl, if necessary. For culturing, test medium was sterilised by autoclaving [SOP/W/56] before addition of the last stock solution: sodium hydrogen carbonate – the buffering ingredient. The stock solutions were renewed on regular basis for *Lemna gibba* culturing and stored in a refrigerator. The composition of 20X AAP medium and concentration of each ingredient are presented in Appendix 3.

3.3. Performance of Lemna Growth Inhibition Test

The *Lemna gibba* growth inhibition test was performed according to the OECD Guideline for Testing of Chemicals No 221 (2006) [1] , [SOP/W/47].

3.3.1. Test conditions

The semi-static test design was applied. The test solutions were renewed twice during 7 days of exposure. At test initiation, at each renewal (before and after renewal) and at test termination pH values were measured (inoLAB pH/Oxi-meter Level 3, WTW, Germany [SOP/W/36]). The pH of fresh solutions was measured before division into replicates and in pooled replicates of the old solutions.
The constant conditions throughout the test were provided with the thermostatic chamber (incubator with built-in shaker, ILW400STD Pol-Eko-Aparatura, Poland [SOP/W/75]). The temperature of the solutions was constantly recorded (thermologger HI 141, Hanna Instruments, USA [SOP/W/51]). The test was performed with constant illumination, a using a fluorescent light source of six 24 W light

bulbs. The light intensity was measured twice during the test (lux-meter with 2 π receptor, Sonopan L-50, Poland [SOP/W/39]),
Each test concentration was prepared in three replicates and the control in six replicates. The 20X AAP medium was used as diluent in test concentrations and in pre-culture. The glass vessels containing 100 mL volume, of 4.5 cm depth and 8 cm diameter were used in order to provide sufficient area for roots and to prevent plants from overlapping at test termination. Transparent covers were applied to minimise evaporation and accidental contamination, allowing necessary air exchange. Replicates were arranged at random and during the test rearranged repeatedly. Time of exposure was 7 days.

In order to quantify test item related effects on vegetative growth over a period of seven days the number of fronds in each replicate was counted. The frond numbers were counted at each renewal (in preliminary test day 2 and day 4, in the definitive test day 2 and day 5 and at the end of exposure. At each renewal and at exposure termination the observations of plant development were performed: frond size, shape and appearance (necrosis, chlorosis or gibbosity), colony break-up or loss of buoyancy, root length and appearance. Growth of plant cultures in the test concentrations was compared with that of the control.
At the start of the test three colonies consisting of three fronds of similar size from the pre-culture were chosen and transferred with an inoculating loop into test vessels. Each replicate was inoculated with a total of 9 fronds.
The dry weight of inoculum in three representative replicates was measured at test initiation.
The dry weight of plants in each test vessel was measured at the end of exposure. For dry weight measurements all colonies (including roots) from replicate were transferred onto glass slides and dried at 60 - 70°C in laboratory oven for 20 h [SOP/W/15] till constant weight. After weighing using an analytical balance [SOP/W/7], the plants were removed and only glass slides were weighed, in order to determine the dry weight of plants on each glass slide.

3.3.2. Preparation of test concentrations

In order to determine the concentrations of the test item Paliogen Violet 5011 in the definitive test, the preliminary range finding test was performed with two concentrations from loading of the test item:

10.0 and 100.0 mg/L based on loading. The test concentrations were prepared by mixing 10.3 and 100.1 mg of the weighed amounts of the test item with 1000 mL of test medium. The mixture was placed on mechanic stirrer for 72 h under heating to 40 °C. After this period of time the mixtures were conditioned at approximately 20 °C for 24 h. Then the mixtures were filtrated through a conditioned cellulose acetate membrane (Filter Sartorius, 0.20 µm pores, pores) [6]. Clear colourless solutions were obtained. A semi-static test was performed.

The test medium for the control was treated in the same way as the mixtures for the test concentrations. All test concentrations and control were tested in three replicates per concentrations and six replicates for the control. The results are given in Table 3. The test solutions were not chemically analyzed.

In the definitive test the test item was used in five concentrations: 1.0, 3.2, 10, 32 and 100 mg/L based on loading. It was performed as semi-static test. The test concentrations 10, 32 and 100 mg/L were prepared separately by weighing the test item into a flask. The appropriate amount of the test medium was added.

The mixtures were stirred over 72 hours at a temperature of 40 °C. After this period of time the mixtures were conditioned at approximately 20 °C for 24 h. Then the control and each test concentration were filtrated through a conditioned cellulose acetate membrane (Filter Sartorius, 0.20 µm pores), [SOP/W/37]. The concentrations 1.0 and 3.2 mg/L were prepared by dilution from the concentration 10.0 mg/L before the filtration. Before the dilution was made the 10 mg/L concentration was checked carefully for homogeneous distribution of the test substance and for presence of precipitated test material. The test solution was clear and without precipitates. The test medium for the control was treated in the same way as the mixtures for the test concentrations: it was stirred for 72 hours, conditioned at 20 °C and was filtrated in the same way as the test concentrations to exclude any influences from the preparation of the test solutions. The preparation of the test solutions as described resulted in homogeneous solution i.e. Water Accommodated Fraction (WAF) which were used for exposure [6, 8].

After a thorough mixing 300 mL of the fresh solutions were transferred for chemical analysis. For each test concentration three replicates of 100 mL were filled in test vessels. On days 2 and 5 as well as at test termination (day 7) samples for chemical analyses were taken from the replicates and from the fresh solutions.

Three replicates of each test concentration and six replicates of the control were prepared. Each replicate was inoculated with three colonies consisting of three fronds from pre-culture. Therefore each replicate contained inoculum of 9 fronds in total.

3.4. Assessments

The toxicity of the test item for duckweed is expressed as its median concentration causing 50% inhibition of average specific growth rate or yield (E_rC_{50}, E_yC_{50}) of *Lemna gibba* cultures in relation to control. The EC_{50} values were estimated based on frond number and dry weight.

The EC_x values were calculated by probit method [5], [SOP/W/68]. The LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) values were estimated based on statistical analysis [5], [SOP/W/68].

3.5. The reference test

The test with reference substance – 3,5 dichlorophenol was performed at a temperature of 24.1 – 24.8 °C with a constant illumination appr. 9.34 klx, in 20X AAP medium [SOP/W/75]. The exposure started on July 27, 2012 and ended on July 03, 2012. The reagent used for test was 97% pure and supplied

by Aldrich. The reference substance was used in five concentrations in the range of 0.32 - 32 mg/L. Every concentration was tested in three replicates and the control in six replicates. The results of the test are given in Appendix 4. The E_rC_{50} value was 12.03 mg/L and E_yC_{50} value was 6.9 mg/L – based on frond number, the respective E_rC_{50} value was 3.8 mg/L and E_yC_{50} value was 2.0 mg/L – based on dry weight [4].

3.6. Analytical measurements

The aim of analytical measurements of the study was to verify concentrations of Paliogen Violet 5011 in the test solutions. The samples of fresh water solutions at test initiation and a each renewal and samples of 48 and 72 hour old solutions at each renewal and at test termination were analysed for in all test concentrations.
The analytical measurements of Paliogen Violet 5011 were performed by spectrophotometric method [SPR/C/205].

3.6.1. Detected substance

Paliogen Violet 5011:

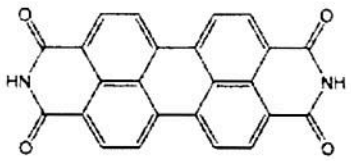
Chemical name: Anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone

CAS No.: 81-33-4

Total formula: $C_{24}H_{10}N_2O_4$

Molecular mass: 390.4 g/mol

Structural formula:



3.6.2. 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 Paliogen Violet 5011 in concentrated sulfuric acid and stock solutions 100.0, 10.0, 5.0, 2.0, 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

3.6.3. Sample preparation for chromatography analysis

The sample of 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. Dry residue was dissolved in an appropriate volume of concentrated sulfuric acid and analyzed spectrophotometrically.

3.6.4. 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 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 analysis of absorbance obtained for control samples of water and fortification samples. Considering the results of analysis no signal of detected

substance was overlapping with matrix signal of control samples in experimental conditions. Therefore specificity of the method is fulfilled.

Precision

Precision is determined as the repeatability (RSD – relative standard deviation [%]). The repeatability for Paliogen Violet 5011 is presented in Table 2.

Extraction recovery level

In order to study the recovery level, the solution of detected substance was added to non-treated samples of water and then analyzed by the method described. The results are presented in Table 2.

Limit of Quantification and Detection

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%). Limit of Detection was estimated as the lowest concentration of the detected substances that the analytical procedure can reliably differentiate from background noise.
Limit of Quantification (LoQ) and Limit of Detection (LoD) for Paliogen Violet 5011 in water is 0.001 mg/L.

4. RESULTS

The effect of Paliogen Violet 5011 on *Lemna gibba* L. growth was estimated. The concentrations of test item in the definitive test were determined based on the preliminary range finding test results.
The growth inhibition was estimated based on counted frond number, measured dry weight and observations of changes in plant development in the definitive test.

4.1. The preliminary test - non GLP

The preliminary test was performed with two concentrations 10 and 100 mg/L of the test item. The initial frond number in each replicate was 9. In order to determine growth, the number of fronds was counted in each replicate on day 2, 4 and 7. Changes in plant development were observed (smaller colonies and roots, slight chlorosis). During the test the light intensity was 6.09 – 9.15 klx. Temperature was in the range of 23.5 – 24.5°C. At test termination the growth rate inhibition (calculated from frond number) compared with the control was 0.0% in 10 mg/L and 6.5% in 100 mg/L test concentration based on loading. The results are presented in Table 3.

4.2. The definitive test

In the definitive semi-static test *Lemna gibba* the initial frond number was 9 (i.e. three plants of three fronds). The test organisms were exposed to the test item for 7 days under conditions required for exponential growth. Five concentrations based on loading of the test item were applied: 1.0, 3.2, 10, 32 and 100 mg/L. The recorded temperature was in the range of 24.2 – 24.7°C (Figure 1) and the light intensity was 8.26 – 9.60 klx.

The pH value measured (Table 4) in fresh solutions varied only slightly among test concentrations and the control by up to 0.37 units. At renewals in old solutions the measured pH increased in comparison with the values for fresh solutions.

The number of fronds distinctly visible in each test vessel was counted and recorded as well as changes in plant development which was observed at each renewal (i.e. day 2 and day 5) and after 7 days of exposure. Frond numbers at each renewal and results of dry weight measurements at test termination are presented in Table 5.

The growth rates, yield and section-by-section growth rate were calculated based on frond numbers counted after 2, 5 and 7 days of exposure (Table 6).

