

## ECOTOXICITY TESTS

- **OECD 207 Earthworm Acute Toxicity Test**

## RESULTS REPORT

### REPORT No. 3

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")*

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## 4. EARTHWORM ACUTE TOXICITY TEST (Eisenia foetida sp.)-OECD 207

### 4.1- TEST SAMPLE

- Sample 1: Ecofire forest

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

### 4.2 –TEST SPECIES

Test species: Eisenia foetida (Michaelsen). Adults over 2 months of age with clitellum and weight 300-600 mg.

Breeding conditions: 20 ± 5°C, humidity: 40-60%, substrate: manure from different livestock species, moss and universal substrate for plants.

### 4.3- TEST CONDITIONS

- According to OECD Standard 207- Test duration: 14 days. Parameters: temperature: 20°C, humidity:40-60%,pH 6.0 ± 0.5, 600 lux continuously. For each study concentration, 4 replicates have been made with 10 worms each.
- Test carried out in 1060 ml containers with a content of 1 kg of substrate. Artificial substrate used as standard in the test: fine sand (content greater than 50% of particles between 50 and 200 microns) 70%, sphagnum moss 10%, kaolin 20%, CaCO<sub>3</sub> (pH = 6.0 ± 0.5).
- The tests have been carried out by uniformly mixing the sample with the standard substrate in each of the concentrations tested. Water has been added until reaching a % humidity of 40 -60%.
- In the negative and positive controls (cloracetamide 15 mg/kg), the same number of replicates as the assay 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)
- The different concentrations tested have been determined by a previous mortality study, in order to obtain greater precision for the calculation of LC(E) 50.
- Indicators:
  - *Mortality (%)*
  - *Decrease in live weight (LW) (%)*

#### 4.4- RESULTS

The tests have been carried out under the indicated conditions, proceeding after 7 days to the preliminary assessment of mortality. At 14 days the worms were collected, quantifying the mortality and the live weight of the survivors. Subsequently, the data analysis has been carried out.

Concentration-g/kg	% Mortality	% LW Variation
Control+	100 ± 0 SD	-
Control -	0 ± 0 SD	22.78 ± 3.94 SD
6.4g/kg	0 ± 0 SD	22.39 ± 3.62 SD
14g/kg	2.5 ± 5.00 SD	19.17 ± 9.38 SD
31g/kg	92.5 ± 6.45 SD (*)	-23.71 ± 1.96 SD (**)
68g/kg	100 ± 0 SD (*)	-
150g/kg	100 ± 0 SD (*)	-
330g/kg	100 ± 0 SD (*)	-

(\*) Mortality is considered significant when the value is greater than 5% compared to the mortality in the negative control.

(\*\*) The decrease in live weight is considered significant when the value is greater than 30% with respect to the variation in live weight in the negative control.

##### 4.4.1- Survival (%)

The influence exerted by different concentrations of the test sample, mixed in 6 different increasing concentrations with the standard substrate, is evaluated. The survival percentages obtained in each replicate are shown below:

ENSAYO	[MUESTRA 1] g/	Réplica 1 (%)	Réplica 2 (%)	Réplica 3(%)	Réplica 4(%)
Control +	0	0	0	0	0
Control -	0	100	100	100	100
C1	6.4	100	100	100	100
C2	14	100	90	100	100
C3	31	10	0	15	5
C4	68	0	0	0	0
C5	150	0	0	0	0
C6	330	0	0	0	0

*% of surviving worms in each replicate of each concentration tested*

*Indicators:*

**IC 50= 22.43g/kg<sup>1</sup>**

**NOEC = 14.00 g/kg**

**LOEC = 31.00 g/kg**

(IC50, EC50; concentration that produces 50% mortality in earthworms)

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

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

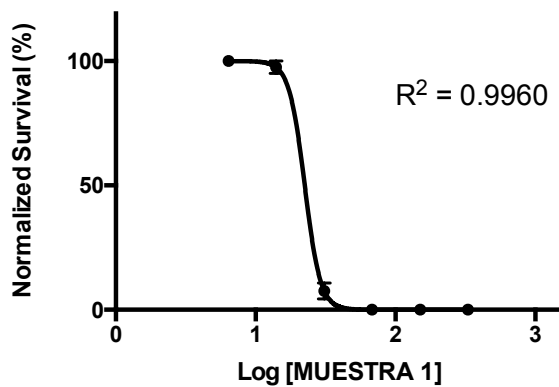
##### ***Soil ecotoxicological assessment***

*Reference:OECD 207 (Earthworm Acute Toxicity 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 207 (Test of acute toxicity in earthworms)...., 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  $X = \text{Log}(X)$  and data normalization:*



