

To:

Eurofins - Dermatest Pty Ltd

***GROWTH INHIBITION OF ALGAE POPULATION WITH A
SAMPLE REFERENCED AS:***

« FSI - ATTACK »

Test report n° 20FYBA1156 – 2020/12/03

This report only concerns the goods submitted to the test. This document's reproduction is permitted only in the form of a full photographic facsimile. This report contains 11 pages.

TABLE

I. REPORT OBJECT	3
II. SAMPLE PRESENTATION	3
I. SIMPLIFIED DESCRIPTION OF THE BIOLOGICAL TEST	3
I.1 TOXICOLOGICAL DESCRIPTORS AND DEFINITIONS.....	3
I.2 REFERENCES	3
I.3 FRESH WATER ALGAL GROWTH INHIBITION TEST (<i>PSEUDOKIRCHNERIELLA SUBCAPITATA</i> , FORMERLY <i>SELENASTRUM CAPRICORNUTUM</i> , NF EN ISO 8692, 2012 – “VESSELS” METHOD).....	4
I.4 TEST ORGANISM AND ENVIRONMENTAL CONDITIONS	4
I.4.1 <i>Unicellular green algae</i>	4
I.4.2 <i>Culture conditions</i>	4
I.4.3 <i>Test conditions</i>	5
I.4.4 <i>Test procedure:</i>	5
I.4.1 <i>Physicochemical measurements</i>	6
I.4.2 <i>Reading</i>	6
I.4.3 <i>Reference substance</i>	6
I.5 DATA PROCESSING.....	6
I.5.1 <i>Comparison of the growth rates</i>	6
I.5.2 <i>EC_{rx} and NOEC calculation</i>	6
II. TEST RESULTS.....	7
II.1 PRELIMINARY TEST – LIMIT TEST.....	7
II.2 FULL TEST - TOXICOLOGICAL DESCRIPTORS RESULTS	7
III. TEST VALIDITY CRITERIA	7

Appendix 1 - Test medium composition.

Appendix 2 - Full test raw data.

I. REPORT OBJECT

Client details:

Name: Eurofins - Dermatest Pty Ltd.

Address: 20 - 22 King St — Rockdale NSW 2216 Australia.

This report gives results obtained on a sample received the 2020/10/29 for the realization of ecotoxicological assay.

II. SAMPLE PRESENTATION

Client sample reference: FSI - Attack

Reception date: 2020/10/29.

Batch number: unknown.

Conservation temperature: Ambient temperature.

EUROFINS Ecotoxicologie France reference: 20G009448-001.

I. SIMPLIFIED DESCRIPTION OF THE BIOLOGICAL TEST

I.1 Toxicological descriptors and definitions

- **NOEC**: “No Observed Effect Concentration”; the highest tested concentration without any effect on test organisms, compared to the controls.

- **EC_x**: effective concentration resulting in X per cent reduction in the growth rate of the population compared to control.

The lower the EC_x, the higher the toxicity.

Growth: increase in cell concentration over the test period.

Growth rate: logarithmic increase in algal cell density per unit of time.

I.2 References

- OECD Guideline for the testing of chemicals – OECD 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.
- French standard NF EN ISO 8692, 2012 – annex A- “Fresh water algal growth inhibition test with unicellular green algae”.

NB : OECD 201 and NF EN ISO 8692 are equivalent methods.

I.3 Fresh water algal growth inhibition test (*Pseudokirchneriella subcapitata*, formerly *Selenastrum capricornutum*, NF EN ISO 8692, 2012 – “vessels” method)

The purpose of this test is to determine the effects of a substance on the growth of a unicellular green algal species after 72hrs of exposure without changing the medium (static assay).

Exponentially-growing cultures of *Pseudokirchneriella subcapitata* are exposed to various concentrations of the test substance and to a control medium. After 72hrs, the cell concentrations are determined in each concentration by microscopy (Malassez counting chamber). The growth rate inhibition percentage is then calculated with comparison to the control.

The test proceeds in two stages:

- 1) A screening test (« range-finding ») to target the concentrations that inhibit, if possible, between 5% and 75% of the algae growth rate,
- 2) A full test to determine, if possible, the following concentrations: EC50-72hrs, EC20-72hrs, EC10-72hrs and NOEC expressed in mg/L of the test substance.

NB: During the screening test, for the concentration corresponding to 100mg/L, 6 vessels are tested in order to consider this condition as a limit-test. If the sample reveals no toxicity at 100mg/L (after statistical analysis using Bonferroni t-test), the sample is considered non-toxic.

