



Study report: GLP study

Study number: LEM 14 003

Test substance: PMV-01 (2014 TOX 026)

Duckweed growth inhibition test (*Lemna minor*) According to OECD Guideline 221

Test facility:

VITO - ABS
Industriezone VLASMEER 7
B 2400 MOL (Belgium)

Contact:




Sponsor:


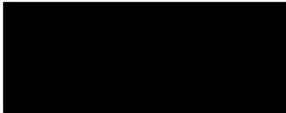
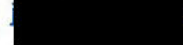
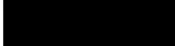




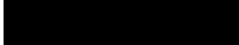

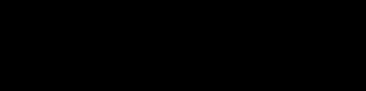
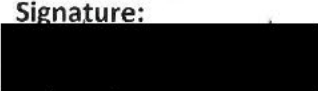
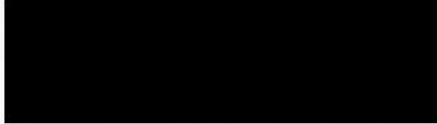
De Ceuster NV
Fortsesteenweg 30
2860 Sint-Katelijne-Waver
Belgium






December 2014
2014/MRG /R /202

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TEST SUBSTANCE	CODE Test Substance
PMV-01	2014 TOX 026

TEST FACILITY MANAGER	STUDY DIRECTOR	QA
 VITO - ABS <i>Vlaamse Instelling voor Technologisch Onderzoek</i> Boeretang 200 B2400 MOL  e-mail:  @vito.be	 VITO - ABS <i>Vlaamse Instelling voor Technologisch Onderzoek</i> Boeretang 200 B2400 MOL  e-mail:  @vito.be	 VITO - ABS <i>Vlaamse Instelling voor Technologisch Onderzoek</i> Boeretang 200 B2400 MOL  e-mail:  @vito.be
Date: 12/12/2014 Signature: 	Date: 12/12/14 Signature: 	Date: 12/12/2014 Signature: 
Sponsor De Ceuster NV Fortsesteenweg 30 2860 Sint-Katelijne-Waver Belgium 		


Personnel:  (toxicity test);  (analyses)

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STATEMENT OF GLP COMPLIANCE BY THE STUDY DIRECTOR

The study described in this report was carried out under my supervision and responsibility and in compliance with the OECD principles of Good Laboratory Practice.

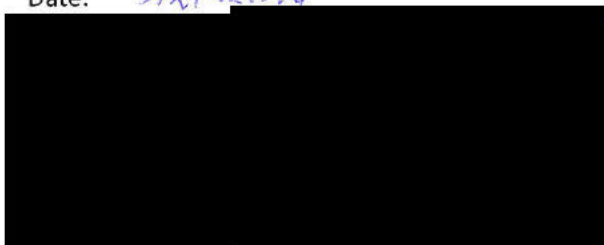
I hereby attest to the authenticity of the study and guarantee that the study was performed according to the procedures described in this report. This study report is a complete and accurate representation of the data obtained.


There were no significant deviations which may have an adverse effect on the quality or integrity of this study.

The study director makes no GLP-compliance claim for the characterization of the test item, which is the responsibility of the sponsor.

Date:

12/12/14



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STATEMENT QA

STUDY LEM 14 003

Lemna growth inhibition test (*Lemna sp.*) Test substance PMV-01 According to OECD Guideline 221

The data contained in this study report were audited by the quality Assurance Unit to assure compliance with the protocol, the standard operating procedure and the pertinent Good Laboratory Practice regulations of the OECD and EEC directives.


The audits took place, were reported to the study director and the management on the following dates:

Date	Audit	Reported findings to Study Director	Reported findings to Test Facility Management
14/08/2014 21/10/2014, 24/10/2014	Study plan Inspection of the preliminary test (test system, preparation of the dilutions, correct use of the appropriate PPE and CPE based on the VITO procedures, waste removal, registration of the instruments)	14/08/2014 27/10/2014	03/11/2014
03/11/2014, 05/11/2014	Inspection of first final test (test system, preparation of the dilutions, manual registration of the fronds, correct use of the appropriate PPE and CPE based on the VITO procedures, waste removal, registration of the instruments)	13/11/2014	
27/11/2014 09/12/2014	Draft report Final report	27/11/2014 09/12/2014	

We declare that the report completely and accurately describes the used materials and methods and that the results and conclusions accurately reflect the raw data that were obtained during the study.

