CHELAB SILLIKER
a Mérieux NutriSciences Company

RISULTATI ANALITICI

GROWTH INHIBITION TEST ON SELENASTRUM CAPRICORNUTUM, ACCORDING TO OECD 201:2006 (CORR. 2011), ON "MIX FANGHI AFO", ID 13.037836.01

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Dates:

- Study starting date: 22/02/2013
- Study completion date: 02/03/2013

INDEX

1. STUDY PURPOSE

Purpose of the study is to evaluate the toxicity of the test substance towards freshwater algae *Selenastrum capricornutum*, by exposing algae in exponential phase of growth to an eluate of the substance. Growth and growth inhibition are quantified as a function of time.
Nature of the study: the study is an acute toxicity study (short term).

2. TEST ITEM

Name: Sludge

Receiving date: 2013/02/20

Chelab ID: 13.037836.01

Composition: unknown

Description: Mix Fanghi AFO

3. REFERENCE ITEMS

Name: Potassium dichromate

CAS nr: 7778-40-9

Molecular Weight: 294.19 g/mol

Molecular Formula: $K_2Cr_2O_7$

Ref.: 207802

Supplier: Sigma

CHELAB ID: 1565

4. TEST SYSTEMS

Several species of non-attached microalgae and cyanobacteria may be used. In this study *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*), ATCC 22662, CCAP 278/4, 61.81 SAG was used, as recommended by OCED 201: 2006 corr. 2011, Annex 2, and by ISO 8692:2004(E), Par. 5.1.
Algal culture stock was obtained from algae beads, as suggested by ISO 8692 “Freshwater algal growth inhibition test with unicellular green algae”, Par. 5.1. Algae beads (Algaltoxkit F) were supplied by ECOTOX LDS SRL.
Algae were exposed to the test substance after a de-immobilization procedure, as recommended in the kit instructions provided by the supplier. The kit provides microalgae which are immobilized in a special matrix in which they survive for several month, without losing their viability. After de-immobilization and transfer into an adequate algal culturing medium, the microalgae resume their growth immediately, and in analogous way to that of algae from stock cultures.
Algal culturing medium was obtained reconstituting and mixing five concentrated solutions of various chemicals, supplied with the Algaltoxkit. Algal culturing medium was used as dilution and culture water, and as negative control, since it has the composition recommended by OECD 201:2006 corr. 2011, Annex 3.

5. METHOD OF ANALYSIS

The purpose of this study is to determine the effects of the test substance on the growth of freshwater microalgae *Pseudokirchneriella subcapitata*.
The system response is the reduction of growth in a series of algal cultures exposed to various concentration of the test substance. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate of the unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures were allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate (see OECD 201:2006 corr. 2011, Par. 4).
Growth and growth inhibition were quantified from indirect measurements of the algal biomass as a function of time.
Optical density was used as surrogate parameter for biomass; a conversion factor between the measured optical density and biomass was applied (see OECD 201:2006 corr. 2011, Par. 5), according to the kit supplier instructions.
Algal biomass was not measured directly through a cell counter, because of the possible interferences due to the non-transparency of the test solutions.

The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period (see OECD 201:2006 corr. 2011, Par. 6). The concentration bringing 50 % inhibition of growth rate is determined using statistical methods, and expressed as E_rC_{50}.
Yield, defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period, was calculated as an additional response. From the yield recorded, the concentration bringing 50 % inhibition of yield was calculated, and expressed as E_yC_{50} (see OECD 201:2006 corr. 2011, Par. 7).
In this study, the test substance was treated to obtain an eluate, according to ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
A preliminary test ("limit test") on a 100 mg/l WAF nominal concentration of test substance was carried out: exponentially growing microalgae were exposed to the test solution over a period of 72 hours (see OECD 201:2006 corr. 2011, Par. 3 and Par. 42), and growth rate was evaluated and compared to that of the negative control.

5.1. Aim of the study

Aim of the method is to evaluate the toxicity of the test substance towards freshwater algae *Selenastrum capricornutum*, by exposing algae in exponential phase of growth to an eluate of the substance. Growth and growth inhibition are quantified as a function of time. The growth inhibition test is an acute toxicity test.

5.2. Reagents and reference solutions

- dH_2O.
- $K_2Cr_2O_7$. Sigma 207802, ID 1565.
- Algal beads of *Selenastrum capricornutum* with Matrix Dissolving Medium. ECOTOX LDS TB41, batch SC041212.
- Media for algal culturing medium. ECOTOX LDS TM31.

5.3. Materials and Apparatus

- Autoclave system 121 ℃ ± 1 ℃, 15 min. SRA 12
- Analytical balance (± 0.0001 g). SRA 48
- Technical balance (± 0.01 g). SRA 46
- Stirrer. SRA 39
- pHmeter. SRA 58
- Incubator. SRA 230
- Centrifuge. SRA 79
- Long cells (10 cm path-length)
- Spectrophotometer Jenway 6300, equipped with a holder for long cells. SRA 229
- 25 ml, 10 ml, 5 ml, 2 ml, 1 ml (nominal value) graduate pipette
- 20-200 µl (SRA 169) and 200-1000 µl (SRA 170) automatic pipette
- Sterile disposable filters 0.45 µm porosity
- Vortex mixer
- Sterile tubes
- Glass bottles (1 litre capacity)
- Volumetric flasks
- Stereomicroscope, SRA 313
- Laminar flux hood, SRA 21

Test vessel and other apparatus which came into contact with the test solutions were made entirely of glass or other chemically inert material (see OECD 201:2006 corr. 2011, Par. 14)

5.4. Eluate preparation

The test was carried out on an eluate of the test substance.
Preparation and treatment of the test substance was carried out according to: ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
WAF was not prepared by serial dilution of a single stock WAF according to ASTM D6081-98 (2004), but the selected concentration was prepared adding the chosen quantity into standard water.
For limit test 200 mg of sample milled (granulometry about 250 μm) was mixed with 2 l of standard water for a period of time of 96 hours. Following cessation of mixing, a 24 hours of settling of the aqueous phase was drawn off for testing.
Part of the aqueous phase was removed and transferred into a separator funnel for 6 hours. The definitive aqueous phases obtained was aerated by stirring for 24 hours and it was considered WAF of test substance.
In this study checks of test substance concentrations in WAF or evaluations about equilibrium between aqueous phase and solid are not applicable due to the nature of test substance.
The WAF was tested for pH and for conductivity at the beginning of the test, and again for pH at the end of the test (see OECD 201:2006 corr. 2011, Par. 35).

