

## ECOTOXICITY TESTS

- **Terrestrial Plant Test: Seedling Emergence and Seedling Growth Tests (OECD 208)**

## RESULTS REPORT

### REPORT No. 4

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

*Applicant: Ignacio Ruiz Ezquerra*

**ECOTOXILAB SL**

*Dr. Technician: Gerardo Mengs*

*June-28<sup>th</sup>, 2023*

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### 3. - TERRESTRIAL PLANT TEST: SEEDLING EMERGENCE AND SEEDLING GROWTH TESTS (*Pisum Sativum* sp.) OECD 208

#### 3.1- TEST SAMPLE

- Sample 1: Ecofire forest

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

#### 3.1 –TEST PLANT SPECIES

Details of the test species: Ervilha Lincoln pea (low bush variety) (*Pisum sativum*)

Class: Dicots / Magnoliopsida Order: Fabales / Fabales Family: Fabaceae / Fabaceae Genus: *Pisum* / *Pisum*

Supplier: Batlle®

Reference: 020101G250

Batch: 002/LI130

Valid until: December/2026

Germination test: 87.5% (07 May 2023)

Storage conditions: 20 ± 5°C, darkness, RH: 60-70%

#### 3.2-TEST CONDITIONS

- According to OECD Standard 208- in a heated greenhouse, 22 ± 2°C, relative humidity 70 ± 20%, photoperiod 16 hours, test duration 18 days. For each study concentration, 4 replicates with 8 seeds each have been made.
- Test carried out in 16 cm<sup>2</sup> pots with a content of 800 g of substrate. Standard substrate: universal substrate (dry weight): composition: blonde sphagnum peat, vegetable substrate, coconut fiber, pine bark, perlite and nutrients (140-250 mg/l (N), 100-160 mg/l (P<sub>2</sub>O<sub>5</sub>), 30-150 mg/l (K<sub>2</sub>O), 120-200 mg/l (MgO), pH 6-6.5, EC 1 mS/cm Supplier: Fertiberia®.
- The tests have been carried out by uniformly mixing the sample substrate with the standard substrate in each of the tested concentrations. Water has been added until reaching a % humidity of 70 ± 20%.
- In the negative and positive controls (boric acid 2400 mg/kg), the same number of replicates as the test replicates has been carried out.
- The different concentrations of the tested sample have been the following:  
6.4g/kg, 14g/kg, 31g/kg, 68g/kg, 150g/kg and 330g/kg. (factor = 2.2)
- Indicators:
  - % germination inhibition (% of non-germinated seeds)
  - Biomass (fresh weight of the seedling)
  - Seedling length (aerial part)
  - root length

### 3.3.RESULTS

The tests have been carried out under the indicated conditions, proceeding after 18 days to the collection of the plants and obtaining data for their subsequent analysis. The averages and their standard deviation are given below.

Concentration- g/kg	% non-germinated seeds	Biomass (g) (fresh weight %)	Seedling H (mm)	Root R (mm)
Check+	100	-	-	-
Control -	12.50 ± 10.21 SD	1.03 ± 0.07 SD (100.00%)	H 102.96 ± 06.31 SD	R 94.57 ± 04.59 SD
6.4g/kg	9.37 ± 11.97 SD	1.05 ± 0.07 SD (102.10%)	H 103.76 ± 05.60 SD	R 95.03 ± 06.22 SD
14g/kg	9.37 ± 11.97 SD	1.05 ± 0.06 SD (101.63%)	H 104.25 ± 05.45 SD	R 93.89 ± 05.56 SD
31g/kg	15.62 ± 6.25 SD	1.05 ± 0.07 SD (101.70%)	H 102.48 ± 05.21 SD	R 94.04 ± 05.36 SD
68g/kg	31.25 ± 7.22 SD	0.97 ± 0.06 SD (94.10%)	H 94.91 ± 05.47 SD	R 88.54 ± 04.19 SD
150g/kg	100	-	-	-
330g/kg	100	-	-	-

#### 3.3.1-Germination inhibition (%)

The influence exerted by different concentrations of the test sample on the germination and early development of the seedlings is evaluated. The test sample is mixed with the standard substrate in a uniform manner, in 6 different increasing concentrations. The germination percentages obtained in each replicate are shown below:

ENSAYO	[MUESTRA 1] g/kg	Réplica 1 (%)	Réplica 2 (%)	Réplica 3(%)	Réplica 4(%)
Control +	0	0	0	0	0
Control -	0	87,5	100	75	87,5
C1	6.4	100	100	87,5	75
C2	14.0	100	100	75	87,5
C3	31.0	87,5	87,5	75	87,5
C4	68.0	62,5	75	62,5	75
C5	150.0	0	0	0	0
C6	330.0	0	0	0	0

*% of seeds germinated in each replicate of each concentration tested*

*Indicators:*

**IC 50= 78.33 g/kg**

**NOEC = 31.00 g/kg**

**LOEC = 68.00 g/kg**

(IC50, EC50; concentration that produces a 50% reduction in seed germination)

(NOEC; the highest concentration tested at which no effect was observed)

(LOEC; the lowest concentration tested at which an effect has been observed)

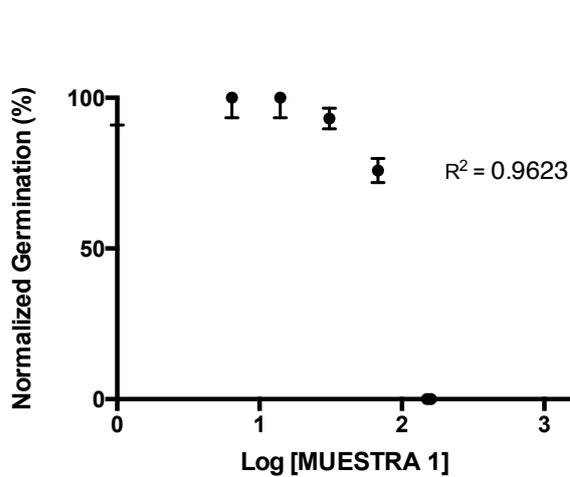
#### Soil ecotoxicological assessment

*Reference:OECD 208 (Plant Seed Emergence and Growth Test).*

<sup>1</sup>**Value greater than 10 mg/g (10 g/kg)**(RD 9/2005 of January 14). Ecotoxicological assessment in contaminated soils.

Royal Decree 9/2005, of January 14, which establishes the list of potentially soil-contaminating activities and the criteria and standards for the declaration of contaminated soils. ANNEX III-Criteria for considering a soil as contaminated. 2. *In those cases in which the protection of ecosystems is considered a priority: -a) That the average lethal or effective concentration, IC(E)50, for soil organisms obtained in toxicity tests..., OECD 208 (Test of emergence and growth of seeds in terrestrial plants)...., is less than 10 mg of contaminated soil/g of soil.*

*IC50 calculation: Statistical analysis performed with the GraphPad Prisma 8.0 Software, by logarithmic transformation  $X = \log(X)$  and data normalization:*



log(inhibitor) vs. normalized response -- Variable slope

**Best-fit values**

LogIC50 1,894  
 HillSlope -8,078  
 IC50 78,33

**Std. Error**

LogIC50 0,06833  
 HillSlope 8,878

**95% Confidence Intervals**

LogIC50 1,753 to 2,034  
 HillSlope -26,33 to 10,18  
 IC50 56,69 to 108,3

**Goodness of Fit**

Degrees of Freedom 26  
 R square 0,9623  
 Absolute Sum of Squares 1996  
 Sy.x 8,762

Number of points

Analyzed 28

*Calculation of NOEC and LOEC: Statistical analysis performed with the GraphPad Prisma 8.0 Software, using "Repeated measures one-way ANOVA" and Sidak's multiple comparison test:*

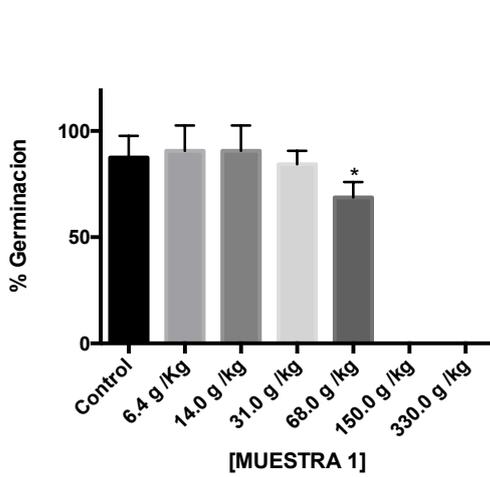


Table Analyzed

Repeated measures one-way ANOVA data

Repeated measures ANOVA summary

Assume sphericity? No  
 F 148,6  
 P value < 0,0001  
 P value summary \*\*\*\*  
 Statistically significant (P < 0.05)? Yes  
 Geisser-Greenhouse's epsilon 0,3766  
 R square 0,9802

Was the matching effective?