The morphology of aquatic plants was observed in each test concentration at each renewal and at test termination. The assessments of changes in plant development are summarized in Tables 7 - 9.
The morphological effects were compared with the appearance of colonies in the control. On day 2 only in the highest test concentration 100 mg/L (loading) fronds of bright green colour were observed.
On day 5 in the test concentrations 1.0, 3.2, 10 and 32 mg/L fronds were similar to the control. In the test concentrations 100 mg/L (loading) fronds of bright green colour were recorded.
On day 7 in test concentrations 1.0, 3.2, 10, 32 and 100 mg/L fronds were similar to the control.
The *Lemna gibba* growth curves based on growth rates for each theoretical test concentration and control are given in Figure 3. The *Lemna gibba* growth curves based on growth rates for each measured test concentration and control are given in Figure 6.

The inhibition of growth rate values and the inhibition of yield values estimated in comparison to the control based on frond number after 7 days are presented in Table 10 and as a graph in Figures 4 and 7. The inhibition of growth rate values and the inhibition of yield values in comparison to the control based on dry weight are presented in Table 10 and as a graph in Figures 5 and 8.
The relation of yield and mean growth rate inhibition based on frond number versus theoretical test concentrations based on loadings after 7 days are given in Figures 9 and 10, respectively.
The relation of yield and mean growth rate inhibition based on dry weight versus theoretical test concentrations based on loadings are given in Figures 11 and 12, respectively.

4.2.1. The validity criterion

The validity criterion of the doubling time of frond number in the control was met according to OECD Guideline No 221. The doubling time of frond number in the control was 1.9 days (i.e. < 2.5 days). The frond number in the control was increased by 12.6 between day 0 and day 7.

4.2.2. Results of analytical measurements

The chemical analyses of Paliogen Violet 5011 were performed in the following samples of the test concentrations. The samples of fresh and old solutions at test initiation, at each renewal and at test termination were analysed in order to determine the contents of Paliogen Violet 5011.
In fresh solutions the concentration from loading from of test item was between 0.0064 – 0.0071 mg/L. The measured values did not correlate with the loading, which demonstrates that the measured concentrations were obviously the solubility limit in the test medium under test conditions. In 48 and 72 hour old solutions the concentration of test item was between 0.0067 – 0.0071 mg/L.
Since the measurements in test solutions demonstrated the presence and stability of the test item in solution, the mean measured concentrations were calculated. The results are given in Tables 15 - 17.
The mean measured concentrations were calculated as the time-weighted means according to the following formula:

* Calculation of Time-weighted mean [6, 7]

$$\text{Area} = \frac{\text{Conc 0} - \text{Conc 1}}{\text{Ln(Conc 0)} - \text{Ln(Conc 1)}} \cdot \text{Days}$$

Days – number of days between renewals
Conc 0 – determined concentration at start of renewal period
Conc 1 – determined concentration at end of renewal period

$$\text{Time-weighted mean} = \frac{\text{Total Area}}{\text{Total Days}}$$

4.2.3. Endpoint values

The EC_x values were determined based on the loadings of concentrations Paliogen Violet 5011 [1]. The E_rC_x values based on growth rates are presented in Table 12. The E_yC_x values based on yield are presented in Table 13. The E_rC_x based on section-by-section growth rates are given in Table 14.
However, analytical results demonstrated that the measured concentrations of the test item were markedly lower than the loading of the test item since these concentrations exceeded the limit of solubility. Thus, the EC_x values were also determined based on time-weighted mean concentrations of test item [1, 6, 7, 8].

The calculations and statistical analysis were performed with commercial software ToxRat Professional [5], [SOP/W/68]. The description below concerns only the endpoint values based on theoretical concentrations of Paliogen Violet 5011.

The median concentration causing 50% inhibition of mean growth rate of *Lemna gibba* culture after 7 days based on frond number (E_rC_{50}/7 d) is above 100 mg/L i.e. the highest applied concentration. The E_rC_{20}/7 d and E_rC_{10}/7 d are above 100 mg/L.
The statistical tests performed with data on growth rate based on frond number were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance

homogeneity (with Residuals) showed that variances are homogeneous, Williams Multiple Sequential t-test Procedure did not show a significant difference between all test concentration compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the growth inhibition effect LOEC/7 d is above 100 mg/L. The determined value of the test item concentration not causing any effect on duckweed growth NOEC/7 d is above or equal to 100 mg/L.

The median concentration causing 50% of yield inhibition of *Lemna gibba* culture after 7 days based on frond number (E_yC_{50}/7 d) is above 100 mg/L i.e. the highest applied concentration.

The E_yC_{20}/7 d and E_yC_{10}/7 d are above 100 mg/L.

The statistical tests performed with data on yield based on frond number were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance homogeneity (with Residuals) showed that variances are homogenous, Williams Multiple Sequential t-test Procedure did not show a significant difference between all test concentrations compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the yield inhibition effect LOEC/7 d is above 100 mg/L. The determined value of the test item concentration not causing any effect on yield NOEC/7 d is above or equal to 100 mg/L.

The median concentration causing 50% inhibition of mean growth rate of *Lemna gibba* culture after 7 days based on dry weight (E_rC_{50}/7 d) is above 100 mg/L i.e. the highest applied concentration. The E_rC_{20}/7 d and The E_rC_{10}/7 d are above 100 mg/L.

The statistical tests performed with data on growth rate based on dry weight were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance homogeneity (with Residuals) showed that variances are homogeneous, Williams Multiple Sequential t-test Procedure did not show a significant difference between all test concentrations compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the growth inhibition effect LOEC/7 d is above 100 mg/L. The determined value of the test item concentration not causing any effect on duckweed growth NOEC/7 d is above or equal to 100 mg/L.

The median concentration causing 50% of yield inhibition of *Lemna gibba* culture after 7 days based on dry weight (E_yC_{50}/7 d) is above 100 mg/L i.e. the highest applied concentration. The E_yC_{20}/7 d and and E_yC_{10}/7 d are above 100 mg/L.

The statistical tests performed with data on yield based on dry weight were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance homogeneity (with Residuals) showed that variances are homogeneous, Williams Multiple Sequential t-test Procedure did not show significant difference between all test concentrations compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the yield inhibition effect LOEC/7 d is above 100 mg/L. The determined value of the test item concentration not causing any effect on yield NOEC/7 d is above or equal 100 mg/L.

In this test the results based on mean measured concentrations are more relevant, since the measured concentrations of the test item in the concentrations were markedly below the the loadings of the test item used for the preparation of the test solutions. The calculations carried out on the basis of time-weighted mean concentrations of test item with Lemna provided the following results:

The median concentration causing 50% inhibition of mean growth rate of *Lemna gibba* culture after 7 days based on frond number (E_rC_{50}/7 d) is above 0.007 mg/L. The E_rC_{20}/7 d is above 0.007 mg/L and E_rC_{10}/7 d is above 0.007 mg/L.
The statistical tests performed with data on growth rate based on frond number were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance homogeneity (with Residuals) showed that variances are homogeneous, Williams Multiple Sequential t-test Procedure did not show a significant difference between all test concentrations compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the growth inhibition effect LOEC/7 d is above 0.0069 mg/L. The determined value of the test item concentration not causing any effect on duckweed growth NOEC/7 d is above or equal 0.0069 mg/L.

The median concentration causing 50% of yield inhibition of *Lemna gibba* culture after 7 days based on frond number (E_yC_{50}/7 d) is above 0.0069 mg/L. The E_yC_{20}/7 d is above 0.0069 mg/L and E_yC_{10}/7 d above 0.0069 mg/L.
The statistical tests performed with data on yield based on frond number were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance homogeneity (with Residuals) showed that variances are homogenous, Williams Multiple Sequential t-test Procedure did not show a significant difference between all test concentrations compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the yield inhibition effect LOEC/7 d is above 0.0069 mg/L. The determined value of the test item concentration not causing any effect on yield NOEC/7 d is above or equal 0.0069 mg/L.

The median concentration causing 50% inhibition of mean growth rate of *Lemna gibba* culture after 7 days based on dry weight (E_rC_{50}/7 d) is above 0.0069 mg/L. The E_rC_{20}/7 d is above 0.0069 mg/L. The E_rC_{10}/7 d is above 0.0069 mg/L.
The statistical tests performed with data on growth rate based on dry weight were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance homogeneity (with Residuals) showed that variances are homogeneous, Williams Multiple Sequential t-test Procedure did not show a significant difference between all test concentrations compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the growth inhibition effect LOEC/7 d is above 0.0069 mg/L. The determined value of the test item concentration not causing any effect on duckweed growth NOEC/7 d is above 0.0069 or equal to 0.0069 mg/L.

The median concentration causing 50% of yield inhibition of *Lemna gibba* culture after 7 days based on dry weight (E_yC_{50}/7 d) is above 0.0069 mg/L. The E_yC_{20}/7 d is above 0.0069 mg/L and E_yC_{10}/7 d is above 0.0069 mg/L.

The statistical tests performed with data on yield based on dry weight were: Shapiro-Wilk's Test on normal distribution confirmed normal distribution of data, Levene's Test on variance homogeneity (with Residuals) showed that variances are homogeneous, Williams Multiple Sequential t-test Procedure did not show a significant difference between all test concentrations compared with the control. Therefore, based on statistical analysis results the lowest concentration of the test item causing the yield inhibition effect LOEC/7 d is above 0.0069 mg/L. The determined value of the test item concentration not causing any effect on yield NOEC/7 d is above or equal 0.0069 mg/L.

The test substance had no effect on the test organisms up to the saturation concentration in the test medium under the test conditions.

5. DEVIATIONS FROM STUDY PLAN

There were no deviations from the OECD Guideline No 221 and the Study plan in the experimental part of the study. The validity criterion as given in OECD Guideline No 221 was met.

In the Study plan the deadline for final report was September 2012. However, due to delay in sending the draft report to sponsor and obligation to acquire sponsor's acceptance of the draft report, the deadline was postponed.

6. REFERENCES

[1] OECD Guidelines For Testing of Chemicals No 221 (2006) „*Lemna sp*. Growth inhibition test".

[2] Directive 2004/10/EC on the harmonization 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).

[3] Polish legislation: 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).
Act of Parliament of 25th February 2011 on chemical substances and mixtures (Dz.U. Nr 63, poz. 322).

[4] I. Sims, P. Whitehouse, R.F. Lacey – Development and ring-test of draft OECD test Guideline, 1999.

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

[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] OECD Guideline for Testing of Chemicals No 211 (2008): "Daphnia magna Reproduction Test", Annex 6, pp. 20-21.

[8] Monograph No.26, Aquatic Toxicity Testing of Sparingly Soluble, Volatile and Unstable Substances, ECETOC, September 1996, Brussels.