Date: 12/12/2014

Signature

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SUMMARY

The aim of this test was to evaluate the effect of the commercial test substance PMV-01 on the growth of the duckweed species *Lemna minor* during 7 days of exposure. The test was performed according to the protocol described in OECD 221 (2006).

Test quality: GLP

Test item: PMV-01 (test substance 2014 TOX 026) [REDACTED]
Pepino mosaic virus, CH2 strain, isolate 1906

Test protocol: OECD 221 (2006)

Test code: LEM 14 003

Test organisms: *Lemna minor*

Test concentrations: The test substance PMV-01 is soluble in water. In the preliminary test the test substance was added directly to Steinberg medium at a concentration of 1000 mg/l. A 1/10 dilution series of this stock solution was tested (1000 – 100 – 10 – 1 – 0.1 mg/l). No negative effects were seen on the growth parameters in this preliminary test.
In the final test a limit test set up was used with 1000 mg/l PMV-01.

Medium: Steinberg mineral medium (OECD guideline 221).

Analyses: the test substance [REDACTED] *Pepino mosaic virus*, CH2 strain, isolate 1906. The viral load was measured using TaqMan RT-qPCR. Samples were taken for analyses of the viral load at the start and at the end of the final test.

Test conditions: incubator (vessels randomly placed), 25.1-25.3°C, continuous light (±4200-4390 lux)

Test vessels: glass vessels 250 ml with translucent lid

Test volumes: 100 ml controls/test solutions

Number of organisms: 12 fronds/test vessel at the start

Set up: 6 replicates
Measurement of number of fronds (at day 2, 5 and 7), wet weight and dry weight (at day 7).
Calculation of yield and specific growth.

Dates: 29/10-5/11/2014 final test

Results:

Test validation: All validity criteria were met.

Results: Results showed that the viral load was below detection limit at the nominal test concentration of 1g/l PMV-01.
Growth rate was not inhibited in the nominal concentration range up to 1000 mg/l PMV-01.

1. PURPOSE OF THE STUDY

The aim of this test was to evaluate the effect of PMV-01 on the growth of the aquatic higher plant species *Lemna minor* during 7 days of exposure.

The method is based upon OECD guideline for testing of chemicals 221 (2006) and described in VITO SOP TLEME001v1.

2. TEST SUBSTANCE

2.1. Identification

VITO code	Sponsor code
2014 TOX 026	PMV-01

10.1.c Wob
juncto 63.2.d
Vo 1107/2009

TEST ITEM INFORMATION


Name:	PMV-01 [REDACTED] <i>Pepino mosaic virus</i> , CH2 strain, isolate 1906)
Batch number:	[REDACTED]
Expiring date:	[REDACTED]
Storage conditions:	Frozen (-20°C ± 2)
Purity:	na; Active ingredient [REDACTED]
Formula:	Active ingredient: <i>Pepino mosaic virus</i> , CH2 strain, isolate 1906
Molecular weight:	na
Appearance:	Green solution with particles
CAS:	na
Solubility in water:	soluble
Stability:	7 d at 20°C 38 d at 4°C
H codes:	Not classified, no additional information on adverse effects available
Methodology for concentration analysis:	TaqMan RT-qPCR

10.1.c Wob juncto
63.2.a Vo
1107/2009 juncto
93.2.a Vo 178/2002

10.1.c Wob
juncto 63.2.d
Vo 1107/2009

na = not applicable

Certificate of Analysis: annex 1.

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2.2. Registration

All handling of the pure product was registered on forms BTEST001-Frm1v02 and Frm3v03. Forms will be kept in BIO1 - 321 and later in the archives.

3. TEST FACILITY

The test was performed at VITO ABS – Industriezone Vlasmeer 7, B2400 MOL Belgium - BIOL – ecotox facility (BIOL 0273 - 0277- 0202) and BIOL 0377 – 0365 - 0384 (analyses).

4. REFERENCE SUBSTANCE

No reference substance was used in the test. A reference substance (3,5-DCP) was tested to assess the sensitivity of the plant culture to toxic substances.