5.5. Reference solutions preparation

Five concentration of $K_2Cr_2O_7$ were used as positive control (see OECD 201:2006 corr. 2011, Par. 12): C1=1.8 mg/l, C2=1 mg/l, C3=0.56 mg/l, C4=0.32 mg/l and C5=0.18 mg/l; concentrations were chosen on the basis of the supplier indications.
100 mg of $K_2Cr_2O_7$ were dissolved in 100 ml of dH_2O, obtaining a 1 g/l stock solution. A 1:100 dilution was prepared, obtaining a 10 mg/l solution. From the 10 mg/l solution, the following concentrations were obtained:
- C1 = 1.8 mg/l: 18 ml of the 10 mg/l were diluted in algal culturing medium (final volume = 100 ml)
- C2 = 1 mg/l: 10 ml of the 10 mg/l were diluted in algal culturing medium (final volume = 100 ml)
- C3 = 0.56 mg/l: 5.6 ml of the 10 mg/l were diluted in algal culturing medium (final volume = 100 ml)
- C4 = 0.32 mg/l: 3.2 ml of the 10 mg/l were diluted in algal culturing medium (final volume = 100 ml)
- C5 = 0.18 mg/l: 1.8 ml of the 10 mg/l were diluted in algal culturing medium (final volume = 100 ml)

Preparation of the positive control solutions was carried out under a laminar flow hood. The correspondence between the EC_{50} indicated in the algal beads certificate and the value obtained from the test was verified. Each positive control concentration was tested in three replicates.
Algal culture medium was used as negative control. It was tested in six replicates (see ISO 8692:2004, Par. 7.5).

5.6. Test solution preparation

A limit test was carried out on a 100 mg/l WAF nominal concentration of test substance (see OECD 201:2006 corr. 2011, Par. 42), in order to verify if the EC_{50} is greater than this concentration. The limit test was carried out in six replicates (see OECD 201:2006 corr. 2011, Par. 42).

5.7. Analytical measurements

The pH of the test solutions was measured at the beginning and at the end of the test. It was verified that the pH of the test solutions and of the negative control has not varied of more than 1.5 during the test (see OECD 201:2006 corr. 2011, Par. 30).
The temperature was recorded at the beginning and at the end of the test period.
Light intensity was measured at the beginning and at the end of the test period.

5.8. Preparation of algal inoculum

Algae of the commercial kit, used in this study, were provided immobilized in a special matrix. They were de-immobilized just before the test, following the supplier instructions:
- Storage liquid was poured out from the tube containing algal beads;
- 5 ml of Matrix Dissolving Medium, supplied with algal beads, were transferred into the tube;
- The tube was caped and shaken vigorously, until the Matrix immobilizing the algae were totally dissolved;
- The tube was centrifuged for 10 minutes at 3000 rpm;
- The supernatant was poured out and replaced by 10 ml of deionized water;
- The tube was centrifuged again for 10 minutes at 3000 rpm, and the supernatant was poured out;
- Algae were resuspended in 10 ml of algal culturing medium;
- Algal suspension was poured into a 25 ml calibrated flask, and algal culturing medium was added to the 25 ml mark;

- Algal stock was transferred into the corresponding long cell, supplied with the kit;
- The Optical Density of the algal stock obtained (OD1) was read at the spectrophotometer (wave-length 670 nm) after the calibration of the spectrophotometer with algal culturing medium;
- According to the OD/N regression formula, given in the OD/N sheet included in each kit, optical density was converted into algal cell number (N1);

$$N1 = OD1 \cdot 1257968 - 38284$$

- With N2 equal to $1 \cdot 10^6$ algae/ml, the dilution factor needed to reach an Optical Density equal to OD2 (corresponding to N2) was calculated from the N1/N2 ratio, as follows;

$$Fd = N1/N2$$

- Total volume of algal culturing was calculated as follows:

$$V_{tot} = 25ml \cdot Fd$$

- The Volume of algal culturing medium to be added, in order to obtain the desired concentration, will be calculated as follows:

$$V = V_{tot} - 25ml$$

- Algal stock was diluted with algal culturing medium, in order to obtain a $1 \cdot 10^6$ algae/ml stock.

5.9. Test procedure

As far as positive control is concerned, 1 ml of the algal stock was added to 100 ml of each positive control concentration, in order to obtain an algal concentration of $1 \cdot 10^4$ algae/ml (see OECD 201:2006 corr. 2011, Par. 21). The inoculated test solutions were shaken and transferred into three long cells for each test solution (25 ml of test solution in each long cell).
As far as negative control and test substances are concerned, 2 ml of the algal stock were added to 200 ml of each positive control concentration, in order to obtain an algal concentration of $1 \cdot 10^4$ algae/ml (see OECD 201:2006 corr. 2011, Par. 21). The inoculated test solutions were shaken and transferred into six long cells for each test solution (25 ml of test solution in each long cell).
The nominal cell density was used as the initial cell density, and no initial cell density measurement was carried out (see ISO 8692:2004, Par. 7.7).
Long cells, slightly open, were placed in a random way in the holding tray, in order to compensate for possible small “site to site” differences during incubation. The holding tray was incubated at 23 ℃ ± 2 ℃, to obtain a satisfactor y algal growth during the test. A constant illumination was provided (10000 lux in the case of sideway illumination) (see ISO 8692:2004, Par. 7.6).
Standard protocols for toxicity tests with algae usually prescribe a continuous algae suspension to facilitate CO_2 transfer and reduce pH variation. Detailed investigation with the kit have, however, revealed that the resuspension of the algae once per day, immediately prior to the OD measurement of all the long cells, largely suffices to obtain the minimum number of algal division prescribed in standard procedures. Continuous shaking of the test vials is therefore not mandatory for the tests carried out with the kit.
The preliminary limit test had a duration of 72 hours (see OECD 201:2006 corr. 2011, Par. 32).
The optical density of each long cell was measured daily during the test period, by introducing long cells into the spectrophotometer, after shaking, and reading optical density at 670 nm wavelength. Calibration of the spectrophotometer was carried out before starting the measuring, and every time a new test solution was analyzed, with a long cell containing 25 ml of algal culturing medium.
Before measuring the optical density, the cells were shaken to homogenize the content, as reported in the kit instructions. The shaking procedure was as standard as possible, in order to ensure maximum reproducibility.
Since data elaboration showed a growth inhibition < 10 % in the limit test, the complete test was not carried out.

