REPORT ON THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT

Renato Baudo

TABLE OF CONTENTS page 1

EXECUTIVE SUMMARY 2
BACKGROUND AND RATIONALE 3
ORGANIZATION OF AN INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT 3
IMPORTANT NOTE 3
PARTICIPATION IN THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT 4
TEST PROTOCOL OF THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT 4
SHIPMENT OF THE TEST KITS 4
RESULTS OF THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT 4
MEASUREMENTS 5
STATISTICAL ANALYSIS 5
VALIDITY CRITERIA 5
DATA TREATMENT 5
REPEATABILITY AND REPRODUCIBILITY 5
HILL MODEL ERC50 MACRO REGTOX 5
CONCLUSIONS 6
ANNEX 1 PROCEDURE FOR THE PERFORMANCE OF A REFERENCE TEST WITH $K_2Cr_2O_7$ 8
ANNEX 2 STATISTICAL ANALYSIS 24

EXECUTIVE SUMMARY

The spectrophotometric measurement in long cells of the marine microalgae growth inhibition test has been proposed to ISO to be included in the Standard 10253, which is presently under revision.

As per ISO rules, the proposal must be backed by data on an International Interlaboratory Comparison.

Therefore, in 2014 twelve labs, from six different Countries, have been invited to take part in such a ringtest, following the Marine Algaltoxkit procedure of MicroBioTests Inc. and using the reference toxicant potassium dichromate.

The mean 72 h ErC50 is 17,87 mg $K_2Cr_2O_7$/L, with a lower and an upper 95 % confidence limit of 9.28 mg/L and 26.46 mg/L respectively.

This interlaboratory comparison revealed that 11 participating labs found consistent results without outliers or stragglers.

The intra-laboratory variability (repeatability) is very low (CV % = 6.22 %), and the inter-laboratory variability (reproducibility) is acceptable (CV % = 24.53 %) for an ecotoxicological test, taking into account the biological variability of living organisms.

The mean ErC50 and the inter-laboratory variability of this ringtest performed in long cells are virtually identical to those reported in ISO standard 10253 for a ringtest performed in 1989/1990 in culture flasks.

.

BACKGROUND AND RATIONALE

The test procedure of the freshwater Algaltoxkit based on the growth measurement of the microalgae in long cells has been accepted by the ISO and was published in 2012 as Annex B of the ISO standard 8692.

A request has been submitted to the ISO in February 2014 by the Italian Standardization Organization UNI to also add the marine Algaltoxkit test procedure in long cells as an Annex to the ISO standard 10253 on the marine algal growth inhibition test which is presently under revision.

This proposal is now under consideration in ISO but a request was made by the working group ISO/TC 147/SC5 that data should be provided on the precision of the marine algal test in long cells generated by an International Interlaboratory Comparison.

A general rule of ISO in this regard is that the data must be provided by laboratories who are not “first time users” of the assay, but already have experience with the specific test procedure (ISO 5725-1: 1994)[1].

ORGANIZATION OF AN INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT

An invitation for participation in this ringtest has therefore been made to laboratories in different countries known to perform marine Algaltoxkit tests with *Phaeodactylum tricornutum*, specifying that the participation was “free of charge” (= no subscription charge).

Based on the positive answer of several of these laboratories, it was then decided to organize the International Interlaboratory Comparison on the marine Algaltoxkit microbiotest in September 2014.

All the participants in this international ringtest received in September – October 2014 “one marine Algaltoxkit”, prepared by the company MicroBioTests Inc.

The specific Data treatment program for Algaltoxkit tests, for calculation of the ErC50 was also sent to all the participants.

The participating laboratories were requested to perform one complete marine Algaltoxkit test on the reference chemical potassium dichromate ($K_2Cr_2O_7$), and to send their results to the organizer within one month of reception of the kit.

NB : Each marine Algaltoxkit allows to perform 2 complete bioassays, but only one test has to be carried out in the framework of this ringtest.

A second assay could be performed in case of problems with the first test.

The Operational Procedure to be followed for the Marine Algaltoxkit ringtest is the test procedure which is described in the “Standard Operational Procedure” manual of the Marine Algaltoxkit, in the section “Reference Test”, but in a test concentration range of 32 mg/L to 3.2 mg/L potassium dichromate.