Results of three tests on 3,5-DCP in our lab, using the same batch of plants that is used for the present study, are shown in table 1. The reported range for EC₅₀ (frond number, yield) for this test species is 1.8-3.6 mg/l 3,5-DCP (ISO 20079). The values measured in our lab are within this range.

Table 1: results on the EC₅₀ value for the number of fronds (yield) for the batch LEM 2014/2

test	EC ₅₀ (frond number, yield) (mg/l 3,5-DCP)
LEM 14 006	1.88
LEM 14 007	1.99
LEM 14 008	1.98


5. ANALYTICAL METHODS

5.1. Standard measurements

pH meter: BIO 1334 (WTW portable)

conductivity: BIO 1539

pH and conductivity were measured using standard laboratory equipment and standard calibration and verification procedures as described in the respective Standard Operating Procedures.

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5.2. Analyses

TaqMan RT-PCR (two-step) procedure for detection of *Pepino mosaic virus* in tomato leaf samples using Gutiérrez-Aguirre primers and probes. The method is described in annex 4.

The analytical protocol was provided by Scientia Terrae and a preliminary validation study was performed where the same samples (n=5) were measured in both the Scientia Terrae lab and the VITO facilities. The results of both labs were completely comparable. Further tests were performed at the VITO test facility.

6. TEST PERFORMANCE

6.1. Test system

6.1.1. Justification of the test system

The duckweed growth inhibition test is a widely accepted standardized test to evaluate the toxicity of products in water for aquatic higher plants (OECD 221, ISO 20079). *Lemna minor* is one of the species recommended by the authorities.

6.1.2. Characterization of the test system

NAME : *Lemna minor* L.,St

Batch LEM 2014/2, delivered by Friedrich-Schiller-Universität Jena with certificate (ordered from: Federal Environment Agency (UBA) via lemna@uba.de).

Lab keeping and breeding: SOP TLEMV001v1.

6.2. Test description

6.2.1. Date of performance

LEM 14 003

Exposure: 21-28/10/2014 (preliminary) and 29/10-5/11/2014 (final)

6.2.2. Principles of the test method (OECD 221)

Exponentially growing plant cultures of the genus *Lemna* are allowed to grow as monocultures in different concentrations of the test substance over a period of seven days. The objective is to quantify substance-related effects on vegetative growth over this period based on assessments of selected variables: frond number (primary measurement variable), wet weight and dry weight. Growth in the test solutions is compared to growth in the controls and the concentration bringing about 50 % inhibition is determined when possible.


The response is evaluated as a function of the nominal concentration of test substance (mg/l).

6.2.3. Method of administration

The test solutions are administered as surrounding water. Exposure route is uptake in the roots and leaves.

6.2.4. Test concentrations

250 ml aliquots of the same test substance batch were kept frozen until use. For the preliminary test the aliquot 2014 TOX 026/2 was used, and aliquot 2014 TOX 026/4 was used for the final test.

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A preliminary range finding test was set up. 1g of the test substance was added to 1 liter of mineral medium (Steinberg medium (StB): see below for composition) and a 1/10 dilution series was used in the test (1000 – 100 – 10 – 1 – 0.1 mg/l). No adverse effects were seen on the growth parameters in this concentration range.

For the final test (LEM 14 003 final) again 1g of the test substance was added to 1 liter of mineral medium (StB medium) and a limit test was set up with 1000 mg/l PMV-01. Samples were taken at the start (from the test solution) and at the end of the test (from different replicates) for analyses of the viral load. The samples were frozen immediately until analyses.

6.2.5. Test conditions (final test)

Set up:

control (StB medium)- 6 replicates: to establish the control growth parameters, needed for test validation (see validity criteria) and as a reference.

Test dilutions - 6 replicates: to establish the inhibiting effect of different concentrations of the test substance when compared to controls.

Fronid number at the start: 2-5 frond colonies are used as test organisms. The total number of fronds is 12 at the start of the test.

Test vessels: glass vessels 250 ml, previously washed in a standard laboratory dishwasher. During the test the vessels were covered with translucent lids.


Test volumes: 100 ml of each dilution or medium (control)

Medium: Freshly prepared StB Medium (SOP TLEMV001v1) was used to provide the nutrients and minerals that are needed for unrestricted algal growth. The composition of the medium is listed in table 2.