7. 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/15	Laboratory oven- instruction manual.
SOP/W/18	Preparing and labelling of chemical reagents.
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 – instruction manual.
SOP/W/39	Luxmeter L-50 – instruction manual.
SOP/W/47	*Lemna* sp. Growth inhibition test.
SOP/W/51	Temperature logger HI 141 – instruction manual.
SOP/W/56	Autoclave sterilizer – instructions manual.
SOP/W/66	*Lemna* sp. culturing.
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/C/205	The method of determination 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. Preparation of test concentrations, definitive test

Theoretical* concentration of test item (loadings) [mg/L]	Amount of the test item [mg]			Volume of 20X AAP medium [mL]
	01.09.12	04.09.12	06.09.12	
100	100.2	100.2	100.2	1000
32	31.98	31.98	32.05	1000
10	10.01	10.01	10.03	1000
3.2	320 mL of test concentration 10 mg/L			680
1.0	100 mL of test concentration 10 mg/L			900
Control	--			1000

*- theoretical concentration as expression used in this report were calculated by dividing the loading of the three highest test concentration. The concentration 10 mg/L was used to prepare further dilutions by the dilution factor used to prepare the test substance concentrations in Tables 1-11

Table 2. Recovery level of Paliogen Violet 5011 in fortified samples (n = 5)

Theoretical* concentration of test item(loadings) [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 3. Frond number and dry weight, preliminary test – non-GLP

Theoretical* concentration of test item (loadings) [mg/L]	Frond number			Dry weight [mg]
	day 2	day 4	day 7	day 7
Control	18	23	57	8.54
	20	25	63	8,08
	16	21	53	9,68
	17	21	55	8,92
	20	26	72	11.43
	17	25	58	7.51
mean	**18.0**	**23.5**	**59.7**	**9.03**
standard deviation	*1.67*	*2.17*	*6.92*	*1.39*
10	19	31	83	13.71
	18	25	61	9.58
	18	23	56	8.73
	13	20	59	10.63
	19	27	*75*	13.06
	13	21	49	8.07
mean	**16.7**	**24.5**	**63.8**	**10.63**
standard deviation	*2.88*	*4.09*	*12.69*	*2.31*
100	13	22	62	8.41
	16	24	*59*	10.85
	13	25	56	8.66
	11	21	45	9.61
	15	26	70	10.43
	15	19	43	10.27
mean	**13.8**	**22.8**	**55.8**	**9.70**
standard deviation	*1.83*	*2.64*	*10.30*	*0.99*
inoculum	day 0		9	1.81
			9	1.67
			9	1.11
mean	--		**9**	**1.53**

Time of exposure was 13.08.2012 – 20.08.2012.

Table 4. pH values, definitive test

Theoretical* concentration of test item (loadings) [mg/L]	day 0	day 2		day 5		day 7
	fresh#	48 h old**	fresh#	72 h old*	fresh#	72 h old**
Control	7.66	8.66	7.54	9.28	7.67	9.16
1.0	7.74	8.70	7.56	9.20	7.62	9.28
3.2	7.89	8.68	7.74	9.20	7.66	9.36
10.0	8.02	8.75	7.68	9.04	7.50	9.36
32.0	8.03	8.68	7.69	9.17	7.74	9.35
100.0	8.03	8.67	7.70	9.15	7.70	9.30

#- pH measured in samples before division into replicates

**- pH measured in samples of pooled replicates

Table 5. Frond number and dry weight, the definitive test

Theoretical* concentration of test item (loadings) [mg/L]	Frond number			Dry weight [mg]
	day 2	day 5	day 7	day 7
Control	21	68	126	20.59
	20	63	107	14.35
	15	64	124	19.81
	18	62	115	17.26
	20	71	106	14.68
	16	51	102	18.86
mean	**18.3**	**63.2**	**113.3**	**17.6**
standard deviation	*2.42*	*6.85*	*9.99*	*2.63*
1.0	21	74	121	19.00
	20	63	108	22.80
	18	62	99	17.00
mean	**19.7**	**66.3**	**109.3**	**19.6**
standard deviation	*1.53*	*6.66*	*11.06*	*2.95*
3.2	17	59	104	15.04
	14	49	87	12.13
	19	57	109	15.44
mean	**16.7**	**55.0**	**100.0**	**14.2**
standard deviation	*2.52*	*5.29*	*11.53*	*1.81*
10.0	19	59	105	16.07
	17	59	112	16.89
	20	61	115	17.67
mean	**18.7**	**59.7**	**110.7**	**16.9**
standard deviation	*1.53*	*1.15*	*5.13*	*0.80*
32.0	21	69	129	22.02
	18	60	118	19.46
	21	75	121	18.42
mean	**20.0**	**68.0**	**122.7**	**20.0**
standard deviation	*1.73*	*7.55*	*5.69*	*1.85*
100.0	13	50	103	15.67
	15	53	105	16.06
	17	61	109	15.69
mean	**15.0**	**54.7**	**105.7**	**15.8**
standard deviation	*2.00*	*5.69*	*3.06*	*0.22*
Inoculum	day 0		9	2.62
			9	2.09
			9	2.33
mean			**9**	**2.35**

Table 6. Section-by-section Growth Rate, Growth Rate and Yield based on frond number, definitive test

Theoretical* concentration of test item (loadings) [mg/L]	Section-by-section Growth Rate			Mean Growth Rate	Yield		
	0 – 2 d	2 – 5 d	5 – 7 d	0 – 7 d	2 d	5 d	7 d
Control	0.424	0.587	0.206	0.377	21.0	68.0	126.0
	0.399	0.574	0.177	0.354	20.0	63.0	107.0
	0.255	0.725	0.220	0.375	15.0	64.0	124.0
	0.347	0.618	0.206	0.364	18.0	62.0	115.0
	0.399	0.633	0.134	0.352	20.0	71.0	106.0
	0.288	0.580	0.231	0.347	16.0	51.0	102.0
mean	**0.352**	**0.620**	**0.196**	**0.361**	**18.3**	**63.2**	**113.3**
standard deviation	*0.068*	*0.057*	*0.035*	*0.012*	*2.42*	*6.85*	*9.99*
1.0	0.424	0.630	0.164	0.371	21.0	74.0	121.0
	0.399	0.574	0.180	0.355	20.0	63.0	108.0
	0.347	0.618	0.156	0.343	18.0	62.0	99.0
mean	**0.390**	**0.607**	**0.167**	**0.356**	**19.7**	**66.3**	**109.3**
standard deviation	*0.039*	*0.030*	*0.012*	*0.014*	*1.53*	*6.66*	*11.06*
3.2	0.318	0.622	0.189	0.350	17.0	59.0	104.0
	0.221	0.626	0.191	0.324	14.0	49.0	87.0
	0.374	0.549	0.216	0.356	19.0	57.0	109.0
mean	**0.304**	**0.599**	**0.199**	**0.343**	**16.7**	**55.0**	**100.0**
standard deviation	*0.077*	*0.043*	*0.015*	*0.017*	*2.52*	*5.29*	*11.53*
10.0	0.374	0.567	0.192	0.351	19.0	59.0	105.0
	0.318	0.622	0.214	0.360	17.0	59.0	112.0
	0.399	0.558	0.211	0.364	20.0	61.0	115.0
mean	**0.364**	**0.582**	**0.206**	**0.358**	**18.7**	**59.7**	**110.7**
standard deviation	*0.041*	*0.035*	*0.012*	*0.007*	*1.53*	*1.15*	*5.13*
32.0	0.424	0.595	0.209	0.380	21.0	69.0	129.0
	0.347	0.602	0.225	0.368	18.0	60.0	118.0
	0.424	0.636	0.159	0.371	21.0	75.0	121.0
mean	**0.398**	**0.611**	**0.198**	**0.373**	**20.0**	**68.0**	**122.7**
standard deviation	*0.044*	*0.022*	*0.034*	*0.007*	*1.73*	*7.55*	*5.69*
100.0	0.184	0.674	0.241	0.348	13.0	50.0	103.0
	0.255	0.631	0.228	0.351	15.0	53.0	105.0
	0.318	0.639	0.193	0.356	17.0	61.0	109.0
mean	**0.252**	**0.648**	**0.221**	**0.352**	**15.0**	**54.7**	**105.7**
standard deviation	*0.067*	*0.023*	*0.024*	*0.004*	*2.00*	*5.69*	*3.06*

Table 7. Results of observations on day 2, definitive test

Theoretical* concentration of test item (loadings) [mg/L]	Observations
Control	normal development, colour, shape of fronds, roots and colonies
1.0	no changes
3.2	no changes
10	no changes
32	no changes
100	fronds of brighter colour

Table 8. Results of observations on day 5, definitive test

Theoretical* concentration of test item (loadings) [mg/L]	Observations
Control	normal development, colour, shape of fronds, roots and colonies
1.0	no changes
3.2	no changes
10	no changes
32	bending colonies
100	fronds of brighter colour

Table 9. Results of observations on day 7, definitive test

Theoretical* concentration (loadings) of test item [mg/L]	Observations
Control	normal development, colour, shape of fronds, roots and colonies
1.0	no changes
3.2	no changes
10	no changes
32	no changes
100	no changes

Table 10. Inhibition of growth rate and yield of *Lemna gibba*, definitive test

Theoretical* concentration of test item (loadings) [mg/L]	Based on frond number		Based on dry weight	
	% Inhibition at 7 d (growth rate)	% Inhibition at 7 d (yield)	% Inhibition at 7 d (growth rate)	% Inhibition at 7 d (yield)
Control	0.0	0.0	0.00	0.00
1.0	1.4	3.8	0.00	0.00
3.2	5.0	12.8	10.48	22.23
10	0.8	2.6	1.63	4.69
32	0.0	0.0	0.00	0.00
100	2.6	7.3	4.86	11.71

Table 11. Concentration and stability of Paliogen Violet 5011, definitive test

Theoretical* concentration of the test item (loadings) [mg/L]	Mean concentration (n=3) of test item measured in samples collected [mg/L]								
	at test initiation	Renewal after 48 h			Renewal after 48 h			at test termination	
	fresh solution	48 h old solution	% of initial concentration	fresh solution	72 h old solution	% of initial concentration	fresh solution	48 h old solution	% of initial concentration
Control	<LoD	<LoD	--	<LoD	<LoD	--	<LoD	<LoD	--
1.0	0.0070	0.0071	101.43	0.0071	0.0690	97.18	0.0070	0.0070	100.00
3.2	0.0069	0.0069	100.00	0.0070	0.0069	98.57	0.0067	0.0069	102.98
10	0.0064	0.0067	104.69	0.0065	0.0070	107.69	0.0067	0.0069	102.98
32	0.0070	0.0071	101.43	0.0067	0.0070	104.48	0.0069	0.0069	100.00
100	0.0070	0.0069	98.57	0.0068	0.0070	102.94	0.0069	0.0069	100.00

Table 12. Endpoint values based on growth rate (frond number and dry weight) of *Lemna gibba* for theoretical test item concentrations based on loading, definitive test