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

**Best-fit values**

LogIC50 1,351  
HillSlope -7,769  
IC50 22,43

**Std. Error**

LogIC50 0,0158  
HillSlope 0,8247

**95% Confidence Intervals**

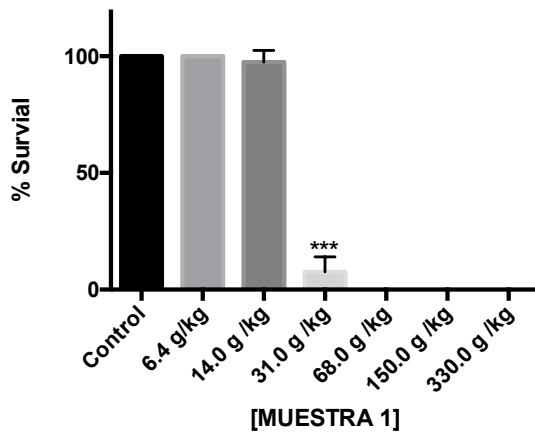
LogIC50 1,318 to 1,384  
HillSlope -9,480 to -6,059  
IC50 20,80 to 24,19

**Goodness of Fit**

Degrees of Freedom 22  
R square 0,996  
Absolute Sum of Squares 200  
Sy.x 3,015

Number of points Analyzed 24

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



**Repeated measures one-way ANOVA data**

Table Analyzed 12,5

Repeated measures ANOVA summary

Assume sphericity? No

F 1302

P value < 0,0001

P value summary \*\*\*\*

Statistically significant (P < 0.05)? Yes

Geisser-Greenhouse's epsilon 0,2188

R square 0,9977

Was the matching effective?

F 2

P value 0,1501

P value summary ns

Is there significant matching (P < 0.05)? No

R square 0,000766

ANOVA table

	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	65093	6	10849	F (1,313, 3,938) = 1302	P < 0,0001
Individual (between rows)	50	3	16,67	F (3, 18) = 2,000	P = 0,1501
Residual (random)	150	18	8,333		
Total	65293	27			

Data summary

Number of treatments (columns) 7

Number of subjects (rows) 4

Number of families 1  
Number of comparisons per family 6  
Alpha 0,05

**Bonferroni's multiple comparisons test**

Comparison	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. 6.4 g/kg				
Control vs. 14.0 g/kg	2,5	-13,08 to 18,08	No	ns
Control vs. 31.0 g/kg	92,5	72,39 to 112,6	Yes	***
Control vs. 68.0 g/kg				
Control vs. 150.0 g/kg				
Control 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								
Control vs. 14.0 g/kg	100	97,5	2,5	2,5	4	4	1	3
Control vs. 31.0 g/kg	100	7,5	92,5	3,227	4	4	28,66	3
Control vs. 68.0 g/kg								
Control vs. 150.0 g/kg								
Control vs. 330.0 g/kg								

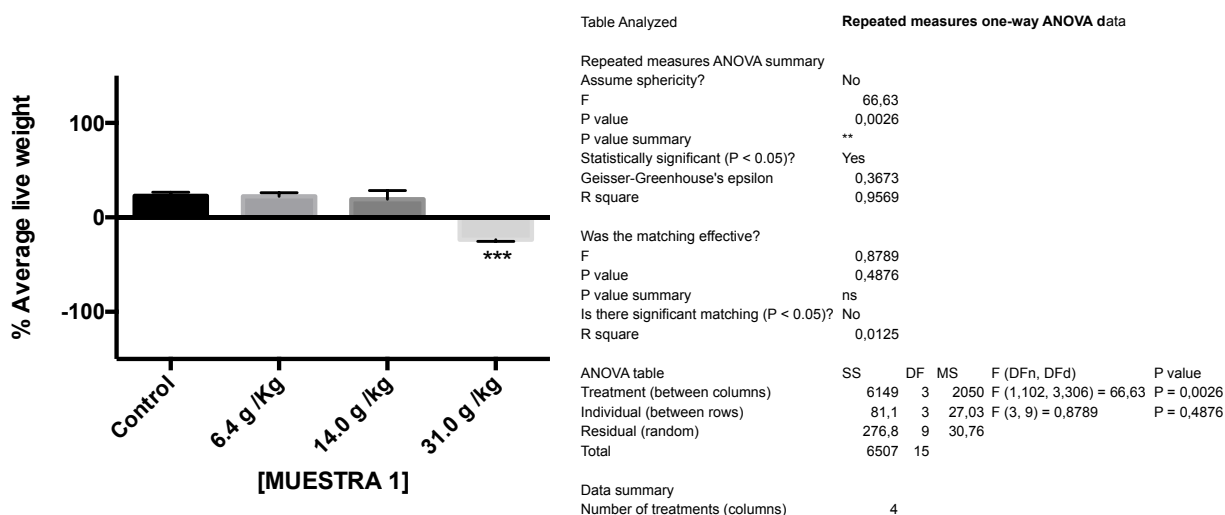
4.4.2-Variation of live weight (g)