Table 2: Composition of the StB medium

compound	mg/L
KNO ₃	350.00
Ca(NO ₃) ₂ · 4H ₂ O	295.00
KH ₂ PO ₄	90.00
K ₂ HPO ₄	12.60
MgSO ₄ · 7H ₂ O	100.00
	µg/L
H ₃ BO ₃	120.00
ZnSO ₄ · 7H ₂ O	180.00
Na ₂ MoO ₄ · 2H ₂ O	44.00
MnCl ₂ · 4H ₂ O	180.00
FeCl ₃ · 6H ₂ O	760.00
EDTA di-sodium-dihydrate	500.00

Conditions: test vessels were placed in incubator BIO 0658 at random places, 25.1-25.3 °C, continuous light (4200-4390 lux).

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6.2.6. Assessments, measurements and analyses (final test)

The number of fronds was counted at day 2, 5 and 7. After 7 days the test was stopped and the wet weight and dry weight of the biomass per vessel was measured.

pH was measured in each dilution at the beginning and in each vessel at the end of the test (see annex 2: raw data). pH was approximately 5.8 at the start of the test in both conditions. This is within the acceptance range. pH increased during the test to 7.66-7.77 in controls.

Samples for analyses were taken at the start of the experiment from control and test solution and from 1 control vessel and 4 replicates of the test solution at the end of the test.

Wet weight was measured for each replicate. After counting the number of fronds the plants were collected per test vessel, dried by absorbing paper and deposited in previously weighed vessels. The vessels were weighed again and the net weight was used as wet weight per replicate. The vessels were then placed in an oven at 60°C during at least 5 hours and weighed again. The net weight was used as dry weight per replicate.

6.2.7. Calculations

The yield and average specific growth rate were calculated as described in OECD 221 for frond number, dry weight (DW) and wet weight (WW). Student t-test was used to evaluate statistic relevant differences when compared to controls ($p < 0.05$).

6.3. Deviations

6.3.1. Deviations from the study protocol

Based on the results of the preliminary test - where no effects on the growth parameters were seen - it was decided to use a limit test set up for the final test. As recommended by the OECD guideline 221 for limit tests the number of replicates was doubled.

6.3.2. Deviations from OECD guideline 221

The light intensity in the incubator used for the current tests is lower than recommended in the guideline (4500 instead of 6500-10000 lux). As the growth parameters were in line with the validation criteria, this was not considered as a confounding factor.

Also in literature test protocols for *Lemna minor* are described where 4000-45000 lux (Moody & Miller, 2005)¹ and 3600-4400 lux (Nautilus Environmental, 2011)² are used, indicating that these conditions are indeed suitable for *Lemna*.

6.4. Standard operating procedures concerning the test and test organisms

- TLEME001v1 (growth inhibition test on algae)
- TLEMV001v2 (culture and medium)
- TTOES062v3 (pH measurement)
- TTOES055v3 (Milli Q)

¹ "Lemna minor growth inhibition test" in "Small scale Freshwater Toxicity Investigations", pp 271-298. Ed Springer.

² "Toxicity Testing on Synthetic Effluent Samples", Nautilus Environmental, 2011, WO#11064-67

- TTOES057v2 (luxmeter)
- analytical protocol (Scientia Terrae)

7. RESULTS

7.1. pH

pH was measured at the start and at the end of the test (annex 2A - raw data).
pH was not a confounding factor.

7.2. Viral load

The viral load was measured but was below detection limit at all test concentrations. Raw data are presented in annex 3. Therefore results are expressed in nominal concentration of PMV-01 (mg/l).

7.3. Test results

Raw data: in annex 2A.

Calculated values: in annex 2B.

