

ECOTOXILAB

ECOTOXICITY TESTS

- **Freshwater Alga and Cyanobacteria, Growth Inhibition Test: OECD 201**

RESULTS REPORT

REPORT No 1

(Toxicity analysis according to the ecotoxicity standards for aquatic organisms **OECD 201** and OECD 202; and on terrestrial organisms OECD 207 and OECD 208. Tests carried out with the sample):

-Sample 1: Ecofire forest ("water-based organic filler solution")

Client name: **SIMONRACK**

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1. - FRESHWATER ALGA AND CYANOBACTERIA, GROWTH INHIBITION TEST (OECD 201)

1.1- SAMPLE

- Sample 1: Ecofire forest

Sample characteristics: wáter-based organic filter solution (data provided by the client).

1.2- OBJECTIVE OF THE STUDY

Establish the risk of toxicity related to the commercial product called ECOFIRE Forest on freshwater green algae cultures *Desmodesmus subspicatus*, through the Cell Growth Inhibition Test included in the OECD Guide No. 201.

1.3- SPECIES USED IN THE TEST

The degree of toxicity exhibited by the commercial product ECOFIRE Forest has been studied, following the 201 OECD guideline for the testing of chemical products on the growth of freshwater microalgae. The *Desmodesmus subspicatus* strain (BEA 0141/1; Banco Español de Algas, Gran Canaria, Spain) was selected to carry out the exposure tests to the product.

1.4- DESCRIPTION OF THE TEST

An initial concentration of 5×10^3 cel mL⁻¹ were exposed to the product on BBM culture medium (Bold's Basal Medium), with an initial volume of 50 mL in 100 mL Erlenmeyer flasks. The culture was kept under continuous movement (150 rpm), constant light conditions ($70 \mu\text{E m}^{-2} \text{s}^{-1}$) and a constant temperature of 21 ± 1 °C. The initial pH of the medium was 7.1, and its control during the exposure time showed that there were no deviations greater than 0.5 in any of the Erlenmeyer flasks. To prevent a possible shading effect by the product in the medium, light absorption measurements (OD627 and OD720) were performed, without obtaining values greater than 0.009 in any step. Biomass measurement was performed by in vivo assessment of chlorophyll fluorescence,

1.5- RESULTS

Preliminary tests to establish the range of concentrations determined the **absence of toxic effects at concentrations of 100 mg L⁻¹**, so a "Limit Test" was carried out as described in the 201 OECD guideline. For this, a negative control assay and 6 replicates treated with a concentration of 100 mg L⁻¹ were subjected to the growth inhibition test.

The general statistical analysis was performed using an Excel spreadsheet. The ANOVA analysis of variance was obtained through the GraphPad Prism v8 software package (GraphPad Software Inc., USA).

The CFU values obtained after the chlorophyll fluorescence measurements, both in the control test and in the 6 treated samples are summarized in appendix 1. The corresponding cell growth rates (μ) during the test time for the control test and the 6 treated are presented in appendix 2. The corresponding performance of both the control trial and the 6 replicates, as well as the estimation of the different variances and their homogeneity are included in appendix 3.

The results obtained through the "Limit Test", exposing the cultures to a concentration of 100 mg L⁻¹ of the commercial product ECOFIRE forest, indicate that this concentration does not present significant adverse effects on the cell growth of the *Desmodesmus subspicatus* strain.. The analysis of variance applied to the treated samples indicates a high degree of homogeneity ($p=0.9679$) among them, and no percentage of inhibition with respect to the control exceeded 10%.

Therefore, it is NOT APPROPRIATE to continue with the search for EC10 or EC50 type response variables.