5.10. Microscope observation

Microscope observation was performed, with a stereomicroscope, at the beginning and at the end of the test. The normal and healthy appearance of the inoculum culture was verified (see OECD 201:2006 corr. 2011, Par. 41).

5.11. Data elaboration

Daily measured optical density data were reported in the appropriate data form, and the conversion factor was applied to obtain biomass data. The conversion factor, specific for each algal batch, was supplied with the algal beads. Calculated biomass data were rounded to the second decimal digit.

5.12. Plotting growth curves

The estimated biomass concentration in test solutions, together with the test concentrations and the times of measurement, were tabulated to produce plots of growth curves. A logarithmic scale was used, since it is mandatory and generally gives a better presentation of variations in growth pattern during the test period (see OECD 201:2006 corr. 2011, Par. 44).

5.13. Growth rate

The section by section specific growth rate was calculated as follows, for each treatment solution and each treatment period:

$$\mu_{i-j} = \frac{\ln Xj - \ln Xi}{tj - ti}$$

Where μ_{i-j} = specific growth rate from time *i* to *j*;
X_i = biomass at time *i*
X_j = biomass at time *j*
For each treatment group and control group, the mean value of growth rate and its variation coefficient were calculated.
The average specific growth rate was also calculated over the entire test duration, with *i* = 0 and *j* = 3 days. All data were rounded to the second decimal digit.

5.14. Percent inhibition of growth rate

The percentage inhibition of growth rate for each treatment replicate was calculated as follows:

$$\%I_r = \frac{\mu_c - \mu_t}{\mu_c} \cdot 100$$

Where $\%I_r$ = percent inhibition in average specific growth rate
μ_c = mean value for average specific growth rate (μ) in the control group
μ_t = average specific growth rate for the treatment replicate
For each treatment group, the mean value of percent inhibition and its variation coefficient were calculated. All data were rounded to the second decimal digit.

5.15. Percent inhibition of yield

Yield was calculated as the biomass at the end of the test minus the starting biomass for each single vessel of controls and treatments.
For each test concentration and control, the mean value of Yield and its variation coefficient were calculated.
The percent inhibition in Yield ($\%I_Y$) for each treatment replicate was calculated as follows:

$$\%I_Y = \frac{Y_c - Y_t}{Y_c} \cdot 100$$

Where $\%I_Y$ = percent inhibition of Yield
Y_c = mean value for Yield (Y) in the control group
Y_t = value for Yield for the treatment replicate
For each treatment group, the mean value of Yield percent inhibition and its variation coefficient were calculated. All data were rounded to the second decimal digit.

5.16. EC_{50} calculation

For positive control, EC_{50} was calculated. EC_{50} is defined as the concentration of test substance (or positive control) which causes an inhibition of growth equal to 50 % compared with the negative control (see OECD 201:2006 corr. 2011, Par. 6).

6. VALIDATION PROCEDURE

Negative control was carried out to verify that both the algal culturing medium and the test apparatus are not toxic to algae.
Positive control was carried out to verify that the batch of algae used in the test is sensitive to toxicant.

7. RESULTS

Table 1: Optical Density Data for positive control – 24 hours

Test solution	Replicate 1	Replicate 2	Replicate 3
0.18 mg/l	0.057	0.052	0.060
0.32 mg/l	0.052	0.051	0.050
0.56 mg/l	0.046	0.048	0.047
1 mg/l	0.054	0.054	0.051
1.8 mg/l	0.047	0.046	0.046

Table 2: Optical Density for negative control and test substance 13.037836.01 - 24 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Negative Control	0.060	0.056	0.058	0.058	0.060	0.056
100 mg/l	0.049	0.043	0.046	0.049	0.043	0.046

Table 3: Specific growth rate µ for negative control - 0-24 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Mean	CV %
Negative Control	1.31	1.17	1.24	1.31	1.17	1.24	1.24	5.05

Table 4: Optical Density Data for positive control – 48 hours

Test solution	Replicate 1	Replicate 2	Replicate 3
0.18 mg/l	0.102	0.099	0.101
0.32 mg/l	0.088	0.087	0.090
0.56 mg/l	0.072	0.072	0.074
1 mg/l	0.055	0.055	0.050
1.8 mg/l	0.047	0.046	0.047

Table 5: Optical Density for negative control and test substance 13.037836.01 - 48 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Negative Control	0.107	0.096	0.102	0.101	0.099	0.103
100 mg/l	0.089	0.093	0.096	0.095	0.091	0.093

Table 6: Specific growth rate µ for negative control - 24-48 hours

Test	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Mean	CV %

solution								
Negative Control	0.95	0.94	0.95	0.94	0.84	1.04	0.94	6.73

Table 7: Optical Density Data for positive control – 72 hours

Test solution	Replicate 1	Replicate 2	Replicate 3
0.18 mg/l	0.179	0.187	0.185
0.32 mg/l	0.118	0.123	0.126
0.56 mg/l	0.105	0.102	0.101
1 mg/l	0.058	0.055	0.052
1.8 mg/l	0.048	0.047	0.048

Table 8: Optical Density for negative control and test substance 13.037836.01 - 72 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Negative Control	0.253	0.266	0.263	0.253	0.266	0.263
100 mg/l	0.167	0.172	0.169	0.169	0.167	0.172

Table 9: Specific growth rate µ for negative control – 48-72 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Mean	CV %
Negative Control	1.07	1.28	1.18	1.15	1.23	1.16	1.18	6.11

Table 10: Specific growth rate µ for negative control – 0-72 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Mean	CV %
Negative Control	1.11	1.13	1.13	1.11	1.13	1.13	1.12	0.92