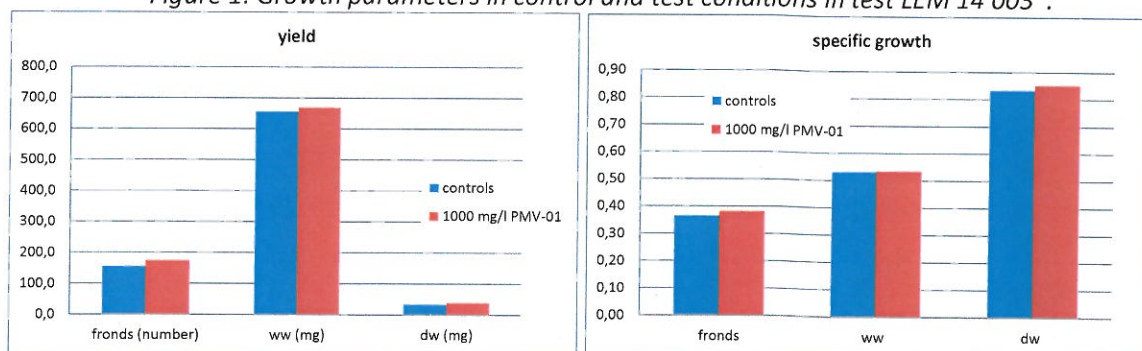
7.3.1. Validity of the test (annex 2A)

In controls at least a seven fold increase of the frond number after 7 days should be seen.
This was indeed the case: during the test LEM 14 003 the frond number increased 14x. The test results are accepted and reported here.

7.3.2. Growth inhibition

Figure 1 shows the growth parameters measured in control and test condition after an exposure period of 7 days in the final test. There was no significant effect on either of the measured end points for specific growth or yield.

Figure 1: Growth parameters in control and test conditions in test LEM 14 003 .




7.4. Interpretation of the results

The test substance has no adverse effects on the Lemna growth parameters in the test range up to 1000 mg/l PMV-01 (nominal concentrations).

8. ARCHIVES

The next items will be kept in archives at the VITO ABS archives for at least 5 years after the issue of the final report.

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- *Dossier*: study protocol, draft versions of the study report, copies of the study report and attached documents, the original raw data,
- *General*: logbooks of the instruments.
- *Test substance*: product registration forms

The archive is located at VITO - BIO L 110 - Industriezone Vlasmeer 7 – B 2400 MOL (Belgium).

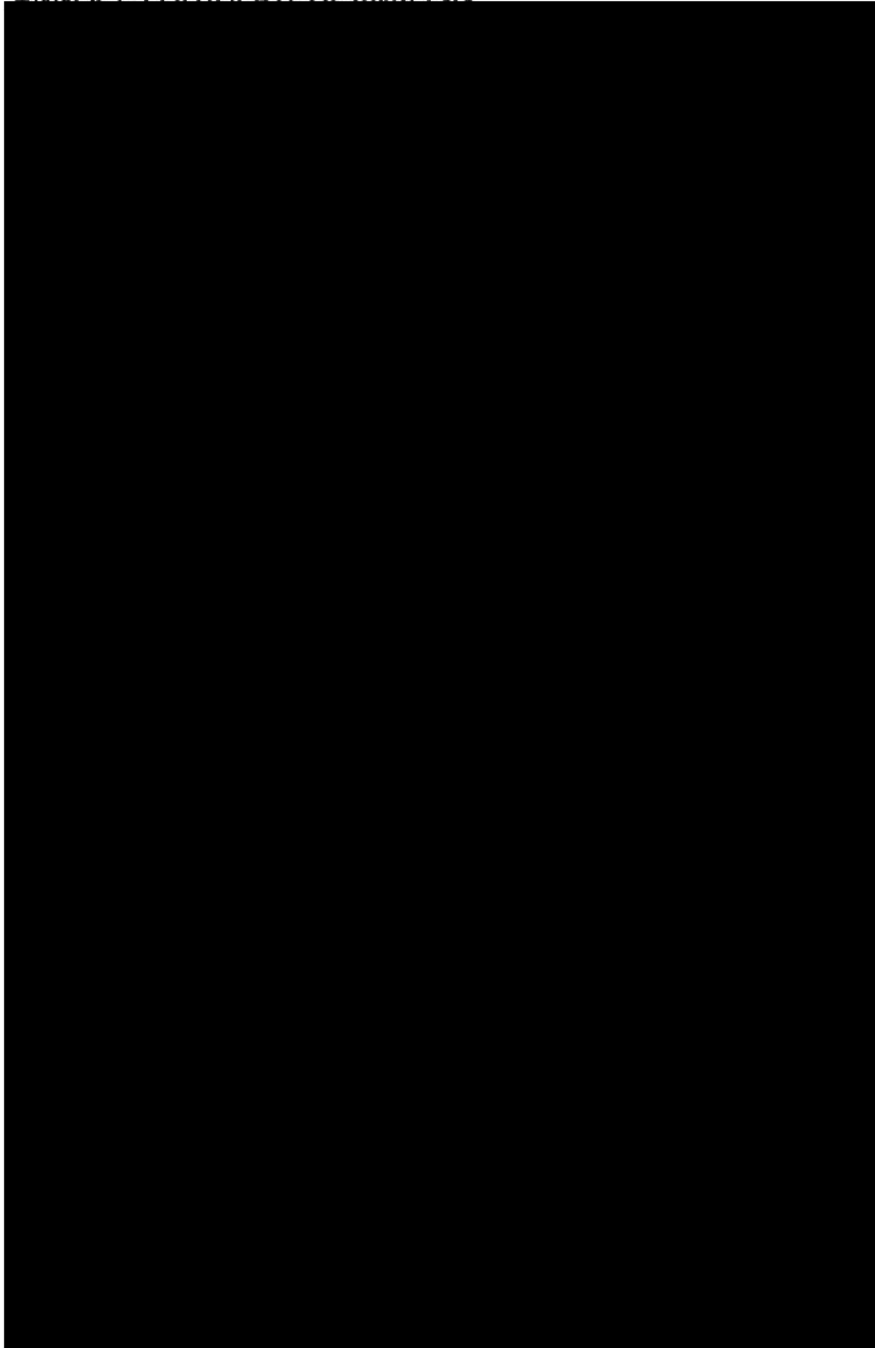
After the period of 5 years the sponsor will be contacted to decide on prolongation of retention, relocation or disposal.