Endpoint	The E_rC_x, LOEC and NOEC values [mg/L] from frond number in intervals:			From dry weight [mg]
	0 - 2 d	0 - 5 d	0 - 7 d	0 - 7 d
E_rC_{50}	> 100.0	> 100.0	> 100.0	> 100.0
E_rC_{20}	> 100.0	> 100	> 100.0	> 100.0
E_rC_{10}	> 100.0	> 100	> 100.0	> 100.0
LOEC	> 100.0	> 100.0	> 100.0	> 100.0
NOEC	≥ 100.0	≥ 100.0	≥ 100.0	≥ 100.0

Table 13. Endpoint values based on yield (frond number and dry weight) of *Lemna gibba* for theoretical test item concentrations based on loading, definitive test

Endpoint	The E_yC_x, LOEC and NOEC values [mg/L] from frond number in intervals:			From dry weight [mg]
	0 - 2 d	0 - 5 d	0 - 7 d	0 - 7 d
E_yC_{50}	> 100.0	> 100.0	> 100.0	> 100.0
E_yC_{20}	> 100.0	> 100	> 100.0	> 100.0
E_yC_{10}	> 100.0	> 100	> 100.0	> 100.0
LOEC	> 100.0	> 100.0	> 100.0	> 100.0
NOEC	≥ 100.0	≥ 100.0	≥ 100.0	≥ 100.0

Table 14. Endpoint values based on section-by-section growth rate (frond number) of *Lemna gibba* for theoretical test item concentrations based on loading, definitive test

Endpoint	The $E_rC_{x,}$, LOEC and NOEC values from frond number [mg/L] in intervals:		
	0 - 2 d	2 - 5 d	5 - 7 d
E_rC_{50}	> 100.0	> 100.0	> 100.0
E_rC_{20}	> 100.0	> 100.0	> 100.0
E_rC_{10}	> 100.0	> 100.0	> 100.0
LOEC	> 100.0	> 100.0	> 100.0
NOEC	≥ 100.0	≥ 100.0	≥ 100.0

Table 15. Endpoint values based on growth rate (frond number and dry weight) of *Lemna gibba* for mean measured test item concentrations, definitive test

Endpoint	The E_rC_x, LOEC and NOEC values [mg/L] from frond number in intervals:			From dry weight [mg]
	0 - 2 d	0 - 5 d	0 - 7 d	0 - 7 d
E_rC_{50}	> 0.0069	> 0.0069	> 0.0069	> 0.0069
E_rC_{20}	> 0.0069	> 0.0069	> 0.0069	> 0.0069
E_rC_{10}	> 0.0069	> 0.0069	> 0.0069	> 0.0069
LOEC	> 0.0069	> 0.0069	> 0.0069	> 0.0069
NOEC	≥ 0.0069	≥ 0.0069	≥ 0.0069	≥ 0.0069

n.d. – not determined due to mathematical reasons [5], [SOP/W/68]

Table 16. Endpoint values based on yield (frond number and dry weight) of *Lemna gibba* for mean measured test item concentrations, definitive test

Endpoint	The E_yC_x, LOEC and NOEC values [mg/L] from frond number in intervals:			From dry weight [mg]
	0 - 2 d	0 - 5 d	0 - 7 d	0 - 7 d
E_yC_{50}	> 0.0069	> 0.0069	> 0.0069	> 0.0069
E_yC_{20}	> 0.0069	> 0.0069	> 0.0069	> 0.0069
E_yC_{10}	> 0.0069	> 0.0069	> 0.0069	> 0.0069
LOEC	> 0.0069	> 0.0069	> 0.0069	> 0.0069
NOEC	≥ 0.0069	≥ 0.0069	≥ 0.0069	≥ 0.0069

n.d. – not determined due to mathematical reasons [5], [SOP/W/68]

Table 17. Endpoint values based on section-by-section growth rate (frond number) of *Lemna gibba* for measured test item concentrations, definitive test

Endpoint	The $E_rC_{x,}$, LOEC and NOEC values from frond number [mg/L] in intervals:		
	0 - 2 d	2 - 5 d	5 - 7 d
E_rC_{50}	> 0.0069	> 0.0069	> 0.0069
E_rC_{20}	> 0.0069	> 0.0069	> 0.0069
E_rC_{10}	> 0.0069	> 0.0069	> 0.0069
LOEC	> 0.0069	> 0.0069	> 0.0069
NOEC	≥ 0.0069	≥ 0.0069	≥ 0.0069

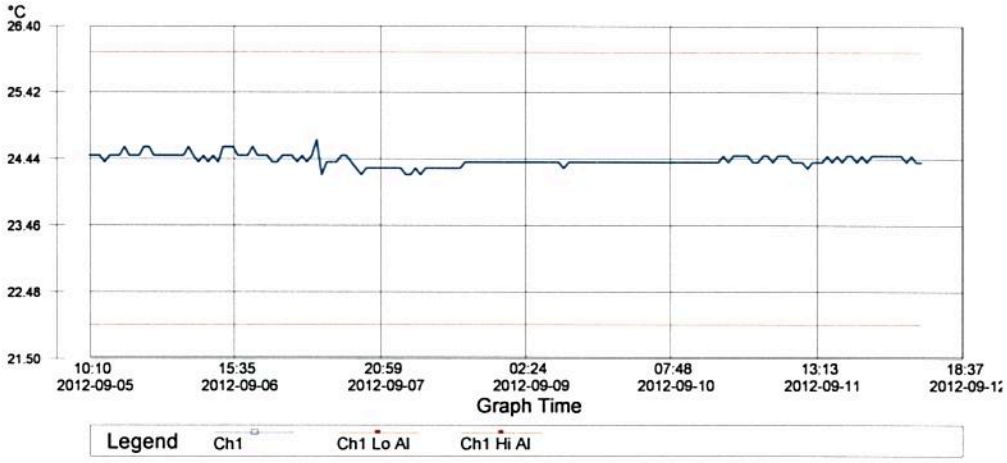


Figure 1. Temperature, definitive test

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

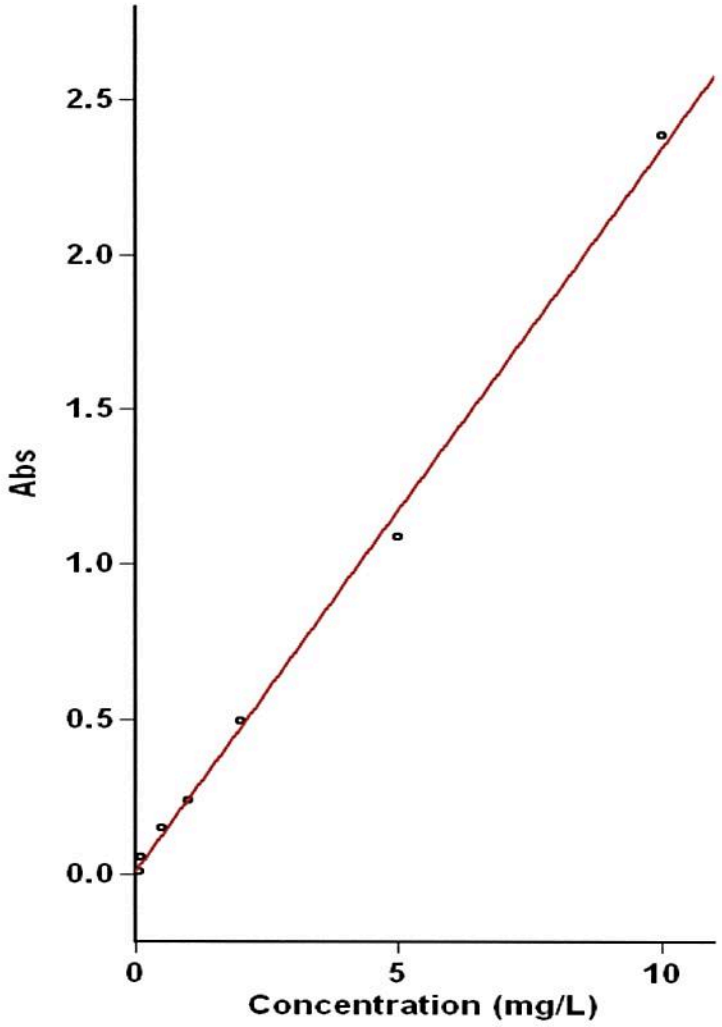


Figure 2. Calibration curve for Paliogen Violet 5011

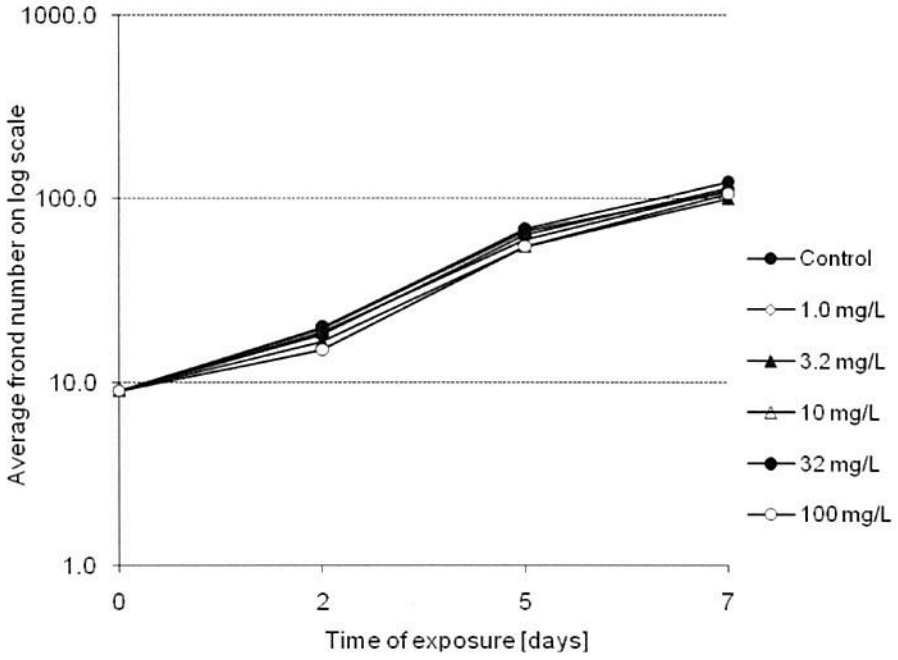


Figure 3. Growth curves for each theoretical test concentration and control, definitive test

