

To:

Eurofins - Dermatest Pty Ltd

***DAPHNIDS ACUTE IMMOBILISATION TEST WITH A
SAMPLE REFERENCED AS:***

« FSI-DEFEND »

Test report n° 20FER6-1074 V2– 2020/11/27

NB: This report cancels and replaces the report 20FER6-1074 – 2020/11/19.

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 13 pages.

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Appendix 1 - Test medium composition.

Appendix 2 - Full test raw data.

Modification: replacement of the sample reference throughout the report.

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 2020/09/17 for the realization of ecotoxicological assay.

II. SAMPLE PRESENTATION

Client sample reference: FSI-Defend

Reception date: 2020/09/17.

Batch number: unknown.

Conservation temperature: Ambient temperature.

EUROFINS Ecotoxicologie France reference: 20G008382-002.

I. SIMPLIFIED DESCRIPTION OF THE BIOLOGICAL TEST

I.1 Toxicological descriptors

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

- ECx: effective concentration resulting in a X per cent reduction in the mobility compared to control.

The lower the ECx, the higher the toxicity.

I.2 References

- Daphnids (test species: *Daphnia magna* Straus), acute immobilisation assay after 48 hours (OECD 202 – April 2004).
- French standard NF EN ISO 6341, 2012- “Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)”.

NB: OECD 202 and NF EN ISO 6341 are equivalent methods.

I.3 *Daphnia magna*, acute immobilisation test (NF EN ISO 6341, 2012)

This guideline describes an acute toxicity test to assess the substance concentration which causes the immobilisation of 50% of Daphnids in 48h. This concentration is called EC50-48h.

Young daphnids, aged less than 24 hours at the start of the test, are exposed to a concentration range of the test substance, and to the test medium (control) for a period of 48 hours. Animals unable to swim within 15 seconds, after a gentle shaking of the test vessel are considered to be immobilised (even if they can still move their antennae).

Immobilisation is recorded at 24 hours and 48 hours and compared to the control values. The results are analysed in order to calculate the EC50 at 48h.

The test proceeds in two stages:

- 1) A screening test (« range-finding ») to target the concentrations that inhibit, if possible, between 0 % and 100 % of daphnids mobility
- 2) A full test to determine the following concentrations: EC100, EC50, EC20, EC10 and EC0 expressed in mg/L of the test substance.

I.4 Test organism and environmental conditions

I.4.1 Daphnids

- Species: *Daphnia magna*
- Batch: *Daphnia magna* Straus clone 5
- Origin: Breeding from Eurofins laboratory

1.4.2 Test conditions

Test duration:	48 hrs
Test solution replacement:	none (static mode)
Test medium:	synthetic medium; the characteristics are defined in standard NF EN ISO 6341 (composition in appendix 1)
Temperature:	18-22°C; thermostatically controlled enclosure; maximum variation of $\pm 1^\circ\text{C}$ compared to the temperature at the beginning of the test
Illumination:	none
Ventilation:	none,
Age of the test organisms:	≤ 24 hrs at the beginning of the test and issued from at least third brood progeny
Acclimatisation:	neonates' ≤ 24 hrs
Vessel and test volume:	10 mL gauged test tubes
Number of animals per concentration:	
- Screening test:	10 daphnia divided into 2 replicates of 5 organisms
- Full test:	20 daphnia divided into 4 replicates of 5 organisms

1.4.3 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 »	Concentration in % of the stock solution (100 mg/L)	Number of replicates « Substance test »
2	100 %, 35 %, 10 %, 3.5 %, 1 %,	2*

*Excepted for 100 mg/L, which is tested in 4 replicates to be used as a limit test.

➤ Full test :

For the full test, concentrations are chosen among those obtained with the screening test. The 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.

Number of replicates « Control test »	Concentrations tested	Number of replicates « Substance test »
4	Minimum 5	4

The daphnids are added to the test solution by mean of a Pasteur pipet and distributed from the lowest to the highest concentration.

1.4.4 Physicochemical measurements

pH and dissolved oxygen (in mg/L) are measured at the beginning and at the end of the full test in the different tested concentrations.

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

1.4.5 Reading

After 24 hours \pm 30 minutes and 48 hours \pm 30 minutes, the daphnia still mobile are counted for each tested concentration.

Apart from mobility, any abnormal sign or behaviour must be recorded.

1.4.6 Reference substance

Potassium dichromate (K₂Cr₂O₇).