F 4,224  
 P value 0,02  
 P value summary \*  
 Is there significant matching (P < 0.05)? Yes  
 R square 0,01374

ANOVA table

	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	42009	6	7001	F (2,260, 6,779) = 148,6	P < 0,0001
Individual (between rows)	597,1	3	199	F (3, 18) = 4,224	P = 0,0200
Residual (random)	848,2	18	47,12		
Total	43454	27			

Data summary

Number of treatments (columns) 7  
 Number of subjects (rows) 4

Number of families 1  
 Number of comparisons per family 21  
 Alpha 0,05

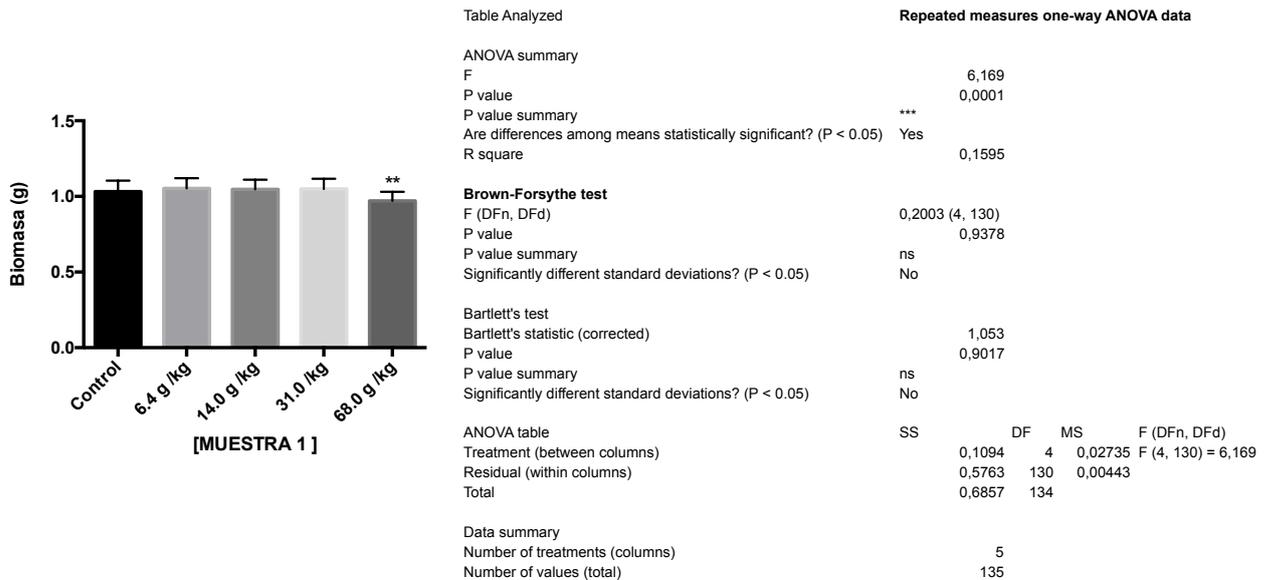
Sidak's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Control vs. 6.4 g /Kg	-3,125	-60,24 to 53,99	No	ns
Control vs. 14.0 g /kg	-3,125	-32,95 to 26,70	No	ns
Control vs. 31.0 g /kg	3,125	-26,70 to 32,95	No	ns
Control vs. 68.0 g /kg	18,75	-15,69 to 53,19	No	ns
Control vs. 150.0 g /kg	87,5	38,79 to 136,2	Yes	**
Control vs. 330.0 g /kg	87,5	38,79 to 136,2	Yes	**
6.4 g /Kg vs. 14.0 g /kg	0	-48,71 to 48,71	No	ns
6.4 g /Kg vs. 31.0 g /kg	6,25	-53,40 to 65,90	No	ns
6.4 g /Kg vs. 68.0 g /kg	21,88	-53,19 to 96,94	No	ns
6.4 g /Kg vs. 150.0 g /kg	90,63	33,51 to 147,7	Yes	*
6.4 g /Kg vs. 330.0 g /kg	90,63	33,51 to 147,7	Yes	*
14.0 g /kg vs. 31.0 g /kg	6,25	-28,19 to 40,69	No	ns
14.0 g /kg vs. 68.0 g /kg	21,88	-35,24 to 78,99	No	ns
14.0 g /kg vs. 150.0 g /kg	90,63	33,51 to 147,7	Yes	*
14.0 g /kg vs. 330.0 g /kg	90,63	33,51 to 147,7	Yes	*
31.0 g /kg vs. 68.0 g /kg	15,63	-14,20 to 45,45	No	ns
31.0 g /kg vs. 150.0 g /kg	84,38	54,55 to 114,2	Yes	**
31.0 g /kg vs. 330.0 g /kg	84,38	54,55 to 114,2	Yes	**
68.0 g /kg vs. 150.0 g /kg	68,75	34,31 to 103,2	Yes	**
68.0 g /kg vs. 330.0 g /kg	68,75	34,31 to 103,2	Yes	**
150.0 g /kg vs. 330.0 g /kg				

Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2	t	DF
Control vs. 6.4 g /Kg	87,5	90,63	-3,125	5,984	4	4	0,5222	3
Control vs. 14.0 g /kg	87,5	90,63	-3,125	3,125	4	4	1	3
Control vs. 31.0 g /kg	87,5	84,38	3,125	3,125	4	4	1	3
Control vs. 68.0 g /kg	87,5	68,75	18,75	3,608	4	4	5,196	3
Control vs. 150.0 g /kg	87,5	0	87,5	5,103	4	4	17,15	3
Control vs. 330.0 g /kg	87,5	0	87,5	5,103	4	4	17,15	3
6.4 g /Kg vs. 14.0 g /kg	90,63	90,63	0	5,103	4	4	0	3
6.4 g /Kg vs. 31.0 g /kg	90,63	84,38	6,25	6,25	4	4	1	3
6.4 g /Kg vs. 68.0 g /kg	90,63	68,75	21,88	7,864	4	4	2,782	3
6.4 g /Kg vs. 150.0 g /kg	90,63	0	90,63	5,984	4	4	15,14	3
6.4 g /Kg vs. 330.0 g /kg	90,63	0	90,63	5,984	4	4	15,14	3
14.0 g /kg vs. 31.0 g /kg	90,63	84,38	6,25	3,608	4	4	1,732	3
14.0 g /kg vs. 68.0 g /kg	90,63	68,75	21,88	5,984	4	4	3,656	3
14.0 g /kg vs. 150.0 g /kg	90,63	0	90,63	5,984	4	4	15,14	3
14.0 g /kg vs. 330.0 g /kg	90,63	0	90,63	5,984	4	4	15,14	3
31.0 g /kg vs. 68.0 g /kg	84,38	68,75	15,63	3,125	4	4	5	3
31.0 g /kg vs. 150.0 g /kg	84,38	0	84,38	3,125	4	4	27	3
31.0 g /kg vs. 330.0 g /kg	84,38	0	84,38	3,125	4	4	27	3
68.0 g /kg vs. 150.0 g /kg	68,75	0	68,75	3,608	4	4	19,05	3
68.0 g /kg vs. 330.0 g /kg	68,75	0	68,75	3,608	4	4	19,05	3

### 3.3.2-Variation of Biomass-fresh weight (g)

Evaluation of the effect of the test sample on the development of the seedling by quantifying the fresh weight of the biomass formed 18 days after sowing:

*Biomass variation:* Statistical analysis performed with the GraphPad Prisma 8.0 Software, using "Repeated measures one-way ANOVA" (Brown-Forsythe test and Bartlett's test) and Dunnett's multiple comparison test.



#### Ordinary one- way ANOVA Multiple comparisons

Number of families	1
Number of comparisons per family	4
Alpha	0,05

#### Dunnett's multiple comparisons test

Mean Diff,	95% CI of diff,	Significant?	Summary
Control vs. 6.4 g /kg	-0,02166 -0,06528 to 0,02195	No	ns
Control vs. 14.0 g /kg	-0,01511 -0,05873 to 0,02850	No	ns
Control vs. 31.0 /kg	-0,0171 -0,06150 to 0,02730	No	ns
Control vs. 68.0 g /kg	0,06088 0,01398 to 0,1078	Yes	**