Table 11: % Growth inhibition for positive control – 72 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Mean	CV %
0.18 mg/l	13.64	11.82	12.73	12.73	7.15
0.32 mg/l	30.91	29.09	28.18	29.39	4.73
0.56 mg/l	36.36	38.18	38.18	37.57	2.80
1 mg/l	72.73	77.27	82.73	77.58	6.45
1.8 mg/l	92.73	96.36	92.73	93.94	2.23

EC_{50} for positive control = 0.8 mg/l

Table 12: % Growth Inhibition for test substance 13.037836.01 - 72 hours

Test solution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Mean	CV %
100 mg/l	15.18	14.29	15.18	15.18	15.18	14.29	14.88	3.09

Table 13: Mean Coefficient of Variation (CV %) for section-by-section specific growth rates

Test solution	0 - 24 hours	24 - 48 hours	48 - 72 hours	Mean
Negative Control	5.05	6.73	6.11	6.0

pH (Negative Control) at the beginning of the test = 8.02
pH (Negative Control) at the end of the test = 8.00
Variation of pH (Negative Control) = 0.02
pH (13.037836.01 - 100 mg/l) at the beginning of the test = 8.05
pH (13.037836.01 - 100 mg/l) at the end of the test = 8.05
Variation of pH (13.037836.01 - 100 mg/l) = 0.00

7.1. Validity criteria

For the test to be valid, the following performance criteria were checked (see OECD 201:2006 corr. 2011, Par. 11):

- The biomass in the control cultures had increased exponentially by a factor of at least 16 within the 72 hours of the test. This corresponds to a specific growth rate of at least 0.92 day^{-1} (see Table 10).
- The mean coefficient of variation (CV %) for section-by-section specific growth rates μ in the control cultures had not exceeded 35 % (see Table 13).
- The coefficient of variation (CV %) of average specific growth rates μ during the whole test period in control cultures had not exceeded 7 % (see Table 10).

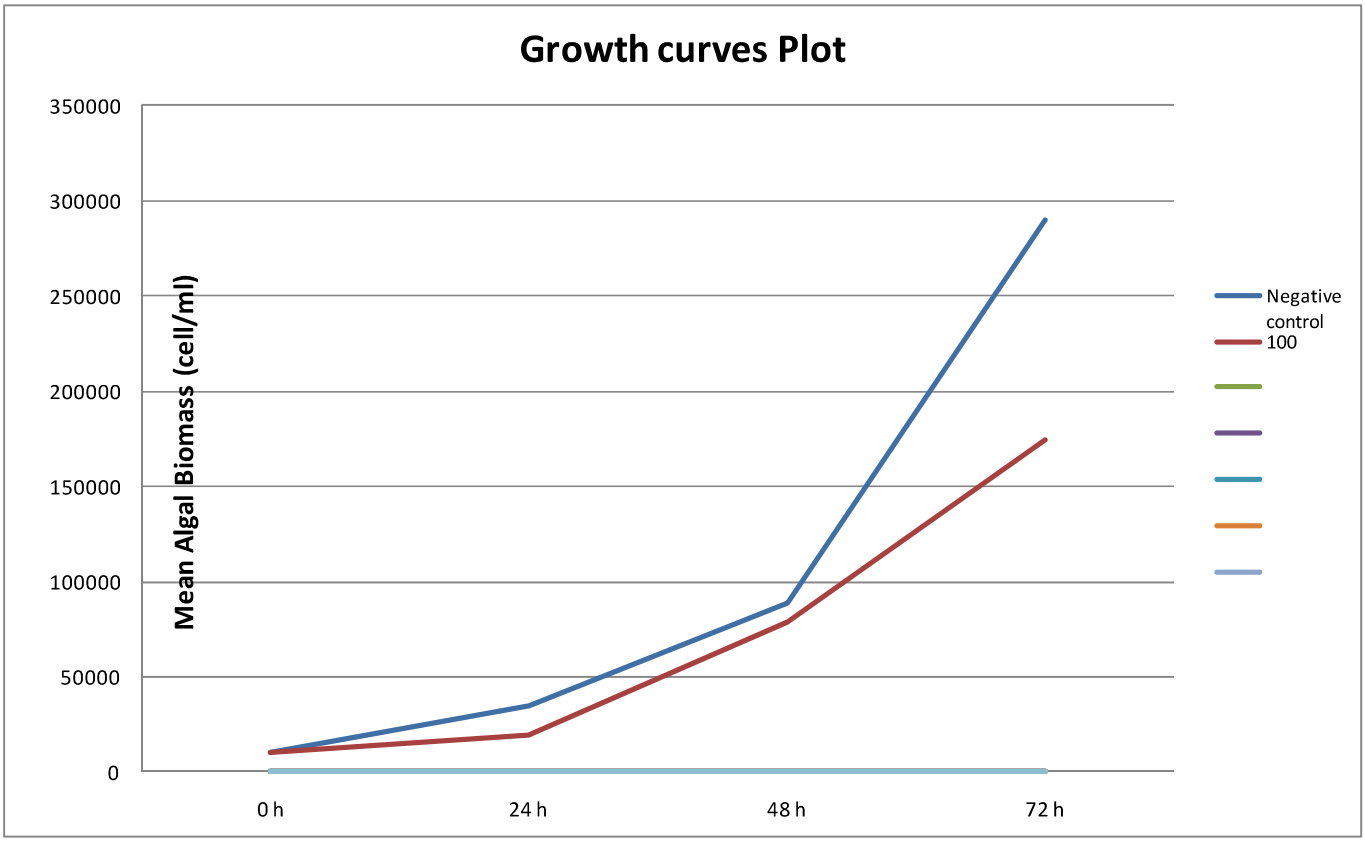

Fig. 1: Algal growth (limit test)

8. REFERENCE AND GUIDELINES

- OECD 201:2006 corr. 2011
- UNI EN ISO 8692:2005
- ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
- ISPRA "La nuova classificazione dei rifiuti e i test ecotossicologici per la caratteristica di pericolo H14" Andrea M. Lanz, Andrea Paina

9. CONCLUSIONS

According to OECD 201:2006 corr. 2011, if the inhibition of growth for the test substance is < 50 %, when testing the 100 mg/l WAF nominal concentration, EC_{50} is considered to be > 100 mg/l. No definitive test is necessary. Test item has no toxic effects at concentrations up to 100 mg/L.