I.4 Test organism and environmental conditions

I.4.1 Unicellular green algae

- Species: *Pseudokirchneriella subcapitata* (*Selenastrum capricornutum*)
- Batch: CCAP278/2
- Origin: SAMS Research Services Ltd, Scotland.

I.4.2 Culture conditions

Culture

Culture medium: LC-Oligo medium (pH 7.1 ± 0.1)
Temperature: 21-24°C
Illumination: 16hrs/8hrs day and night cycle; intensity of about 4500 lux.
Ventilation: bubbling

Pre-culture

A pre-culture incubated for about 3 days in test conditions (exponential growing phase) is used for the test substances inoculation.
Medium used for pre-culture: test medium.

Test duration: 72 hrs

Test solution replacement: none (static mode)

Test medium: composition is given in appendix 1

Temperature: 21-24°C; test performed in a temperature controlled chamber; a maximum of $\pm 2^\circ\text{C}$ variation is allowed with reference to the chamber temperature at the beginning of the test.

Illumination: constant, daylight type (400-700 nm), about 7500 Lux (55 $\mu\text{E}/\text{m}^2/\text{s}$)

Ventilation: none,

Initial cellular concentration: about 10^4 cells per mL from the pre-culture

Vessel and test volume: glass vessels, 20ml per vessel

I.4.4 Test procedure:

Sample preparation: a 100mg/L solution (prepared in test medium) was agitated for 24 hours in a closed flask, on a magnetic stirrer (200 rpm) at $20^\circ\text{C} \pm 2^\circ\text{C}$.

➤ Screening test :

Number of replicates « Control test »	Nominal concentrations (mg test item/L)	Number of replicates « Substance test »
6	100mg/L, 32mg/L, 10mg/L, 3.2mg/L, 1mg/L	1*

*Excepted for 100mg/L, which is tested in 6 replicates to be used as a limit test.

➤ Full test :

For the full test, concentrations are chosen basing on the results obtained with the screening test. The full test comprises at least five concentrations part of a geometric series and separated from each other by a factor comprised between 1,3 et 2,2.

The concentration rate must in preference comprise concentration values leading to, at least, a 5 to 75% inhibition of the algae growth rate.

Number of replicates « Control test »	Concentrations tested	Number of replicates « Substance test »
6	Minimum 5	3

The algae are added in the wells respecting the initial cellular concentration of 10^4 cells per mL.

pH is measured at the beginning and at the end of the test in the different tested concentrations.

During the full test temperature is measured continuously in the chamber.

I.4.2 Reading

After 72hrs +/-2hrs, the cell concentrations are determined in each concentration by microscopic examination (Malassez counting chamber).

I.4.3 Reference substance

Potassium dichromate ($K_2Cr_2O_7$): Fluka, batch n°SZBG0050V (expiry date: 20th December 2018).

I.5 Data processing

I.5.1 Comparison of the growth rates

After cellular density measurement (number of cells/mL) in the test and control cultures at 72h, the specific growth rate (μ) is calculated as follow

$$\mu = \frac{\ln N_n - \ln N_o}{t_n - t_o}$$

Where:

N_n = number of cells/mL at t_n (72 hrs),

N_o = number of cells/mL at t_o .

The percent of specific growth inhibition ($I_{\mu t}$) for each concentration is calculated as follow:

$$I_{\mu t} = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

Where:

μ_c = mean growth rate in controls,

μ_t = mean growth rate for a test concentration at time t.

I.5.2 EC_x and NOEC calculation

Determination of the EC_x: logistic model based on Hill equation (Regtox_ev6.6.2.xls macro). NOEC is determined by statistic modelling using the Bonferroni's t test (Toxcalc software).

II. TEST RESULTS

II.1 Preliminary test – Limit test

Inhibition growth rate measured during the screening test:

- 100 mg/L: 0%
- 32 mg/L: 0%
- 10 mg/L: 0%
- 3.2 mg/L: 0%
- 1 mg/L: 0%

Concentrations tested in the full test: 100 mg/L (limit test).

II.2 Full test - Toxicological descriptors results

The raw results are annexed (appendix 2).