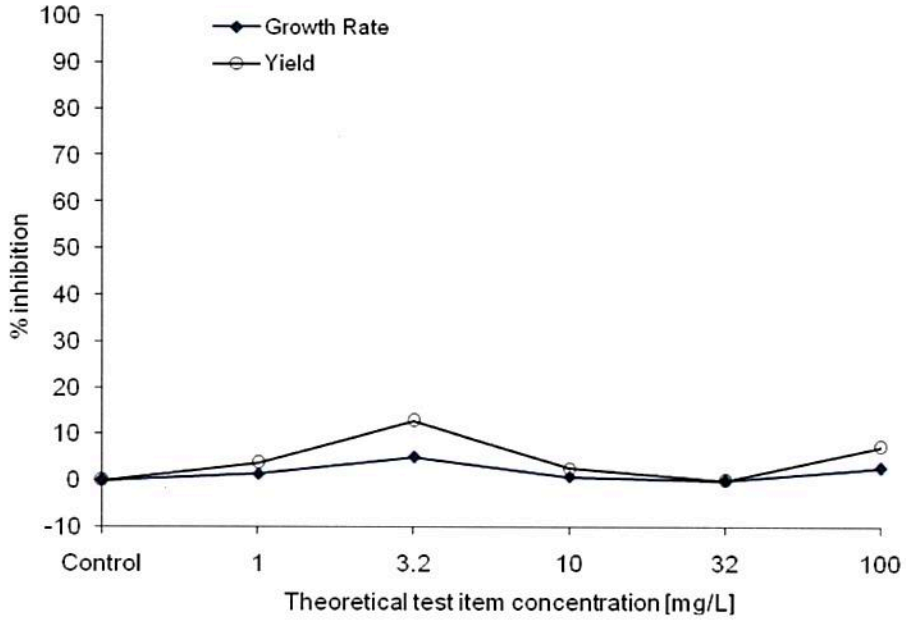


Figure 4. Effect on the growth of *Lemna gibba* (based on frond number), definitive test

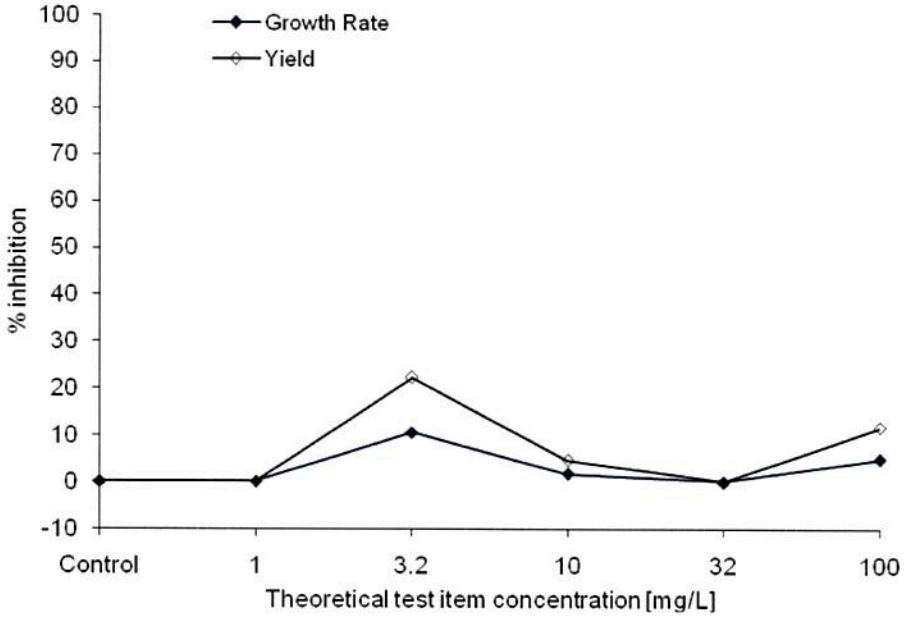


Figure 5. Effect on the growth of *Lemna gibba* (based on dry weight), definitive test

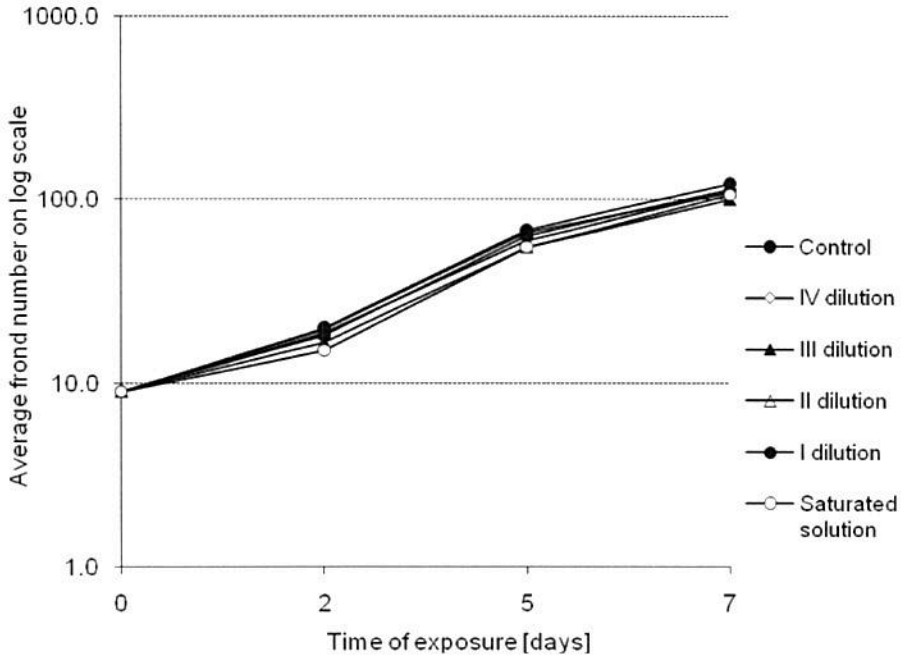


Figure 6. Growth curves for each measured test concentration and control, definitive test

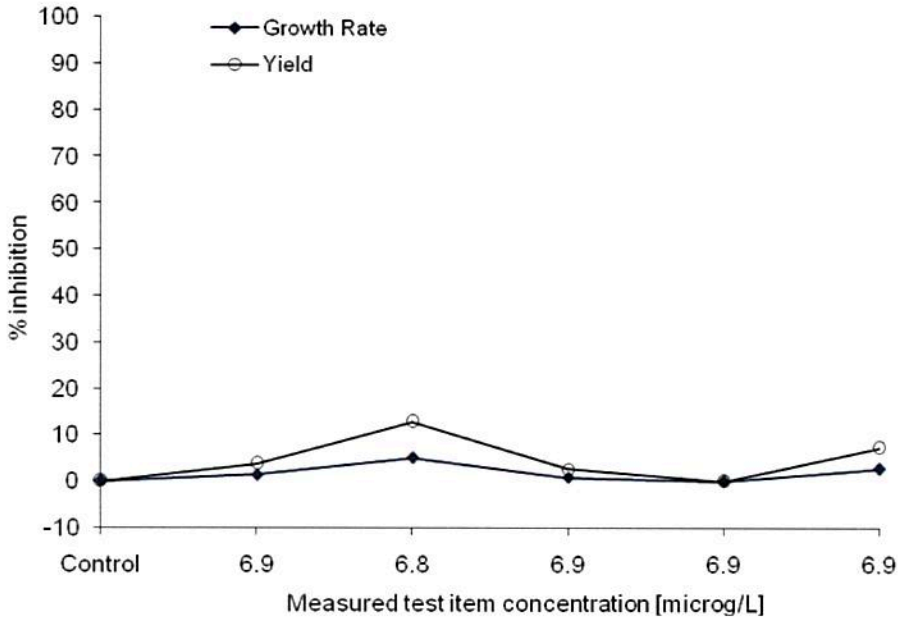


Figure 7. Effect on the growth of *Lemna gibba* (based on frond number) from measured concentrations, definitive test

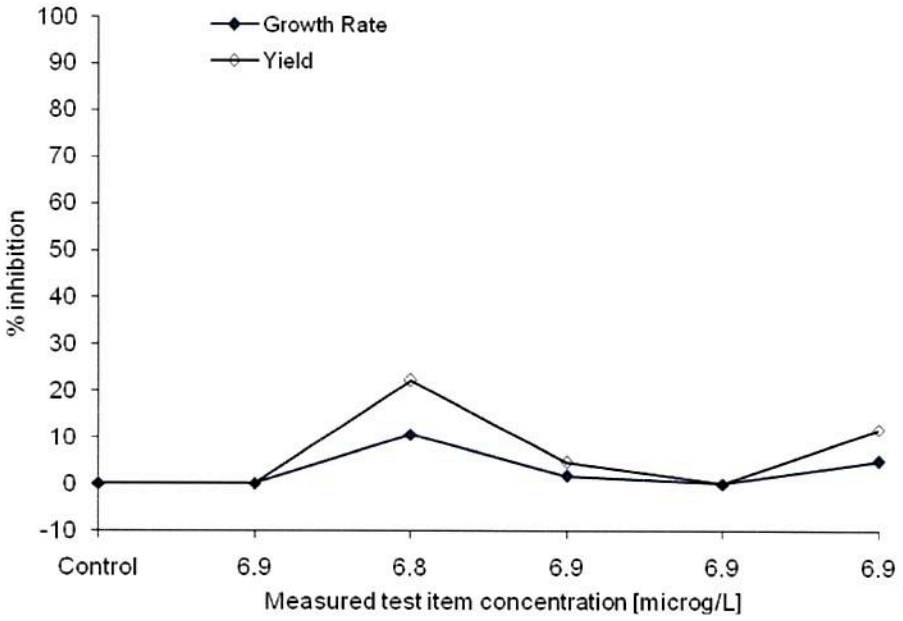


Figure 8.Effect on the growth of *Lemna gibba* (based on dry weight) from measured concentrations, definitive test

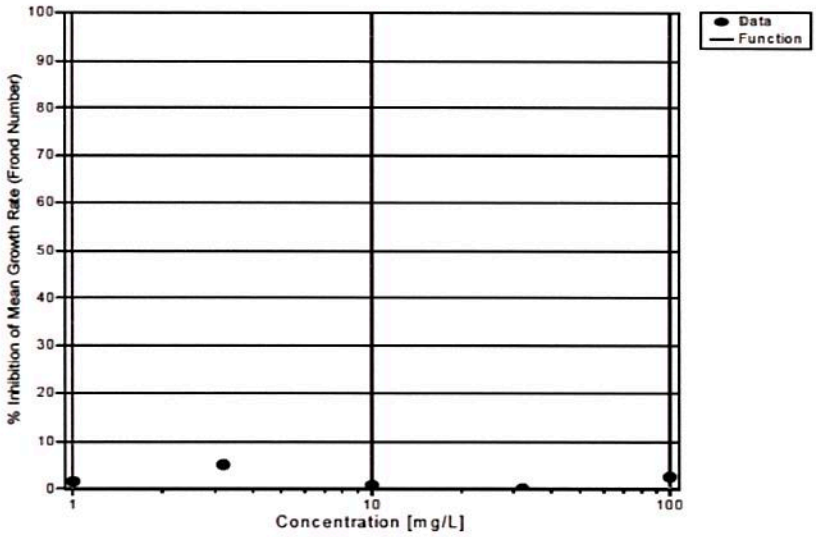


Figure 9. Inhibition of mean growth rate (frond number) versus theoretical concentration of test item on day 7, definitive test