ACUTE IMMOBILIZATION TEST ON DAPHNIA MAGNA, ACCORDING TO OECD 202:2004, ON "MIX FANGHI AFO"
ID 13.037836.01

Sponsor:
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74100 Taranto (TA)
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Test Facility:
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Study Director: *Federica Cattapan*

Dates:

- Study starting date: 21/02/2013
- Study completion date: 02/03/2013

INDEX

10. STUDY PURPOSE

Purpose of the study is to evaluate the toxicity of the test substance towards young daphnids, by exposing young daphnids aged less than 24 hours to an eluate of the substance. Immobilization of daphnids was quantified as a function of time.
Nature of the study: the study is an acute toxicity study (short term).

11. TEST ITEM

Name: Sludge

Receiving date: 2013/02/20

Chelab ID: 13.037836.01

Composition: unknown

Description: Mix Fanghi AFO

12. REFERENCE ITEMS

Name: Potassium dichromate

CAS nr.: 7778-50-9

Molecular Weight: 294.19 g/mol

Molecular Formula: $K_2Cr_2O_7$

Batch: 05406KJ

Supplier: Sigma

Purity: 99.9 %

CHELAB ID: 1564

13. TEST SYSTEMS

Daphnia magna Straus was used in the test, as it is the preferred test species (see OECD 202:2004, Par. 9). The daphnids were obtained from a kit (Supplier: ECOTOX LDS SRL), and were exposed to the test solutions when they were less than 24 hours aged (see OECD 202:2004, Par. 9).
Since any water which conforms to the chemical characteristics of an acceptable dilution water is suitable as test water (see OECD 202:2004, Par. 10 and Annex 2), culturing and dilution water was obtained by adding concentrated solutions of salts, supplied with the ephippia (*i.e.* Calcium chloride, Magnesium sulphate, Sodium bicarbonate and Potassium chloride) to distilled water (see OECD 202:2004, Annex 3).
The same water was used as negative control.
Hatching of ephippia was carried out at 20 ℃ - 22 ℃, with at least 6000 lux of light intensity, for 80 hours, as indicated from the supplier.

14. METHOD OF ANALYSIS

The purpose of this study is to determine the effects of the test substance on the surviving ability of *Daphnia magna*, aged less than 24 hours. The system response is the immobilization of daphnids exposed to various concentrations of the test substance. The response is evaluated as a function of the exposure concentration in comparison with the average immobilization of replicates of unexposed control daphnids.
Young daphnids, aged less than 24 hours at the start of the test, were exposed to an eluate of the test substance for a period of 48 hours. Immobilization was recorded at 24 and 48 hours, and compared with control values. The test endpoint is immobilization of daphnids, expressed as a percentage, during the exposure period.
A reference substance ($K_2Cr_2O_7$) was tested for EC_{50} as a means of assuring that the test conditions are reliable (see OECD 202:2004, Par. 3 and Par. 5).
Test vessels and other apparatus that came into contact with the test solutions were made entirely of inert materials. Test vessels were loosely covered to reduce the loss of water due to evaporation, and to avoid the entry of dust into the solutions (see OECD 202:2004, Par. 7).
Test vessels were filled with 10 ml of test solution each, providing 2 ml of test solution for each animal (as recommended by OECD 202:2004, Par. 14), since 5 daphnids were transferred in each vessel.

In this study, the test substance was treated to obtain an eluate, according to ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
A preliminary test ("limit test") on a 100 mg/l WAF nominal concentration of test substance was carried out: young daphnids, aged less than 24 hours, were exposed to the test solution over a period of 48 hours (see OECD 202:2004, Par. 3), and immobilization percentage was evaluated and compared to that of the negative control.

14.1. Aim of the study

The purpose of this study is to determine the effects of the test substance on the surviving ability of *Daphnia magna*, aged less than 24 hours.

14.2. Reagents and reference solutions

- dH_2O.
- $K_2Cr_2O_7$. Sigma 207802. Batch 05406KJ
- Ephippia of Daphnia magna Straus. ECOTOX LDS TB33. Batch DM090812
- Media for daphnids culturing medium. ECOTOX LDS TM23.

14.3. Materials and Apparatus

- Autoclave system 121 ℃ ± 1 ℃, 15 min. SRA 12
- Analytical balance (± 0.0001 g). SRA 48
- Technical balance (± 0.01 g). SRA 46
- Stirrer. SRA 39
- pHmeter. SRA 58
- Incubator. SRA 230
- Incubation cells
- 25 ml, 10 ml, 5 ml, 2 ml, 1 ml (nominal value) graduate pipette
- Sterile disposable filters 0.45 µm porosity
- Vortex mixer
- Sterile tubes
- Glass bottles (1 litre capacity)
- Volumetric flasks
- Stereomicroscope, SRA 313
- Laminar flux hood. SRA 21

Test vessel and other apparatus which came into contact with the test solutions were made entirely of glass or other chemically inert material.

14.4. Eluate preparation

The test was carried out on an eluate of the test substance.
Preparation and treatment of the test substance was carried out according to: ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
WAF was not prepared by serial dilution of a single stock WAF according to ASTM D6081-98 (2004), but the selected concentration was prepared adding the chosen quantity into standard water.
For limit test 200 mg of sample milled (granulometry about 250 µm) was mixed with 2 l of standard water for a period of time of 96 hours. Following cessation of mixing, a 24 hours of settling of the aqueous phase was drawn off for testing.
Part of the aqueous phase was removed and transferred into a separator funnel for 6 hours. The definitive aqueous phases obtained was aerated by stirring for 24 hours and it was considered WAF of test substance.

In this study checks of test substance concentrations in WAF or evaluations about equilibrium between aqueous phase and solid are not applicable due to the nature of test substance.
The WAF was tested for pH and for conductivity at the beginning of the test, and again for pH at the end of the test (see OECD 201:2006 corr. 2011, Par. 35). pH has to be between 6 and 9 to be suitable for daphnids surviving.