Evaluation the effect of the test sample on the variation of the live weight of the surviving worms at 14 days. The percentages of this variation in the different concentrations tested are shown below:

ENSAYO	[MUESTRA1] g/kg	Réplica 1 (%)	Réplica 2 (%)	Réplica 3(%)	Réplica 4(%)
Control +	0	0	0	0	0
Control -	0	17,7489177	23,1527094	22,8571429	27,3809524
C1	6.4	21,0300429	23,7288136	18,1818182	26,6409266
C2	14.0	26,8041237	27,7777778	10,7744108	11,3445378
C3	31.0	-23,8636364		-25,581395	-21,6730038
C4	68.0				
C5	150.0				
C6	330.0				

% variation of the live weight of the surviving worms in each replicate of each concentration tested

Live weight variation: Statistical analysis performed with the GraphPad Prisma 8.0 Software, using "Repeated measures one-way ANOVA" and Sidak's multiple comparison test.



Number of families 1  
 Number of comparisons per family 6  
 Alpha 0,05

Sidak's multiple comparisons test	Mean Diff,	95% CI of diff,	Significant?	Summary
Control vs. 6.4 g /Kg	0,39	-9,856 to 10,64	No	ns
Control vs. 14.0 g /kg	3,613	-34,45 to 41,68	No	ns
Control vs. 31.0 g /kg	46,49	36,03 to 56,95	Yes	***
6.4 g /Kg vs. 14.0 g /kg	3,223	-27,55 to 34,00	No	ns
6.4 g /Kg vs. 31.0 g /kg	46,1	39,51 to 52,69	Yes	***
14.0 g /kg vs. 31.0 g /kg	42,88	13,29 to 72,47	Yes	*

Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2	t	DF
Control vs. 6.4 g /Kg	22,79	22,4	0,39	1,657	4	4	0,2354	3
Control vs. 14.0 g /kg	22,79	19,17	3,613	6,155	4	4	0,5869	3
Control vs. 31.0 g /kg	22,79	-23,71	46,49	1,691	4	4	27,5	3
6.4 g /Kg vs. 14.0 g /kg	22,4	19,17	3,223	4,976	4	4	0,6476	3
6.4 g /Kg vs. 31.0 g /kg	22,4	-23,71	46,1	1,065	4	4	43,27	3
14.0 g /kg vs. 31.0 g /kg	19,17	-23,71	42,88	4,785	4	4	8,961	3

#### 4.5- COMMENT ON RESULTS AND INCIDENTS

- The mortality of the earthworms in the negative control tests was 0% (less than 5%) and the loss of live weight at 14 days did not exceed 30% of the registered initial weight. This assay was carried out with the standard substrate (OECD 207) as the only growth substrate.
- Mortality of earthworms in positive control trials was 100% in all trials. This assay was carried out with the standard substrate (OECD 207) as the only growth substrate, to which chloracetamide (15mg/Kg) was added.
- Humidity has been maintained between 40-60% values at the beginning and at the end of the test in all replicates tested, as well as in the controls. The initial pH was  $6.0 \pm 0.5$  in all replicates and controls.
- The 6 concentrations tested have been estimated by means of a previous test to coincide with the real mortality (for a better adjustment of the data), constituting a geometric series of factor (2.2). Four replicates of each concentration have been made.
- The mortality of 50% of the worms (IC50) has occurred at a concentration of 22.43 g/kg. A statistical analysis has been carried out with a dose-response curve, by means of logarithmic transformation and data normalization. The goodness of fit is good ( $R^2= 0.996$ ) so we can conclude that there are significant differences in the increasing concentrations tested.
- The calculation of NOEC and LOEC has been estimated by performing the analysis of variances (Repeated measures ANOVA one-way) and Bonferroni multiple comparison test. We can confirm that the comparison between the different concentrations is statistically significant in the concentration of 31 g/kg.
- At the beginning of the analyses, when the earthworms were deposited on the surface of the substrate, they proceeded to bury them in the first four minutes, in a similar way in all the concentrations tested. At 30 minutes, an escape attempt was observed in all replicates of the different concentrations of 68 g/kg and higher. Peripheral location in the containers was observed in all replicates of concentrations of 68 g/kg and higher.
- At 7 days, mortality was observed in all replicates of concentrations of 31 g/kg and higher, as well as physical and behavioral alterations in surviving worms at these concentrations.
- The live weight of the worms was maintained uniformly until the concentration of 31 g/kg in which it decreased significantly. As we can see in the data analysis, the difference in weight of the worms in the concentration of 31 g/kg is statistically significant.

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, CI-50, for soil organisms obtained in OECD 207 (Earthworm Acute Toxicity Test) toxicity tests, it should be less than 10 mg/g soil (10g/kg); in our case it is higher, being 22.43 g/kg.

Dr. Technician

**ECOTOXILAB**

Sgd. Gerardo Mengs González