Dr. Technician

ECOTOXILAB

Sgd. Gerardo Mengs González

TIME 72 h

GENios; Serial number: 12900400638; Firmware: V 4.62 - 07/01 GENios; XFLUOR4 Version: V 4.50																					
Date:																					8/6/23
Time:																					10:10
Measurement mode:																					Fluorescence Top
Excitation wavelength:																					485 nm
Emission wavelength:																					670 nm
Gain:																					75
Number of flashes:																					3
Lag time:																					0 µs
Integration time:																					40 µs
Plate definition file:																					GRE96fb.pdf
Shake duration (Orbital Normal):																					5 s
Rawdata																					Temperature: 26.3 °C
<>	1	2	BG-11	CONTROL	S1	S2	S3	S4	S5	S6	11	12									
A	56	46	69	73	71	56	54	56	70	85	65	66									
B	56	49	113	17505	16505	17505	15233	16505	17505	17505	78	59									
C	50	72	177	16188	16188	16188	16188	17188	16188	17188	64	65									
D	44	49	188	18963	16963	15963	16963	16963	15963	16863	71	71									
E	38	26	147	16553	16553	16553	17553	16553	16553	17553	50	75									
F	95	49	169	16255	16255	16255	14255	17255	16255	16255	61	79									
G	84	84	155	17405	16405	15405	16405	15405	17005	16405	41	64									
H	49	49	42	54	57	42	49	46	91	55	56	66									
			158,17	17144,83	16311,50	16311,50	16099,50	16644,83	16578,17	16961,50											

APPENDIX 2

Cell growth ratio exhibited by the control trial and the 6 treated at 24 hour intervals up to the final 72 hour exposure time, and throughout the exposure interval. The values represent the logarithmic increase in biomass, obtained from the equation:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \text{ (day}^{-1}\text{)}$$

where:

- μ_{i-j} is the average specific cell growth rate in the ij time interval;
- X_i is the biomass at time i;
- X_j is the biomass at time j.

Time interval	Growth rate (μ)						
	Control	S1	S2	S3	S4	S5	S6
0h vs 24h	1.13	1.12	1.12	1.11	1.12	1.10	1.11
24h vs 48h	1.13	1.12	1.12	1.10	1.14	1.13	1.12
48h vs 72h	1.12	1.12	1.11	1.12	1.11	1.12	1.14
0h vs 72h	1.13	1.12	1.11	1.11	1.12	1.11	1.12

Percentage inhibition of the cell growth rate ($\% I_t$) with respect to the control:

	S1	S2	S3	S4	S5	S6
$\% I_t$	0,97	1,23	1,35	0,52	1,25	0,42

APPENDIX 3

Percentage inhibition of the yield rate (% I_y) with respect to the control.

	S1	S2	S3	S4	S5	S6
% I_y	4,86	4,86	6,10	2,92	3,31	1,07

And analysis of variance homogeneity between the 6 results obtained for the trials treated.

Ordinary one-way ANOVA					
Table Analyzed	homogeneidad de varianza				
Data sets analyzed	A-E				
ANOVA summary					
F	0.1351				
P value	0.9679				
P value summary	ns				
Significant diff. among means (P < 0.05)?	No				
R square	0.02116				
Brown-Forsythe test					
F (DFn, DFd)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
Bartlett's test					
Bartlett's statistic (corrected)	5.023				
P value	0.2849				
P value summary	ns				
Are SDs significantly different (P < 0.05)?	No				
ANOVA table					
	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	11.64	4	2.909	F (4, 25) = 0.1351	P=0.9679
Residual (within columns)	538.3	25	21.53		
Total	549.9	29			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	30				

1.6- COMMENT ON RESULTS AND INCIDENTS

The commercial product called **ECOFIRE forest** is outside the classification criteria for **Acute Aquatic Toxicity of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS)**, which establishes its lowest category (Category Acute 3 for algae) between 10 and 100 mg L⁻¹. The same criteria is applied from Regulation (EC) No. 1272/2008 of the European Parliament and of the Council, of December 16, 2008, on the classification, labeling and packaging of substances and mixtures, commonly known as the CLP Regulation.

Test based on the OECD 201 standard. During the test there were no notable technical incidents.