14.5. Hatching of ephippia

80 hours before the beginning of the test, the ephippia were hatched following the supplier instruction: the content of each vial of ephippia was poured into a microsieve and rinsed with tap water, in order to eliminate the conservation medium. Ephippia were transferred into a Petri dish with 15 ml of pre-aerated freshwater, and the dishes were incubated for 80 hours at 20 ℃ - 22 ℃ under continuous illumination (min. 6000 lux). Two vials of ephippia were used.
The organisms were collected 80 hours after the start of the incubation.

14.6. Reference solutions preparation

Daphnia culture medium was used as negative control, to verify that the leaching solution is not toxic to daphnids, 40 daphnids were used (8 groups of 5 daphnids each, see Tables 1 and 2) (see OECD 202:2004, Par. 15).
Five concentration of $K_2Cr_2O_7$ were used as positive control, to verify that the batch of daphnids used in the test is sensitive to toxicant (see OECD 202:2004, Par. 12): C1=3.2 mg/l, C2=1.8 mg/l, C3=1 mg/l, C4=0.56 mg/l and C5=0.32 mg/l. Concentrations were chosen to cover a wide range in which the EC_{50} has been evaluated: the results of the inter laboratory tests and a Technical Corrigendum to ISO 6341 give an EC_{50} – 24 h of $K_2Cr_2O_7$ within the range 0.6 mg/l – 2.1 mg/l (see OECD 202:2004, Par. 5). Dilutions of the positive control were prepared in Daphnia culturing medium; preparation of the positive control solutions were carried out under a laminar flow hood.
The correspondence between the EC_{50} indicated in the ephippia certificate and the value obtained from the test was verified.
20 daphnids were exposed to each concentration of the positive control (see Table 1).

14.7. Test solution preparation

A limit test was carried out on a 100 mg/l WAF nominal concentration of test substance (see OECD 202:2004, Par. 24), in order to verify if the EC_{50} is greater than this concentration.
Since OCED 202:2004 suggests the use of 20 daphnids for the limit test (see OECD 202:2004, Par. 24), 40 daphnids were exposed to the test substance, divided into 8 groups of 5, to increase the statistical significance of results (see Table 2).

14.8. Analytical measurements

The dissolved oxygen and pH were measured at the beginning and at the end of the test in the negative control and in the highest test concentration. It was verified that the pH of the eluate had not varied of more than 1.5 during the test (see OECD 202:2004, Par. 22).
The temperature was recorded at the beginning and at the end of the test.
Light intensity was measured at the beginning and at the end of the hatching period.

14.9. Pre-feeding of ephippia

78 hours after the incubation of the ephippia, as recommended by the ephippia supplier, a 2 hours pre-feeding was applied with a suspension of *Spirulina* microalgae, in order to provide the neonates hatched from the ephippia with food prior to the test. One vial of *Spirulina* powder per hatching dish was filled with culture medium; the vial was shaken thoroughly to homogenize the contents, and the content was poured into each hatching dish.

14.10. Beginning of the test

Two hours after the pre-feeding, daphnids were collected for the test. A light table was used to increase the visibility of young daphnids. Using a micropipette, daphnids were transferred from the hatching dish to the rinsing wells. About 20 daphnids were transferred in each rinsing well, trying to carry over as little as possible culture medium from the hatching dish to the wells, and rinsing the micropipette thoroughly after each transfer. Then, exactly 5 neonates were transferred from each rinsing well into the 4 wells of each row (see Tables 1 and 2). Plates were incubated in the darkness for 48 hours; the temperature was within the range 18 ℃ - 22 ℃, constant within ± 1 ℃. The test vessels was not ae rated during the test, and the daphnids were not fed during the test (see OECD 202:2004, Par. 18, 19 and 20).

	A	B	C	D
NC	NC	NC	NC	NC
C1 PC	C1 PC	C1 PC	C1 PC	C1 PC
C2 PC	C2 PC	C2 PC	C2 PC	C2 PC
C3 PC	C3 PC	C3 PC	C3 PC	C3 PC
C4 PC	C4 PC	C4 PC	C4 PC	C4 PC
C5 PC	C5 PC	C5 PC	C5 PC	C5 PC
Rinsing wells (about 20 daphnids each)	TEST WELLS (5 daphnids and 10 ml of test solution each)			

Table 2: Seeding scheme of test solutions (positive control)

	A	B	C	D
NC	NC	NC	NC	NC
100 mg/l	100 mg/l	100 mg/l	100 mg/l	100 mg/l
100 mg/l	100 mg/l	100 mg/l	100 mg/l	100 mg/l
Rinsing wells (about 20 daphnids each)	TEST WELLS (5 daphnids and 10 ml of test solution each)			

Table 3: Seeding scheme of test solutions (test substance)

14.11. Scoring of results

Each test vessel was checked for immobilized daphnids after 24 and 48 hours of treatment. The number of dead and immobilized neonates was recorded in the proper data form. The neonates which were not able to swim after gentle agitation of the liquid for 15 seconds were considered to be immobilized, even if they can still move their antennae. In addition to immobility, any abnormal behavior or appearance was reported (see OECD 202:2004, Par. 21).

14.12. Data elaboration

Data were summarized in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilization at each observation.
Immobilization percentage was calculated at 24 and 48 hours, as follows:

$$\%I = (n / tot) \cdot 100$$

Where *%I* is the immobilization percentage, n is the number of immobilized daphnids for a test solution, and tot is the number of daphnids exposed to the test solution.
The number of immobilized daphnids in the test substance was compared to that obtained in the negative control.
For the positive control data of immobilization at 24 hours were analyzed by Probit analyses (see OECD 202:2004, Par. 25), to calculate the slopes of the curves and the EC_{50} with 95 % confidence limits (p = 0.95).

14.13. Validity criteria

For the test to be valid, the following performance criteria were verified:

- Immobilization percentage in the control < 10 %;
- COD at the end of the test ≥ 3 mg/l in control and in test vessels (see OECD 202:2004, Par. 6).

15. VALIDATION PROCEDURE

Negative control was carried out to verify that both the daphnia culturing medium and the test apparatus are not toxic to daphnids.
Positive control was carried out to verify that the batch of daphnids used in the test is sensitive to toxicant.