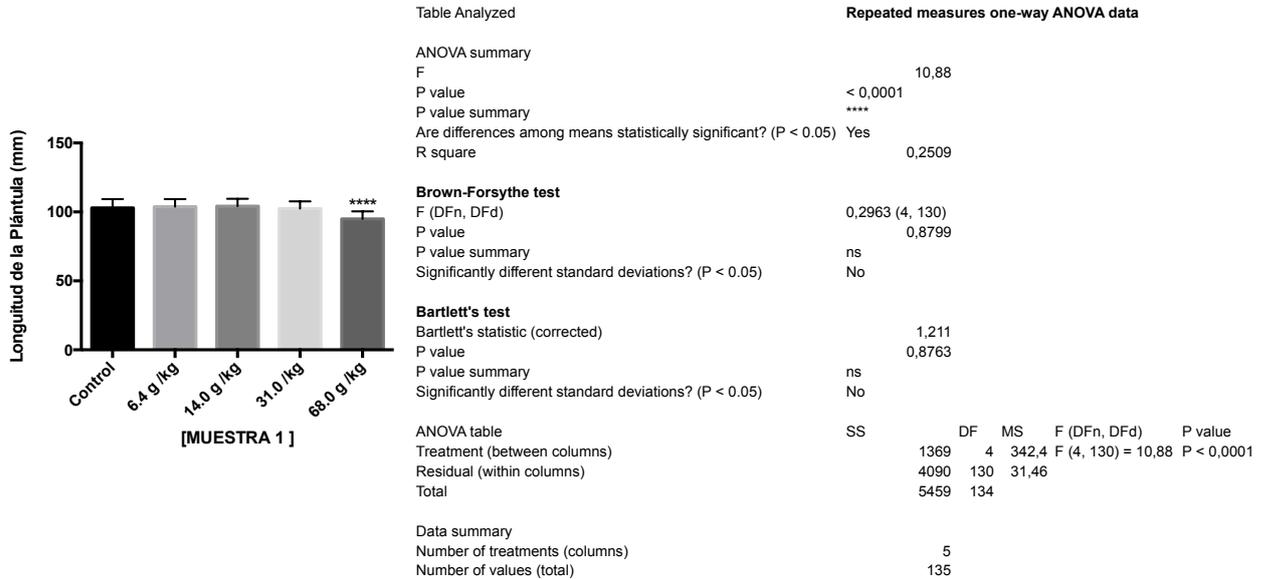
#### Test details

Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2	q	DF
Control vs. 6.4 g /kg	1,032	1,053	-0,02166	0,01764	28	29	1,228 130
Control vs. 14.0 g /kg	1,032	1,047	-0,01511	0,01764	28	29	0,8566 130
Control vs. 31.0 /kg	1,032	1,049	-0,0171	0,01796	28	27	0,9523 130
Control vs. 68.0 g /kg	1,032	0,9709	0,06088	0,01897	28	22	3,209 130

### 3.3.3-Variation in the length of the seedling (mm)

Evaluation of the effect of the test sample on the development of the seedling by quantifying the length of the aerial part formed 18 days after sowing:

*Seedling length variation:* Statistical analysis performed with the GraphPad Prisma 8.0 Software, using "Repeated measures one-way ANOVA" (Brown-Forsythe test and Bartlett's test) and Dunnett's multiple comparison test.



#### ORDINARY ONE-WAY ANOVA-Multiple comparisons

Number of families	1
Number of comparisons per family	4
Alpha	0,05

#### Dunnett's multiple comparisons test

	Mean Diff,	95% CI of diff,	Significant?	Summary
Control vs. 6.4 g /kg	-0,7943	-4,468 to 2,880	No	ns
Control vs. 14.0 g /kg	-1,243	-4,917 to 2,431	No	ns
Control vs. 31.0 /kg	0,4828	-3,257 to 4,223	No	ns
Control vs. 68.0 g /kg	8,055	4,105 to 12,01	Yes	****

#### Test details

	Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2	q	DF
Control vs. 6.4 g /kg	103	103,8	-0,7943	1,486	28	29	0,5345	130
Control vs. 14.0 g /kg	103	104,2	-1,243	1,486	28	29	0,8362	130
Control vs. 31.0 /kg	103	102,5	0,4828	1,513	28	27	0,3191	130
Control vs. 68.0 g /kg	103	94,91	8,055	1,598	28	22	5,041	130

### 3.3.4-Variation of root length (mm)

Evaluation of the effect of the test sample on seedling development by quantifying the length of the root formed 18 days after sowing:

*Root length variation: Statistical analysis performed with the GraphPad Prisma 8.0 Software, using "Repeated measures one-way ANOVA" (Brown-Forsythe test and Bartlett's test) and Dunnett's multiple comparison test.*