9. PRINCIPAL INVESTIGATORS


Not applicable.

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ANNEX 1: CERTIFICATE OF ANALYSIS



10.1.c Wob juncto
63.2.a en d Vo
1107/2009 juncto
39.2.a Vo 278/2002

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ANNEX 2A: RAW DATA (FINAL TEST)

Testcode **LEM14003 final** test substance **2014TOX026/4** **PMV-01**

Start	rec	empty (g)	wet(g)	dry (g)	WW (mg)	DW (mg)
	1	9,6482	9,6528	9,6239	4,6	-24,3
	2	9,3038	9,3278	9,3009	24	-2,9
	3	9,638	9,6398	9,6237	1,8	-14,3
	4	9,3079	9,3384	9,3233	30,5	15,4
	5	9,6303	9,6483	9,6256	18	-4,7
				gem	15,8	0,1

fronds	rec	conc mg/l	29/okt day 0	31/okt 3	3/nov 6	5/nov day 7		pH day 7
	1	0	12	28	98	185		7,77
	2	0	12	25	90	176	controle	7,66
	3	0	12	24	87	176		7,68
	4	0	12	26	82	146		7,66
	5	0	12	27	79	153		7,69
	6	0	12	27	93	166		7,66
	7	1000	12	28	81	174		7,72
	8	1000	12	25	75	189		7,75
	9	1000	13	27	69	156		7,74
	10	1000	12	26	86	186		7,76
	11	1000	12	29	76	202		7,73
	12	1000	12	28	87	217		7,75

weight day7	rec	conc mg/l	empty (g)	wet (g)	dry (g)	WW (mg)	mean WW	DW (mg)	mean DW
	1	0	9,6105	10,2942	9,6488	683,7	672,4	38,3	34,60
	2	0	9,3001	10,0143	9,3348	714,2		34,7	
	3	0	9,3175	9,9714	9,3506	653,9		33,1	
	4	0	9,2951	9,8831	9,326	588,0		30,9	
	5	0	9,6364	10,2642	9,6669	627,8		30,5	
	6	0	9,2988	10,0653	9,3389	766,5		40,1	
	7	1000	9,6117	10,2297	9,6477	618,0	684,3	36,0	39,42
	8	1000	9,3282	10,028	9,367	699,8		38,8	
	9	1000	9,6527	10,3114	9,6908	658,7		38,1	
	10	1000	9,3037	10,0162	9,3459	712,5		42,2	
	11	1000	9,6177	10,2152	9,6539	597,5		36,2	
	12	1000	9,6162	10,4355	9,6614	819,3		45,2	