1.5 Data processing

1.5.1 Immobilisation percentage calculation

For a given concentration, the immobilisation percentage P is calculated as follow:

$$P = 100 - 5(n_1 + n_2 + n_3 + n_4)$$

where n₁, n₂, n₃ and n₄ are the numbers of mobile daphnia at the end of the full test in each of the 4 test tubes corresponding to a given concentration.

1.5.2 ECx calculation

EC₅₀, EC₂₀ and EC₁₀ (and their 95% confidence limit) are calculated by mean of a Log-Probit statistical model (Toxcalc software).

EC₁₀₀ is considered as equal to the lowest tested concentration causing a 100% immobilisation.

EC₀ is considered as equal to the highest concentration causing no immobilisation.

II. TEST VALIDITY CRITERIA

- In the control, not more than 10 per cent of the daphnia should have been immobilised after 48hrs (0%).
- The dissolved oxygen concentration at the end of the test should be superior or equal to 2 mg/l (see appendix 2).
- Reference substance: ($K_2Cr_2O_7$) EC50-24hrs = 0.96 mg/L (value in compliance with results formerly obtained at the laboratory and comprised between 0.60 mg/L and 2.1 mg/L – acceptable range of sensibility for Daphnia as stated in standard NF EN ISO 6341: 2012).

III. BIOLOGICAL TEST RESULTS

III.1 Preliminary test

Mobility inhibition rate measured during the 48h screening test (unit in % of the 100 mg/L stock solution):

- 100% : 0 %
- 35% : 0 %
- 10% : 0 %
- 3.5% : 0%
- 1% : 0%

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

III.2 Full test

The raw results are annexed (appendix 2).

Test	Effect	Toxicological descriptors	FSI-Defend
Daphnids	Immobilization	EC 100-24h	>100 mg/L
		EC 50-24h	>100 mg/L
		EC 20-24h	>100 mg/L
		EC 10-24h	>100 mg/L
		EC 0-24h	100 mg/L
Daphnids	Immobilization	EC 100-48h	>100 mg/L
		EC 50-48h	>100 mg/L
		EC 20-48h	>100 mg/L
		EC 10-48h	>100 mg/L
		EC 0-48h	100 mg/L

Results in mg/L of FSI-Defend for the immobilization test

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

In Maxéville, the 2020/11/27
 Eloïse Renouf, Ecotoxicology Group Leader



APPENDIX 1

TEST MEDIUM COMPOSITION

TEST MEDIUM PREPARATION

	Amounts for 10 L
Sodium hydrogen carbonate, NaHCO ₃	2.00 g ± 0.1 g
Calcium chloride, CaCl ₂ , 2H ₂ O	2.60 g ± 0.1 g
Magnesium chloride, MgCl ₂ , 6H ₂ O	1.48 g ± 0.1 g
Potassium sulfate, K ₂ SO ₄	0.26 g ± 0.01 g
Ultra-pure water <i>quantum satis</i>	10 Litres

Ventilate for at least 12 hours

The test medium is kept for maximum 30 days at room temperature

The water is analysed (pH, hardness, Ca, Mg). Characteristics must be as follow:

- pH 7.8 ± 0.5
- total hardness of 225 mg/L ± 50 mg (CaCO₃)
- $\frac{Ca}{Mg} = \frac{4}{1} \pm 0.5$
- Dissolved [O₂] > 7 mg/L (control at each use)

APPENDIX 2

FULL TEST RAW DATA

Daphnia magna immobilisation test

(NF EN ISO 6341-2012)

Sample : 20G008382-002
(FSI-Defend)

pH : 7.8

Organisms obtained from laboratory culture, aged under 24h at the beginning of the test. Initial number of organisms per vessel : 5.

I. PRELIMINARY TEST

Start of analysis : 5th October 2020 12h00

Counting : 7th October 2020 12h00

Concentrations mg/L	100 x	35	10	3.5	1	0.35	0.1				
I	5	5	5	5	5						
II	5	5	5	5	5						
III	5										
IV	5										

II. FULL TEST - 24H and 48H RESULTS

Start of analysis : 5th October 2020 12h00

Counting : 7th October 2020 12h00

Concentrations mg/L	100											Control
I	5											5
II	5											5
III	5											5
IV	5											5
Total living organisms	20											20
Immobilization %	0%											0%

Minimal concentration inducing 100 % of immobilisation : > 100 mg/L

Maximal concentration inducing 0 % of immobilisation : 100 mg/L

EC 50-48h : >100 mg/L (confidence range 95 % : mg/L - mg/L) - (evaluated by statistical model Log-Probit).