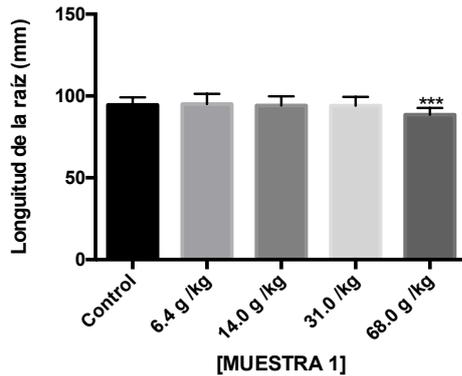


Table Analyzed		Repeated measures one-way ANOVA data				
ANOVA summary						
F						5,874
P value						0,0002
P value summary						***
Are differences among means statistically significant? (P < 0.05)		Yes				
R square						0,1531
<b>Brown-Forsythe test</b>						
F (DFn, DFd)						0,8750 (4, 130)
P value						0,4809
P value summary						ns
Significantly different standard deviations? (P < 0.05)		No				
<b>Bartlett's test</b>						
Bartlett's statistic (corrected)						4,723
P value						0,3169
P value summary						ns
Significantly different standard deviations? (P < 0.05)		No				
ANOVA table		SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)		660	4	165	F (4, 130) = 5,874	P = 0,0002
Residual (within columns)		3652	130	28,09		
Total		4312	134			
Data summary						
Number of treatments (columns)			5			
Number of values (total)			135			

#### ORDINARY ONE-WAY ANOVA-Multiple comparisons

Number of families	1
Number of comparisons per family	4
Alpha	0,05

#### Dunnett's multiple comparisons test

	Mean Diff,	95% CI of diff,	Significant?	Summary
Control vs. 6.4 g /kg	-0,4631	-3,935 to 3,009	No	ns
Control vs. 14.0 g /kg	0,4335	-3,038 to 3,905	No	ns
Control vs. 31.0 /kg	0,5344	-3,000 to 4,069	No	ns
Control vs. 68.0 g /kg	6,026	2,293 to 9,759	Yes	***

Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2	q	DF
Control vs. 6.4 g /kg	94,57	95,03	-0,4631	1,404	28	29	0,3298	130
Control vs. 14.0 g /kg	94,57	94,14	0,4335	1,404	28	29	0,3087	130
Control vs. 31.0 /kg	94,57	94,04	0,5344	1,43	28	27	0,3738	130
Control vs. 68.0 g /kg	94,57	88,55	6,026	1,51	28	22	3,991	130

### 3.4.-COMMENT ON RESULTS AND INCIDENTS

- Germination in the negative control trials was 87.50% (average of all replicates). The emerging plants did not show signs of phytotoxicity or any other visible symptoms of morphological or growth alteration. The mean survival of control plants during the trial was 100%. Seedling (H) and root (R) evolution parameters are considered normal.
- The standard substrate used in the control mixtures and in the different test concentrations was similar throughout the test. The environmental conditions were also similar in all replicates of the controls and of the different concentrations tested.
- Germination in the positive control trials was 0% in all replicates (boric acid 2400 mg/Kg).
- The germination inhibition of 50% of the seeds (IC50) has been produced in a concentration of 78.33 g/kg. A statistical study has been carried out using a regression equation on a dose-response curve by logarithmic transformation and data normalization. The goodness of fit is good ( $R^2=0.9623 \geq 0.7$ ), so we can conclude that there are significant differences in the increasing concentrations tested.
- The calculation of NOEC and LOEC has been estimated using the analysis of variances (Repeated measures ANOVA one-way) and the Sidak multiple comparison test. We can verify that the Sidak test confirms that the comparison between the different concentrations is statistically significant.
- During the germination and growth of the seedlings, no abnormality has been observed. After carrying out a statistical analysis of the data of the rest of the analyzed parameters of the seedlings, we can conclude that the biomass, the length of the seedling and the length of the root have varied significantly in the concentration of 68 g/kg and higher.
- In the emerging seedlings, no symptoms of chlorosis, discoloration, mortality, or any other effect that would affect the normal development of the seedling have been observed.

The commercial product called **ECOFIRE forest is outside the criteria and standards for the declaration of contaminated soils, as established in RD 9/2005 of January 14, on ecotoxicological assessment of contaminated soils**. In its ANNEX III-Criteria for considering a soil as contaminated, it is established that: In those cases in which the protection of ecosystems is considered a priority, the lethal or effective mean concentration, IC(E)50, for soil organisms obtained in the OECD 208 (Terrestrial Plant Seed Emergence and Growth Test) toxicity tests, it should be less than 10 mg/g soil (10g/kg); in our case it is higher as it is 78.33 g/kg.

Dr. Technician

**ECOTOXILAB**

Sgd. Gerardo Mengs González