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ANNEX 2B: CALCULATED VALUES

Testcode LEM14003 final test subst 2014TOX026/4

yield


rec	conc mg/l	fronds	WW mg	DW mg		mean			inhibition (% vs control)		
						fronds (n)	ww (mg)	dw (mg)	fronds	WW	DW
1	0	173	667,9	38,2	mean	155,0	656,6	34,5	0,00	0,00	0,00
2	0	164	698,4	34,6	SD	15,0	63,5	3,9			
3	0	164	638,1	33	VC	9,7	9,7	11,4			
4	0	134	572,2	30,8							
5	0	141	612,0	30,4							
6	0	154	750,7	40							
7	1000	162	602,2	35,9	mean	175,2	668,5	39,3	-13,01	-1,82	-13,96
8	1000	177	684,0	38,7	SD	21,6	79,8	3,6			
9	1000	143	642,9	38	VC	12,3	11,9	9,2			
10	1000	174	696,7	42,1							
11	1000	190	581,7	36,1							
12	1000	205	803,5	45,1							
ttest						0,09	0,78	0,05			

Testcode LEM14003 final test subst 2014TOX026/4

specific growth

	fronds	WW (mg)	DW (mg)
dag 0	12	15,78	0,1

rec	conc mg/l	fronds	WW mg	DW mg	spec growth				mean specific growth				inhibition (% van control)			
					fronds	ww	dw		fronds	ww	dw		fronds	WW	DW	
1	0	173	667,9	38,2	0,38	0,54	0,85	mean	0,36	0,53	0,83		0,00	0,00	0,00	
2	0	164	698,4	34,6	0,37	0,54	0,84	SD	0,01	0,01	0,02					
3	0	164	638,1	33	0,37	0,53	0,83	VC	3,86	2,59	1,92					
4	0	134	572,2	30,8	0,34	0,51	0,82									
5	0	141	612,0	30,4	0,35	0,52	0,82									
6	0	154	750,7	40	0,36	0,55	0,86									
7	1000	162	602,2	35,9	0,37	0,52	0,84	mean	0,38	0,53	0,85		-4,69	-0,43	-2,27	
8	1000	177	684,0	38,7	0,38	0,54	0,85	SD	0,02	0,02	0,01					
9	1000	143	642,9	38	0,35	0,53	0,85	VC	4,69	3,11	1,51					
10	1000	174	696,7	42,1	0,38	0,54	0,86									
11	1000	190	581,7	36,1	0,39	0,52	0,84									
12	1000	205	803,5	45,1	0,41	0,56	0,87									
ttest									0,10	0,80	0,05					

	<p align="center">Study Report GLP study Environment assessment study Duckweed: Growth inhibition test <i>Lemna minor</i></p>	<p>LEM 14 003 Page 17 of 19 Date: 12-12-2014 9:30 print</p>
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ANNEX 3: RAW DATA TAQMAN RT-PCR

Experiment: 141113_PepMV_RW Selected Filter: FAM (465-510)

Include	Color	Pos	Name	Cp	Status
TRUE	255	B1	LEM Start 1000	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	B2	LEM Start 1000	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	B3	LEM Start 1000	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	65280	C1	LEM FIN Co		
TRUE	65280	C2	LEM FIN Co		
TRUE	255	C3	LEM FIN Co	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	D1	LEM FIN 1000 1	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	D2	LEM FIN 1000 1	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	65280	D3	LEM FIN 1000 1		
TRUE	255	E1	LEM FIN 1000 2	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	E2	LEM FIN 1000 2	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	65280	E3	LEM FIN 1000 2		
TRUE	255	F1	LEM FIN 1000 3	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	F2	LEM FIN 1000 3	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	F3	LEM FIN 1000 3	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	F7	Pos Co	26.05	
TRUE	255	F8	Pos Co	25.98	
TRUE	255	F9	Pos Co	25.90	
TRUE	255	G1	LEM FIN 1000 4	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	G2	LEM FIN 1000 4	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	255	G3	LEM FIN 1000 4	35.00	> - Late Cp call (last five cycles) has higher uncertainty
TRUE	65280	G7	NTC		
TRUE	65280	G8	NTC		
TRUE	65280	G9	NTC		