Test	Effect	Toxicological descriptors	FSI - ATTACK
Algae	Growth rate	ECr 50-72h	>100 mg/L
		ECr 20-72h	>100 mg/L
		ECr 10-72h	>100 mg/L
		NOEC-72h	≥100 mg/L

Results in mg/L of FSI - ATTACK for the growth inhibition test

In brackets: 95% confidence limits of ECx (if estimable)

III. TEST VALIDITY CRITERIA

- In the control, the mean growth rate at 72hrs is above 1.4 day⁻¹ (1.83 day⁻¹).
- In the control, the variation coefficient is below 5 % (1.59 %).
- In the control, the variation of pH from 0 to 72hrs is below 1.5 (+ 0.5 pH unit).
- Reference substance realized from 26th and 29th September 2020: (K₂Cr₂O₇) EC50r-72hrs = 1.26 mg/L (value in accordance with results formerly obtained at the laboratory and comprised between 0.92 mg/L et 1.46 mg/L – acceptable range of sensibility for *P. subcapitata* as stated in standard ISO 8692:2012).

The test is thus valid.

In Maxéville, the 2020/11/19

Yves Barthel, Responsable of ecotoxicology Unit



APPENDIX 1

TEST MEDIUM COMPOSITION

Stock solutions:
Stock solution 1: macroelements

Consumables	Amount/liter
NH ₄ Cl	1.5g
MgCl ₂ ,6H ₂ O	1.2g
MgSO ₄ ,7H ₂ O	1.5g
CaCl ₂ ,2H ₂ O	1.8g
KH ₂ PO ₄	0.16g

Stock solution 2: Fe-EDTA

FeCl ₃ ,6H ₂ O	0,064 g
Na ₂ EDTA,2H ₂ O	0,100 g

Stock solution 3: trace elements

H ₃ BO ₃	0.185g
MnCl ₂ ,4H ₂ O	0.415g
ZnCl ₂	0.003g
CoCl ₂ ,6H ₂ O	0.0015g
CuCl ₂ ,2H ₂ O	0.00001g
Na ₂ MoO ₄ ,2H ₂ O	0.007g

Stock solution 4: NaHCO₃

NaHCO ₃	50g
--------------------	-----

Reagents must be of analytical grade. The stock solutions are sterilized by retorting (125°C +/- 5°C – 15 min) immediately after preparing, excepting stock solution 4, sterilized by filtration on a 0.2µm porosity membrane.

Conservation: 12 months at 4°C ± 3°C.

Test medium

For 1000ml of test medium, proceed as follow:

Ad to about 800ml of ultrapure water:

10 ml of stock solution 1

1 ml of stock solution 2

1 ml of stock solution 3

1 ml of stock solution 4.

Complete to 1000ml with ultrapure water.

Aerate the medium during about 30 minutes.

Final pH: 8.1 ± 0.2

APPENDIX 2

FULL TEST RAW DATA

Raw data sheet: Growth rate inhibition of *Pseudokirchneriella subcapitata* - NF EN ISO 8692 (2012) Annexe A

Sample: 20G009448-001

Start of test: 03/11/20

Reading at t+: 3 days

density at t0: 0.01

End of test: 06/11/20

Concentrations mg/L	Cellular concentration $\times 10^6$						Mean number of cells	Cellular inhibition
	1	2	3	4	5	6		
0	2.5	2.56	2.56	2.48	2.34	2.04	2.41	
100	1.96	2.5	2.58	2.3	2.48	2.3	2.35	2%

Concentrations mg/L	Growth rate						Mean growth rate	Inhibition	SD	Variation coefficient (%)
	1	2	3	4	5	6				
0	1.84	1.85	1.85	1.84	1.82	1.77	1.83		0.029	1.59
100	1.76	1.84	1.85	1.81	1.84	1.81	1.82	0%	0.033	1.82

Phytoplankton Test-Growth-Biomass						
Start Date:	Test ID: 9448-001		Sample ID:			
End Date:	Lab ID:		Sample Type:			
Sample Date:	Protocol:		Test Species: SC-Selenastrum capricornutum			
Comments:						
Conc-mg/L	1	2	3	4	5	6
B-Control	1.8405	1.8484	1.8484	1.8378	1.8184	1.7727
100	1.7594	1.8405	1.8510	1.8127	1.8378	1.8127

Conc-mg/L	Mean	N-Mean	Transform: Untransformed				N	t-Stat	1-Tailed	
			Mean	Min	Max	CV%			Critical	MSD
B-Control	1.8277	1.0000	1.8277	1.7727	1.8484	1.592	6	0.484	1.812	0.0326
100	1.8190	0.9952	1.8190	1.7594	1.8510	1.819	6			

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Shapiro-Wilk's Test indicates normal distribution ($p > 0.01$)	0.82157	0.805	-1.2888	0.74228						
F-Test indicates equal variances ($p = 0.78$)	1.29339	14.9396								
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test	100	>100			0.0326	0.01784	0.00023	0.00097	0.6391	1, 10

Dose-Response Plot