16. RESULTS

Test Solution	Immobilized daphnids 24 h (Tot 40 exposed)								Total Immobilized daphnids	% Immobilized daphnids
	replicate 1	replicate 2	replicate 3	replicate 4	replicate 5	replicate 6	replicate 7	replicate 8		
Negative Control	0	0	0	0	1	0	0	0	1	2,50
100 mg/l	0	0	0	0	0	0	0	0	0	0,00

Table 4: Immobilization % for Negative Control and test substance 13.037836.01 - 24 hours

Test Solution	Immobilized daphnids 48 h (Tot 40 exposed)								Total Immobilized daphnids	% Immobilized daphnids
	replicate 1	replicate 2	replicate 3	replicate 4	replicate 5	replicate 6	replicate 7	replicate 8		
Negative Control	0	0	0	0	1	0	0	0	1	2,50
100 mg/l	0	0	0	0	0	0	0	0	0	0,00

Table 5: Immobilization % for Negative Control and test substance 13.037836.01 – 48 hours

Validity criteria was verified: Immobilization % in the negative control < 10 %

EC_{50} for test substance 13.037836.01 > 100 mg/l

Positive Control	Immobilized daphnids 24 h (Tot 20 exposed)				Total Immobilized daphnids	% Immobilized daphnids
	replicate 1	replicate 2	replicate 3	replicate 4		
C1 PC	5	4	5	4	18	90,00
C2 PC	3	2	4	3	12	60,00
C3 PC	3	2	2	2	9	45,00
C4 PC	2	2	2	1	7	35,00
C5 PC	1	1	1	2	5	25,00

Table 6: Immobilization % for Positive Control - 24 hours

Positive Control	Immobilized daphnids 48 h (Tot 20 exposed)				Total Immobilized daphnids	% Immobilized daphnids
	replicate 1	replicate 2	replicate 3	replicate 4		
C1 PC	5	5	5	5	20	100,00
C2 PC	4	4	3	3	14	70,00
C3 PC	3	3	3	3	12	60,00
C4 PC	2	2	2	2	8	40,00
C5 PC	1	1	1	2	5	25,00

Table 7: Immobilization % for Positive Control - 48 hours

EC_{50} for Positive Control = 1.070 mg/l (0.684 mg/l – 1.608 mg/l)

17. REFERENCE AND GUIDELINES

- OECD 201:2006 corr. 2011
- UNI EN ISO 8692:2005
- ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
- ISPRA "La nuova classificazione dei rifiuti e i test ecotossicologici per la caratteristica di pericolo H14" Andrea M. Lanz, Andrea Paina

18. CONCLUSIONS

According to OECD 202:2004, if the immobilization percentage for the test substance is < 50 %, when testing the 100 mg/l WAF nominal concentration, EC_{50} is considered to be > 100 mg/l. No definitive test is necessary. Test item has no toxic effects at concentrations up to 100 mg/L.

FISH, ACUTE TOXICITY TEST ACCORDING TO OECD 203:1992 ON "MIX FANGHI AFO" ID 13.037836.01

Sponsor:
ILVA spa
S.S. Appia, Km 648
74100 Taranto (TA)
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Test Facility:
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Study Director: *Federica Cattapan*

Dates:

- Study starting date: 22/02/2013
- Study completion date: 04/03/2013

CHELAB SILLIKER
a Mérieux NutriSciences Company

INDEX

19. STUDY PURPOSE

Purpose of the study is to evaluate the acute toxicity of the test substance towards the fish *Brachydanio rerio*, by exposing fishes to an eluate of the substance. Endpoints of the test are mortality of the fishes or other abnormal behaviours.
Nature of the study: the study is an acute toxicity study (short term).

20. TEST ITEM

Name: Sludge

Receiving date: 2013/02/20

Chelab ID: 13.037836.01

Composition: unknown

Description: Mix Fanghi AFO

21. TEST SYSTEMS

One or more species may be used, the choice being at the discretion of the testing laboratory.

It is suggested that the species used be selected on the basis of such important practical criteria as, for example, their ready availability throughout the year, ease of maintenance, convenience for testing and any relevant economic, biological or ecological factors. The fishes should be in good health and free from any apparent malformation.

Organism chosen: *Brachydanio rerio* (Teleostei, Cyprinidae) (Hamilton Buchanan)

Length: 2.0 ±1.0 cm.

All fishes were held in the laboratory for at least 12 days before they are used for testing. They were held in water of the quality to be used in the test for at least seven days immediately before testing and under the following conditions:

- Light: 12 to 16 hours photoperiod daily;
- Temperature: 22 ±1°C
- Oxygen concentration: at least 80 per cent of air saturation value;
- Feeding: daily until 24 hours before the test is started.

22. METHOD OF ANALYSIS

The purpose of this study is to determine the acute lethal toxicity of a substance to fish in fresh water . Effects of the test substance on the surviving ability of *Brachydanio rerio* was tested for a period of 96 hours. Fishes were considered dead if touching of the caudal peduncle gives no reaction, and no breathing movements were visible. Records were kept of visible abnormalities (e.g. loss of equilibrium, changes in swimming behaviour, respiratory function, pigmentation, etc.). The response is evaluated as a function of the exposure concentration in comparison with the average immobilization of replicates of unexposed control fishes.
Test vessels and other apparatus that came into contact with the test solutions were made entirely of inert materials.
In this study, the test substance was treated to obtain an eluate, according to ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
A preliminary test ("limit test") on a 100 mg/l nominal concentration of test substance was carried out: seven fishes were exposed to 1000 ml the test solution over a period of 96 hours (see OECD 203:1992, Par. 20), and the effects of the test substance on the surviving ability of *Brachydanio rerio* was evaluated and compared to that of the negative control.

22.1. Aim of the study

The purpose of this study is to determine the effects of the test substance on the surviving ability of *Brachydanio rerio*.

22.2. Reagents and reference solutions

- dH_2O.
- Standard Water used for tests and WAF was prepared from following stock solutions:

A. $NaHCO_3$ 2.59 g/l

B.	$CaCl_2 \times 2H_2O$	11.76	g/l	
C.	$MgSO_4 \times 7H_2O$	4.93	g/l	
D.	KCl	0.23	g/l.	

25 ml of each of the four stock solutions were mixed and make up to 1 litre with deionized water (conductivity < 5 μScm^{-1}). The obtained solution was aerated until the dissolved oxygen concentration equals the air-saturation value. The pH was 7,8 ± 0,2; the ratio of Ca:Mg ions was 4:1 and of Na:K ions is 10:1. The total alkalinity of this solution was 0,8 mmol per litre.