LEM Start 1000: test solution at the start of the experiment

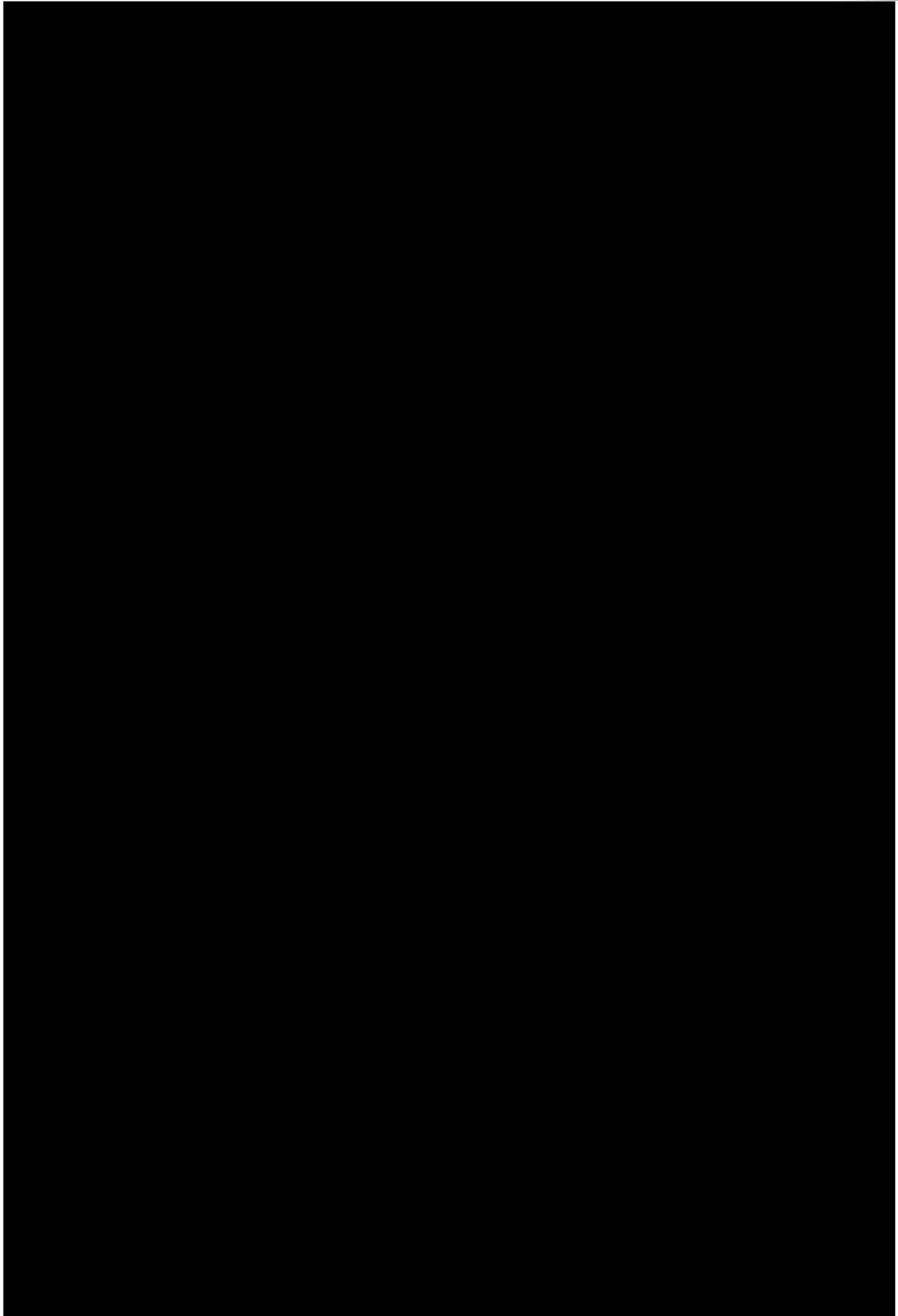
LEM FIN 1000 x: test solution at the end of the experiment from replicate x


LEM FIN Co: control test solution at the end of the experiment

Pos Co: positive control

NTC: negative control

 <p>vito value in technology</p>	<p>Study Report GLP study Environment assessment study Duckweed: Growth inhibition test <i>Lemna minor</i></p>	<p>LEM 14 003 Page 18 of 19 Date: 12-12-2014 9:30 print</p>
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