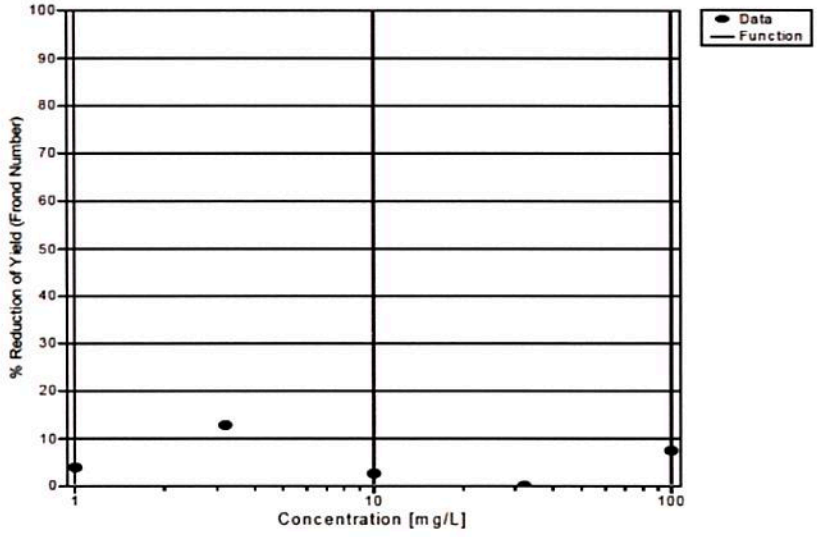


Figure 10. Inhibition of yield (frond number) versus theoretical concentration of test item on day 7, definitive test

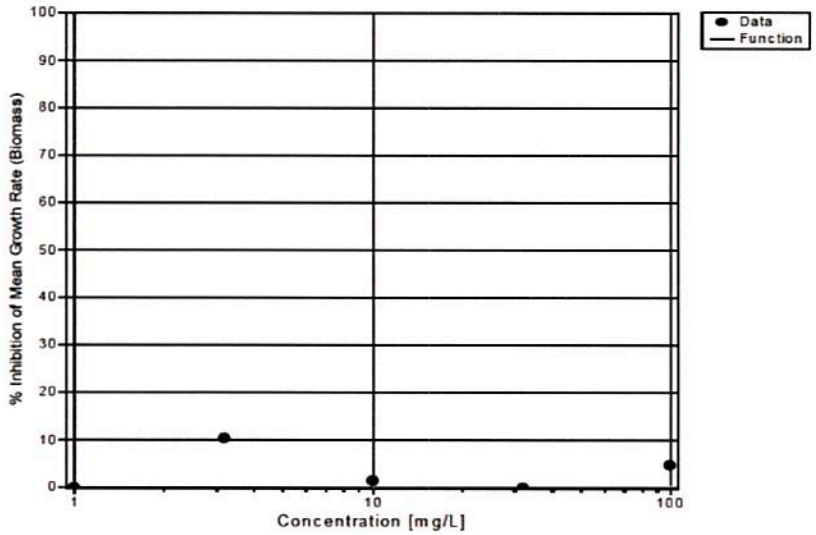


Figure 11. Inhibition of growth rate (dry weight) versus theoretical concentration of test item on day 7, definitive test

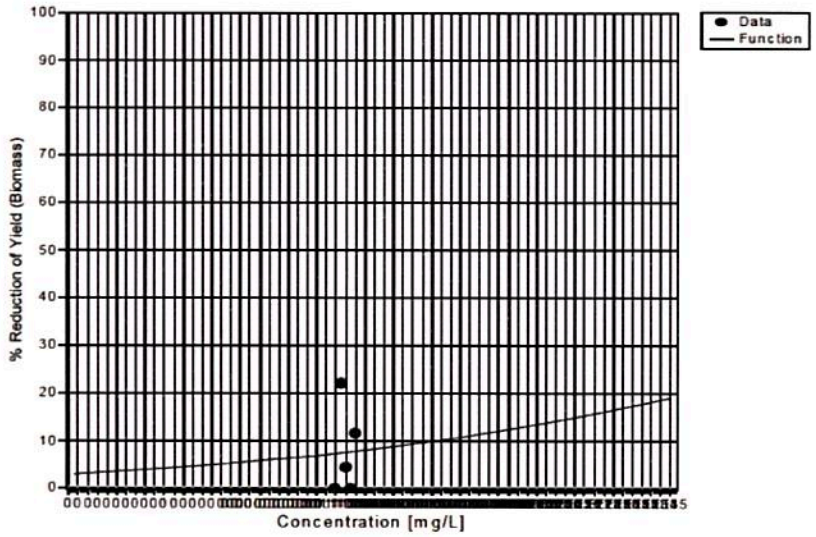


Figure 12. Inhibition of mean yield (dry weight) versus theoretical concentration of test item, definitive test

Appendix 1. Statistics for determination of endpoint values base on theoretical concentration of test item (examples)

Effective Concentrations (ECx) for Yield (Frond Number) at 7 d

Probit analysis using linear max. likelihood regression

Tab. 10: Probit analysis using linear max. likelihood regression: Determination of the concentration/response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [mg/L]	Log(x)	% Inhibition	n	Emp. Probit	Weight	Reg. Probit
Control		0.00	6			excluded
1.0	0.000	3.83	3	-1.1572	0.102	-1.510
3.2	0.505	12.78	3	-0.9330	0.086	-1.566
10.0	1.000	2.56	3	-1.1892	0.072	-1.621
32.0	1.505	0.00	3	-1.4775	0.060	-1.677
100.0	2.000	7.35	3	-1.0691	0.050	-1.733

excluded: value not in line with the chosen function

Inhibition greater than 100% or lower than 0% were replaced by 100% and 0%, respectively.

Parameters of the probit analysis

Tab. 11: Parameters of the probit analysis: Results of the regression analysis

Parameter	Value
Computation runs:	7
Slope b:	-0.11139
Intercept a:	-1.50974
Variance of b:	5.64788
Goodness of Fit	
$Chi_{\downarrow}$:	0.05935
Degrees of freedom:	3
$p(Chi_{\downarrow})$:	0.99622
Log EC50:	-13.55311
SE Log EC50:	307.09508
g-Criterion:	91.16527
Residual Variance ($Chi_{\downarrow}$/df):	0.01978
$r_{\downarrow}$:	0.036
F:	0.111
p(F) (df: 1;3):	0.239

$Chi_{\downarrow}$ is a goodness of fit measure. If the probability, $p(Chi_{\downarrow})$, is lower or equal than 0.100, data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (= are made wider; so, check whether these results are reasonable!). The coefficient of determination, $r_{\downarrow}$ ($0 <= r_{\downarrow} <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level, (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No meaningful concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significant different from zero).
Due to the lacking concentration/response the shown ECx are not valid.

No meaningful Concentration/response was found (slope <= 0). Therefore, the program refrained from deriving Concentration/response plots.

Results of the probit analysis

Tab. 12: Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%- and 99%-confidence limits (according to Fieller`s theorem).

Parameter	EC10	EC20	EC50
Value [mg/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.
lower 99%-cl	n.d.	n.d.	n.d.
upper 99%-cl	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons or inappropriate data

Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The effect parameters and confidence limits can be meaningless. Slope function after Litchfield and Wilcoxon: 0.000

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

Statistical Characteristics of the Sample

Tab. 22: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	104.3	102.0	93.0	117.0	6	9.99	9.6	4.08	3.9	93.8	114.9
1.0	100.3	99.0	90.0	112.0	3	11.06	11.0	6.39	6.4	71.7	128.9
3.2	91.0	95.0	78.0	100.0	3	11.53	12.7	6.66	7.3	61.2	120.8
10.0	101.7	103.0	96.0	106.0	3	5.13	5.0	2.96	2.9	88.4	114.9
32.0	113.7	112.0	109.0	120.0	3	5.69	5.0	3.28	2.9	99.0	128.4
100.0	96.7	96.0	94.0	100.0	3	3.06	3.2	1.76	1.8	88.8	104.6

Shapiro-Wilk´s Test on Normal Distribution

Tab. 23: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [mg/L]	Mean	s	n
Control	104.3	9.99	6
1.0	100.3	11.06	3
3.2	91.0	11.53	3
10.0	101.7	5.13	3
32.0	113.7	5.69	3
100.0	96.7	3.06	3

Results:

Number of residues = 21; Shapiro-Wilk´s W = 0.972; p(W) = 0.767; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).
Levene´s test is chosen for variance homogeneity testing.

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 24: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	24990.70	5	4998.14	1.823	0.169
Residuals	41125.78	15	2741.72		
Total	66116.48	20			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity !

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

Williams Multiple Sequential t-test Procedure

Tab. 25: Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [mg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	104.3	8.741						
1.0	100.3	8.741	15	95.7	-10.4	-1.40	-1.75	-
3.2	91.0	8.741	15	95.7	-10.8	-1.40	-1.83	-
10.0	101.7	8.741	15	101.7	-10.9	-0.43	-1.85	-
32.0	113.7	8.741	15	105.2	-11.0	0.13	-1.86	-
100.0	96.7	8.741	15	105.2	-11.1	0.13	-1.87	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 100.0 mg/L.

Overview over the Effect-Thresholds of the Test Item on Yield (Frond Number)

Overview over the LOEC and NOEC Determination

Tab. 26: Overview over the LOEC and NOEC Determination: Arithmetic means and significance marks as computed for yield (frond number) for all inspection intervals (top); ; bottom part: obtained LOEC and NOEC with indication of statistical test used; *wl: Williams multiple sequential t-test procedure.

Treatm. [mg/L]	0-2 d	0-4 d	0-7 d
1.0	10.7 -	57.3 -	100.3 -
3.2	7.7 -	46.0 -	91.0 -
10.0	9.7 -	50.7 -	101.7 -
32.0	11.0 -	59.0 -	113.7 -
100.0	6.0+	45.7+	96.7 -

Appendix 2.Statistics for determination of endpoint values based on measured concentrations of test item in test vessels with Lemna (examples)

Effective Concentrations (ECx) for Yield (Frond Number) at 7 d

Probit analysis using linear max. likelihood regression

Tab. 10: Probit analysis using linear max. likelihood regression: Determination of the concentration/response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [microg/L]	Log(x)	% Inhibition	n	Emp. Probit	Weight	Reg. Probit
Control		0.00	6			excluded
7.0	0.845	3.83	3	-1.1572	0.093	-1.541
6.9	0.839	12.78	3	-0.9330	0.076	-1.605
6.7	0.826	2.56	3	-1.1892	0.049	-1.736
6.9	0.839	0.00	3	-1.4775	0.076	-1.605
6.9	0.839	7.35	3	-1.0691	0.076	-1.605

excluded: value not in line with the chosen function

Inhibition greater than 100% or lower than 0% were replaced by 100% and 0%, respectively.