22.3. Materials and Apparatus

- Autoclave system 121 ℃ ± 1 ℃, 15 min. SRA 12
- Analytical balance (± 0.0001 g). SRA 48
- Technical balance (± 0.01 g). SRA 46
- Stirrer. SRA 39
- pHmeter. SRA 58
- Incubator. SRA 230
- Incubation cells
- 25 ml, 10 ml, 5 ml, 2 ml, 1 ml (nominal value) graduate pipette
- Sterile disposable filters 0.45 µm porosity
- Vortex mixer
- Sterile tubes
- Glass bottles (1 litre capacity)
- 1 litre glass test tanks
- Volumetric flasks
- Stereomicroscope, SRA 313
- Laminar flux hood. SRA 21

Test vessel and other apparatus which came into contact with the test solutions were made entirely of glass or other chemically inert material.

22.4. Eluate preparation

The test was carried out on an eluate of the test substance.
Preparation and treatment of the test substance was carried out according to: ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
WAF was not prepared by serial dilution of a single stock WAF according to ASTM D6081-98 (2004), but the selected concentration was prepared adding the chosen quantity into standard water.
For limit test 200 mg of sample milled (granulometry about 250 µm) was mixed with 2 l of standard water for a period of time of 96 hours. Following cessation of mixing, a 24 hours of settling of the aqueous phase was drawn off for testing.
Part of the aqueous phase was removed and transferred into a separator funnel for 6 hours. The definitive aqueous phases obtained was aerated by stirring for 24 hours and it was considered WAF of test substance.
In this study checks of test substance concentrations in WAF or evaluations about equilibrium between aqueous phase and solid are not applicable due to the nature of test substance.
The WAF was tested for pH and for conductivity at the beginning of the test, and again for pH at the end of the test (see OECD 201:2006 corr. 2011, Par. 35). pH has to be between 6 and 9 to be suitable for daphnids surviving.

22.5. Fishes stabilization

Fishes stabilization was carried out in a stalling aquarium with: carbon water recycle, suitable oxygenation rate (60÷100%) obtained by air bubbling , temperature control system and programmable illumination.

22.6. Test solution preparation

A limit test was carried out on a 100 mg/l nominal concentration of test substance (see OECD 203:1992, Par. 20), in order to verify if the EC_{50} is greater than this concentration.
Since OCED 203:1992 suggests the use of 7 fishes for the limit test (see OECD 203:1992, Par. 20), 7 fishes were exposed to the test substance.

22.7. Analytical measurements

The dissolved oxygen was measured at the beginning and at the end of the test in the negative control and in the test concentration.
DO ≥60% throughout the test was verified.

22.8. Beginning of the test

8 Fishes were exposed in static conditions to 1 litre of WAF (nominal concentration 100 mg/l) of the test item, as described below

- Duration: 96 hours.
- Illumination: 12 to 16 hours illumination daily,
- Temperature 22 ±1℃.
- dissolved oxygen concentration: not less than 60 % of the air-saturation value at the selected temperature,
- feeding: none.

The fishes were inspected after the first 2 to 4 hours and at least at 24-hour intervals. Fishes were considered dead if touching of the caudal peduncle gives no reaction, and no breathing movements were visible. Records were kept of visible abnormalities (e.g. loss of equilibrium, changes in swimming behaviour, respiratory function, pigmentation, etc.).
Measurements of pH, dissolved oxygen and temperature were carried out daily.
One control without the test substance was run, with 7 fishes.

22.9. Results

The fishes were inspected after the first 2 to 4 hours and at least at 24-hour intervals. All fishes in the first 4 hours did not exhibit visible abnormalities like loss of equilibrium and changes in swimming behaviour. In particular they remained well distributed in the tank, swimming with correct movements and always breathing.
After 24 hours all fishes were alive and they did not showed visible abnormalities. At the end of the test (after 96 hours) all fishes were alive and in good health.

22.10. Validity criteria

For the test to be valid, the following performance criteria were verified:

- Immobilization percentage in the control < 10 %;
- DO ≥60% throughout the test

23. VALIDATION PROCEDURE

Negative control was carried out to verify that the standard water and the test apparatus are not toxic to fishes.

24. RESULTS

Test Solution	Total dead fishes					
	After 2h	After 4h	After 24h	After 48h	After 72h	After 96h
Negative Control	0	0	0	0	0	0
100 mg/l	0	0	0	0	0	0

Table 1: Mortality for Negative Control and test substance

Validity criteria was verified: Immobilization % in the negative control < 10 %

EC_{50} for test substance 13.037836.01 > 100 mg/l

25. REFERENCE AND GUIDELINES

- OECD 203:1992
- ENV/JM/MONO(2000)6; OECD SERIES ON TESTING AND ASSESSMENT Number 23 GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURE": Water accommodated fraction (WAF) procedure.
- ISPRA "La nuova classificazione dei rifiuti e i test ecotossicologici per la caratteristica di pericolo H14" Andrea M. Lanz, Andrea Paina
- OECD/OCDE The Threshold Approach for Acute Fish Toxicity Testing
- B. Hoeger1, S. Jeram2, M. Holt3, P. Douben4 and M. Halder1 1European Commission - DG Joint Research Centre, IHCP, ECVAM, Ispra, Italy; 2Institute of Public Health of the Republic of Slovenia, Ljubljana, Slovenia; 3ECETOC AISBL, Brussels, Belgium; 4Unilever, Sharnbrook, UK, now: Cefic, Brussels, Belgium "REDUCTION OF ANIMAL USE IN ACUTE AQUATIC TOXICITY TESTING: FURTHER DEVELOPMENT OF THE THRESHOLD APPROACH AND ITS APPLICATION TO EXISTING CHEMICALS AND PLANT PROTECTION PRODUCTS

26. CONCLUSIONS

According to OECD 203:1992, if the mortality percentage for the test substance is < 50 %, when testing the 100 mg/l nominal concentration, EC_{50} is considered to be > 100 mg/l. No definitive test is necessary. Test item has no toxic effects at concentrations up to 100 mg/L.

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