Parameters of the probit analysis

Tab. 11: Parameters of the probit analysis: Results of the regression analysis

Parameter	Value
Computation runs:	8
Slope b:	10.24136
Intercept a:	-10.19577
Variance of b:	85 736.97656
Goodness of Fit	
$Chi_{\downarrow}$:	0.05978
Degrees of freedom:	3
$p(Chi_{\downarrow})$:	0.99618
Log EC50:	0.99555
SE Log EC50:	4.48652
g-Criterion:	164.92120
Residual Variance ($Chi_{\downarrow}$/df):	0.01993
$r_{\downarrow}$:	0.020
F:	0.061
p(F) (df: 1;3):	0.180

$Chi_{\downarrow}$ is a goodness of fit measure. If the probability, $p(Chi_{\downarrow})$, is lower or equal than 0.100, data is much scattering round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (= are made wider; so, check whether these results are reasonable!). The coefficient of determination, $r_{\downarrow}$ ($0 <= r_{\downarrow} <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level, (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

No meaningful concentration/response was found (p(F) > 0.05; i.e. slope of the relationship is not significant different from zero).
Due to the lacking concentration/response the shown ECx are not valid.

Results of the probit analysis

Tab. 12: Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%- and 99%-confidence limits (according to Fieller`s theorem).

Parameter	EC10	EC20	EC50
Value [microg/L]	n.d.	n.d.	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.
lower 99%-cl	n.d.	n.d.	n.d.
upper 99%-cl	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons or inappropriate data

Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). The p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The effect parameters and confidence limits can be meaningless. Slope function after Litchfield and Wilcoxon: 1.252

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

Threshold Concentrations (NOEC) for Yield (Frond Number) at 7 d

Statistical Characteristics of the Sample

Tab. 22: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [microg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	104.3	102.0	93.0	117.0	6	9.99	9.6	4.08	3.9	93.8	114.9
7.0	100.3	99.0	90.0	112.0	3	11.06	11.0	6.39	6.4	71.7	128.9
6.9	91.0	95.0	78.0	100.0	3	11.53	12.7	6.66	7.3	61.2	120.8
6.7	101.7	103.0	96.0	106.0	3	5.13	5.0	2.96	2.9	88.4	114.9
6.9	113.7	112.0	109.0	120.0	3	5.69	5.0	3.28	2.9	99.0	128.4
6.9	96.7	96.0	94.0	100.0	3	3.06	3.2	1.76	1.8	88.8	104.6

Shapiro-Wilk´s Test on Normal Distribution

Tab. 23: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [microg/L]	Mean	s	n
Control	104.3	9.99	6
7.0	100.3	11.06	3
6.9	91.0	11.53	3
6.7	101.7	5.13	3
6.9	113.7	5.69	3
6.9	96.7	3.06	3

Results:

Number of residues = 21; Shapiro-Wilk´s W = 0.972; p(W) = 0.767; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).
Levene´s test is chosen for variance homogeneity testing.

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 24: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	24990.70	5	4998.14	1.823	0.169
Residuals	41125.78	15	2741.72		
Total	66116.48	20			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity !

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

Williams Multiple Sequential t-test Procedure

Tab. 25: Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [microg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	104.3	8.741						
7.0	100.3	8.741	15	95.7	-10.4	-1.40	-1.75	-
6.9	91.0	8.741	15	95.7	-10.8	-1.40	-1.83	-
6.7	101.7	8.741	15	101.7	-10.9	-0.43	-1.85	-
6.9	113.7	8.741	15	105.2	-11.0	0.13	-1.86	-
6.9	96.7	8.741	15	105.2	-11.1	0.13	-1.87	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 6.9 microg/L.

Appendix 3. Composition of the 20X AAP medium

Substance	Concentration in 20XAAP medium [mg/L]
$NaNO_3$	510
$MgCl_2 \cdot 6H_2O$	240
$CaCl_2 \cdot 2H_2O$	90
$MgSO_4 \cdot 7H_2O$	290
$K_2HPO_4 \cdot 3H_2O$	30
$NaHCO_3$	300
Microelements	
H_3BO_3	3.7
$MnCl_2 \cdot 4H_2O$	8.3
$FeCl_3 \cdot 6H_2O$	3.2
$Na_2EDTA \cdot 2H_2O$	6.0
$ZnCl_2$	0.066
$CoCl_2 \cdot 6H_2O$	0.029
$Na_2MoO_4 \cdot 2H_2O$	0.145
$CuCl_2 \cdot 2H_2O$	0.00024

Appendix 4. Reference test

Table A. 3,5-dichlorophenol *Lemna gibba* L. GROWTH INHIBITION TEST
Frond number and dry weight, reference test

Nominal concentration of reference substance [mg/L]	Frond number			Dry weight [mg]
	day 3	day 5	day 7	day 7
Control	46.0	94.0	141.0	16.6
	47.0	89.0	134.0	18.4
	45.0	90.0	130.0	21.1
	48.0	94.0	125.0	19.5
	42.0	84.0	120.0	17.5
	45.0	75.0	114.0	15.8
mean	**45.5**	**87.7**	**127.3**	**18.1**
standard deviation	*2.07*	*7.23*	*9.75*	*1.93*
0.32	44.0	84.0	113.0	17.8
	40.0	68.0	103.0	15.2
	41.0	73.0	99.0	13.6
mean	**41.7**	**75.0**	**105.0**	**15.5**
standard deviation	*2.08*	*8.19*	*7.21*	*2.13*
1.0	35.0	65.0	88.0	13.2
	42.0	79.0	116.0	14.0
	40.0	76.0	115.0	13.9
mean	**39.0**	**73.3**	**106.3**	**13.7**
standard deviation	*3.61*	*7.37*	*15.89*	*0.44*
3.2	39.0	66.0	105.0	15.9
	36.0	66.0	96.0	14.6
	37.0	68.0	111.0	14.0
mean	**37.3**	**66.7**	**104.0**	**14.8**
standard deviation	*1.53*	*1.15*	*7.55*	*0.96*
10	22.0	35.0	52.0	7.8
	25.0	45.0	62.0	6.9
	23.0	40.0	55.0	8.2
mean	**23.3**	**40.0**	**56.3**	**7.6**
standard deviation	*1.53*	*5.00*	*5.13*	*0.65*
32	9.0	9.0	9.0	0.5
	9.0	9.0	9.0	0.7
	9.0	9.0	9.0	0.6
mean	**9.0**	**9.0**	**9.0**	**0.6**
standard deviation	*0.00*	*0.00*	*0.00*	*0.08*
inoculum	day 0		9	0.88
			9	1.51
			9	1.29
mean	--		**9**	**1.23**

Time of exposure was 27.07 – 03.08.12

Table B. 3,5-dichlorophenol *Lemna gibba* L. GROWTH INHIBITION TEST
Endpoint values based on growth rate (frond number and dry weight) of *Lemna gibba* for nominal concentrations of the reference substance

The E_rC_x values [mg/L] with 95% confidence interval	Frond number			dry weight
	0 - 3 d	0 - 5 d	0 - 7 d	0 - 7 d
E_rC_{50}	10.95 (n.d.)	12.01 (n.d.)	12.03 (n.d.)	3.80 (n.d.)
E_rC_{10}	4.55 (n.d.)	6.48 (n.d.)	7.49 (n.d.)	2.72 (n.d.)

Table C. 3,5-dichlorophenol *Lemna gibba* L. GROWTH INHIBITION TEST
Endpoint values based on yield (frond number and dry weight) of *Lemna gibba* for nominal concentrations of the reference substance

The E_yC_x values [mg/L] with 95% confidence interval	Frond number			dry weight
	0 - 3 d	0 - 5 d	0 - 7 d	0 - 7 d
E_yC_{50}	6.49 (n.d.)	5.77 (n.d.)	6.86 (n.d.)	1.96 (n.d.)
E_yC_{10}	1.40 (n.d-3.59)	0.93 (n.d.-2.92)	1.61 (n.d.)	0.32 (n.d.)

n.d. – not determined due to mathematical reasons [5], [SOP/W/68]

Table D. 3,5-dichlorophenol *Lemna gibba* L. GROWTH INHIBITION TEST
Endpoint values based on section-by-section growth rate (frond number) of *Lemna gibba* for nominal concentrations of the reference substance

The E_rC_x values [mg/L] with 95% confidence interval	Frond number		
	0 - 3 d	3 - 5 d	5 - 7 d
E_rC_{50}	10.95 (n.d.)	12.54 (n.d.)	13.19 (n.d.)
E_rC_{10}	4.55 (n.d.)	9.12 (n.d.)	10.28 (n.d.)

n.d. – not determined due to mathematical reasons [5], [SOP/W/68]

Appendix 5. 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 6. Copy of the Study plan

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY
BRANCH PSZCZYNA

STUDY PLAN

PALIOGEN VIOLET 5011

Lemna gibba L. CPCC 310 Growth inhibition test

According to OECD No 221 (2006)

STUDY CODE: W/120/12

Study director:	[redacted]
Test facility:	Institute of Industrial Organic Chemistr Branch Pszczyna Department of Ecotoxicology [redacted]
Sponsor:	BASF SE [redacted]
BASF Project No.	99E0223/11X541
BAS Order No.	1089966059

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the original document
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Copy No 1
[redacted]

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
DEPARTMENT OF ECOTOXICOLOGY

Paliogen Violet 5011, *Lemna gibba* L. CPCC 310 Growth inhibition test
Study code: W/120/12

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Paliogen Violet 5011, *Lemna gibba* L. CPCC 310 Growth inhibition 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 the true copy of the study plan at the latest.
The duty of the sponsor is to provide a 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:

date signature
[redacted]

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
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Paliogen Violet 5011, *Lemna gibba* L. CPCC 310 Growth inhibition test
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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

24.08.2012
date

Chemical analysis: [redacted]

24.08.2012
date

Head of Departament of Ecotoxicology: [redacted]

24.8.12
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 221 (2006): '*Lemna sp. Growth inhibition test*' [1], SOP/W/47 '*Lemna*, growth inhibition test' and in compliance with GLP principles [4, 5], [SOP/PJ/1].

[redacted]

24.08.2012
date

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
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Paliogen Violet 5011, *Lemna gibba* L. CPCC 310 Growth inhibition test
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DISTRIBUTION

Original: BASF SE,
[redacted]

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

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

24.08.12 [redacted] 24.08.12 [redacted]
24.08.12
24.08.12
24.08.12
24.08.12
24.08.12 [redacted]

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

Copy No. 1 [redacted]
Copy No. 2

INSTITUTE OF INDUSTRIAL ORGANIC CHEMISTRY BRANCH PSZCZYNA
DEPARTMENT OF ECOTOXICOLOGY

Paliogen Violet 5011, *Lemna gibba* L. CPCC 310 Growth inhibition test
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1. BASIS FOR PERFORMING THE STUDY

The toxicity assessment of Paliogen Violet 5011 for *Lemna gibba* will be carried out according to the order from BASF SE, GV/TC – [redacted] dated July 24, 2012.

2. INTRODUCTION

Toxicity evaluation to *Lemna gibba* will be performed according to OECD Guideline No 221 '*Lemna* sp., Growth Inhibition Test' (2021) [1] and according to SOP/W/47 '*Lemna*, growth inhibition test'.
The study was coded: W/120/12 [SOP/W/4]
The study will be performed in compliance with the principles of Good Laboratory Practice OECD 1997 with exception of non-GLP preliminary test. 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].
The SOPs mentioned in the study plan contain additional specific details concerning functioning of the testing facility and instructions for study performers, in order to fulfill the requirements of the GLP system, and to remain compliant with the study plan. In case of any discrepancies between SOPs and study plan, information in the study plan is superior.

3. AIM OF THE STUDY

The aim of the study is the determination of acute toxicity of the test item for *Lemna gibba* L. and the determination of the EC_{50}, EC_{20} and EC_{10} values after 7 days, as well as LOEC and NOEC (if possible). Cultures of *Lemna gibba* 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 221 are met.

4. TERMS

Start of the study:	August 2012
Proposed starting date of definitive study:	September 2012
Proposed termination date of definitive study:	September 2012
Proposed end of the study :	September 2012

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5. MATERIAL AND METHODS

5.1. 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 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 provided a Characterization of the test substance. According to sponsor's information the solubility of detected substance is 10 µg/L. The sample is stored at room temperature in 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.

5.2. Test species

The test organism *Lemna gibba* L. originates from standard culture maintained at the Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, Laboratory of Aquatic Toxicology [SOP/W/66]. The sensitivity of the culture is monitored on regular basis with the reference substance 3,5- dichlorophenol [SOP/W/72].
Plants for the test should be kept at least 3 weeks in the same medium as used for the test. Young rapidly growing plants without visible lesions or discoloration are used. Plant material of colonies with 2-7 fronds should be used. At least 7 day before start of the exposure sufficient colonies are transferred aseptically into fresh sterile medium and cultured for 7-10 days under the conditions of the test.

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

Test suspensions with a concentrations of 10 and 100 mg/L were stirred over 72 h at 40 °C (in the incubator), then conditioned to 20 °C over 24 h on a mechanic stirrer. Before filtration an intensive grey, non-transparent mixture was obtained. The test item was visibly not dissolved.
The test concentration was filtrated through a conditioned nitrocellulose membrane (Filter type HAWG, 0.20 µm pores, Millipore) [6], [SOP/W/37].The filtrates were a clear transparent solutions.

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

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In the preliminary test glass beakers of 150 mL capacity were used containing 100 mL test solution. The test vessels were covered with transparent plastic lids in order to minimize evaporation and to prevent accidental contamination. The test concentrations and the control were tested in six replicates. In each test vessel three colonies of three fronds of *Lemna gibba* were exposed to the test item for 7 days. The preliminary test was of semi-static design.

5.3.2. Test medium

20X AAP medium recommended by OECD Guideline No 221 (2006) was used as diluent/solvent of the test item. The same medium is used for culturing.

5.3.2.1. 20X AAP medium preparation

The test medium was 20X AAP, the same as used for culturing of *Lemna* sp. The test medium was used to provide the necessary ingredients for duckweed growth.

The 20X AAP medium is prepared based on deionised water by adding appropriate amounts of stock solutions of reagent grade chemicals.

The stock solutions are renewed on regular basis for *Lemna gibba* culturing and stored for maximum a one month in a refrigerator [SOP/W/18, SOP/W/71]. The composition of stock solutions is presented in Table 1.

Table 1. Test medium

Stock solution	Ingredient	Concentration in stock solution [g/1 L]	Volume of each ingredient in 20X AAP medium [mL/1 L]
A1	$NaNO_3$	26	20
	$MgCl_2 \cdot 6\,H_2O$	12	
	$CaCl_2 \cdot 2\,H_2O$	4.4	
A2	$MgSO_4 \cdot 7\,H_2O$	15	20
A3	$K_2HPO_4 \cdot 2\,H_2O$	1.4	20
B	H_3BO_3	0.19	20
	$MnCl_2 \cdot 4H_2O$	0.42	
	$FeCl_3 \cdot 6H_2O$	0.16	
	$Na_2EDTA \cdot 2H_2O$	0.30	
	$ZnCl_2$	0.0033	
	$CoCl_2 \cdot 6H_2O$	0.0014	
	$Na_2MoO_4 \cdot 2H_2O$	0.0073	
	$CuCl_2 \cdot 2H_2O$	$12 \cdot 10^{-6}$	
C	$NaHCO_3$	15	20

In order to prepare 1 L of 20X AAP medium 20 mL of each stock solution is added into 900 mL of deionised water. After 24 h of aeration the pH of test medium is measured and adjusted to 7.5 + 0.1 with 0.1 M HCl, if necessary [SOP/W/47, SOP/W/66].

For culturing, the test medium is sterilized by autoclaving before addition of stock solution C (this solution was sterilized by membrane filtration before using).

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5.3.3. Test conditions

The preliminary test was performed under constant illumination and temperature conditions in an incubator [SOP/W/51, SOP/W/75]. The temperature was in the range 23.5 - 24.5°C and the illumination was in the range 6092 – 9147 lx.

The test vessels were covered with transparent plastic lid in order to minimize evaporation and to prevent accidental contamination. The test vessels and control vessels were arranged at random and repositioned during the test. The exposure period was 7 days.

5.3.4. Measurements and observations

At test initiation fronds and colony numbers were recorded. In order to determine growth, the number of fronds was counted in each replicate on the days of renewal and at test termination. Changes in plant development were observed and reported. The temperature was constantly recorded.

At the end of the preliminary test the inhibition of fronds number of duckweed was 6.5% in the highest concentration compared the control. There was not occurred inhibition of yield in the test concentrations compared to the control. The results of inhibition of *Lemna gibba* are given in Table 2.

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

Nominal concentration of test item [mg/L]	Inhibition of fronds number over the exposure time:		
	2 days	4 days	7 days
Control	--	--	--
10.0	7.4	0.0	0
100.0	23.2	3.0	6.5

5.3.5. Chemical analysis

In the preliminary test the test concentrations were not chemically determined.

5.4. The definitive test

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

5.4.1. Test design

The test will be conducted in a semi-static design The test will be performed with five concentrations: 1.0; 3.2; 10.0; 32.0, 100.0 mg/L.

All test concentrations will be prepared separately by weighing the test item into glass vessels and mixing with test medium, stirring and heating over 72 h (40 °C) and next conditioned over 24 h (20 °C)

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and filtrated over a 0.20 µm milipore membrane disc. Two lowest concentrations must be prepared by dilution before filtration because of dosing problems. The dilutions will be made from the dispersion of the 10 mg/L concentration before filtration. The 10 mg/L dispersion will be carefully checked for homogeneous distribution of unsolved test item. The volume used to prepare the dilution will be high enough to ensure that the loading is close to the nominal concentration. The diluted dispersion will be filtered in the same way as described for the 3 higher concentrations to prepare the test solutions. The control also will be filtered over a milipore membrane disc [6], [SOP/W/37].. Each test concentration will be prepared separately in the same way using different weights of the test substance (except concentrations 1.0 and 3.2 mg/L).

Test glass vessels of 150 mL capacity will be used containing 100 mL of each test concentration. The test vessels will be covered with transparent plastic lids in order to minimize evaporation and to prevent accidental contamination. Three replicates with three colonies with three fronds in each will be used for the concentration and the control. The total time of exposure will be 7 days.

5.4.2. Test medium

20X AAP medium recommended by OECD Guideline No 221 (2006) will be 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 221 [1], [SOP/W/47]. The test will be performed in an incubator. The temperature will be maintained in the range of 24 ± 2°C. The test will be conducted with constant illumination, using a fluorescent light source (six 24 W light bulbs) with a light intensity in the range of 6500 – 10000 lx. Any differences of the selected light intensity over the test area should not exceed the range of ± 15%. The test vessels and control vessels will be arranged at random and repositioned during the test.

5.4.4. Measurements and observations

The total frond number of *Lemna gibba* in each replicate of the test concentrations and the control will be counted and recorded at start of the exposure, on two occasions during exposure and at test termination. Only well developed (visible) fronds will be counted, other changes in plants appearance will be observed and recorded i.e. frond size, necrosis, chlorosis, colony break-up, gibbosity, presence or absence of roots, loss of buoyancy.

The dry weight of the inoculum sample representative for the culture used to start the test will be measured at test initiation. The dry weights of all test plants from each replicate will be measured at test termination. All colonies (including roots) will be rinsed with deionised water, transferred onto glass slides and dried at 60°C in a laboratory oven [SOP/W/15] till constant weight. After weighing with an analytical balance, the plants will be removed and the glass slides without plants will be weighed, in order to determine the dry weight of the plants on each glass slide [SOP/W/7].

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The pH of each test concentration and control will be measured [SOP/W/36] before division into replicates and at test termination in pooled replicates. The temperature will be constantly recorded by an electronic device in an additional test vessel filled with test medium [SOP/W/51]. The light intensity will be measured at the beginning and at the end of exposure with a lux-meter [SOP/W/39].

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 7 day old solutions will be analyzed in all test concentrations [SOP/W/83].
The content of test item will be determined by a validated spectrophotometric method [SOP/C/205]. The validation process will be described in the report.

5.5. Analysis of the results

The 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 the EC_{50} 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 the 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 additional costs.
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,
- Experimental starting and completion date,
- Dates of start and end of preliminary and definitive test,
- Description of the test item,
- Description of the test species,
- Description of the 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,

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- Deviations from the study plan,
- GLP compliance statement – copy,
- Study plan - copy.

7. ARCHIVING

The following documents concerning the experiment labeled with the study code W/09/12 will be archived till the end of the study e.i. till 2022 in Archive of Documentation and Test Samples [SOP/W/35, SOP/PB/2]:

- Raw data – true copies,
- Quality assurance control protocols - originals,
- Correspondence with sponsor - copy,
- Study plan (true copy),
- Final report (true copy).

After the archiving period all documents will be destroyed after notifying the sponsor.
The originals of raw data will be sent to the 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 to November 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 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,

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- 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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10. REFERENCES

[1] OECD Guideline for Testing of Chemicals No 221 (2006): '*Lemna*, growth inhibition 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] Act of Parliament of 25th February 2011 on chemical substances and mixtures (Dz.U. Nr 63, poz. 322).

[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/15	Laboratory oven- instruction manual
SOP/W/18	Preparing and labelling of chemical reagents
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/47	*Lemna* sp. Growth inhibition test
SOP/W/51	Temperature logger HI 141 – instruction manual
SOP/W/57	– Mechanic shaker – instructions manual
SOP/W/66	*Lemna* sp. culturing
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 samples
SOP/C/205	Analytical method for determination of Paliogen Violet 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

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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
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]