In order to avoid as much as possible operational errors, a detailed Operational Procedure for the reference test with potassium dichromate, which was to be strictly followed, was sent to the participating labs. This Operational Procedure is reported in Annex 1.

IMPORTANT NOTE

The preparation of the concentrated algal suspension is based on the use of the OD/N sheet included in each Algaltoxkit , and the OD/N relationship is established with a “Jenway 6300 spectrophotometer”.

[1] ISO 5725-1:1994/TECHNICAL CORRIGENDUM 1:1998. Accuracy (trueness and precision) of measurement methods and results -- Part 1: General principles and definitions

The OD measurements for the reference test must therefore basically also be made with a Jenway 6300 spectrophotometer (which will be the case for most participants in this ringtest).

In case a different type of spectrophotometer is used, the OD values measured may indeed not correspond exactly with the algal numbers of the OD/N regression and have in this case to be checked first by algal counting (e.g. with an hemocytometer or a Bürker chamber).

PARTICIPATION IN THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT

The twelve participating laboratories, institutes, organisations and companies are given hereunder, per country.

Belgium
- EPAS – Ghent
- Laboratorium ECCA - Merelbeke
- MicroBioTests Inc. – Mariakerke (Ghent)

France
- Laboratoire ECOMERS - Nice
- TOTAL SA - Pôle d'Etudes et de Recherche de Lacq - Lacq

Italy
- C.A.D.A. s.n.c. - Menfi (AG)
- Shoreline Soc. Coop. - Trieste
- Chelab Srl - Resana (TV)

Poland
- Lab. Biochemical Ecology of Microorganisms Institute of Oceanography University of Gdansk - Gdynia

Spain
- IK4-TEKNIKER, Polo Tecnologico De Eibar - Eibar. Gipuzkoa
- XENOBIOTICS - Valencia

UK
- Opus - Orkney

TEST PROTOCOL OF THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT

The detailed test protocol which was sent to all those participating in the ringtest is added in Annex 1 of this Report.

The test kit also contained an Excel programme and Results sheets for the data storage and treatment and calculation of the ErC50.

SHIPMENT OF THE TEST KITS

All the test kits were prepared in MicroBioTests Inc. and sent to the 12 participants in September – October 2014 by courier service.

According to the conditions for participation, this meant that the results of the test had to be sent to the organizer by end of November 2014 "at the latest".

RESULTS OF THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE MARINE ALGALTOXKIT

All 12 participants performed the algal toxicity test and sent their results. Five labs repeated a second time the test, so that eventually a total of 17 data sets have been received.

One laboratory, however, did not perform the assay according to the prescribed test procedure, and had carried out the assay in 1 cm spectrophotometric cells instead of the 10 cm long cells. The outcome

furthermore did not abide by all the validity criteria, so the results of this laboratory could not be taken into consideration.

In order “to put the same weight” on the results of each participating laboratory for the ErC50 estimate, it was decided to make the statistical analysis (and the discussions) on “only one” result from the 5 labs that had carried out 2 assays, i.e. the data of their second test.

For this International Interlaboratory Comparison the considered endpoint has been the ErC50 at 72 h.

MEASUREMENTS

As indicated in the test protocol (see Annex 1), the participants were requested to measure the OD in each long cell after 24 h, 48 h and 72 h incubation, and score the results on the Results Sheet

The Excel Sheet of the “Algaltoxkit Data Treatment programme – Calculation of the 72h ErC50” then calculates the 72h ErC50 and the 72h ErC10 and 72h ErC20, along with the respective 95 % confidence limits.

STATISTICAL ANALYSIS

It was stipulated by the organiser that the statistical analysis of all the data would be made by I.S.E., as specified in Annex 2, to determine the repeatability and reproducibility of this International Interlaboratory Comparison, according to the ISO 5725-2[2] procedure.

VALIDITY CRITERIA

For the reference test to be valid, the following 3 conditions must be met :

1. The mean algal density in the control replicates must have increased by a factor of at least 16 after 3 days incubation. This increase corresponds to a specific growth rate of 0.9 per day.

2. The variation coefficient of the specific growth rates in the controls should not be higher than 7 %.

3. The mean pH value in the control replicates should not have increased by more than 1 pH unit by the end of the test.

The 11 results that could be taken into consideration all respect this validity criterion.

DATA TREATMENT

As indicated in Annex 2 – Statistical Analysis, it had been agreed with the participants that their results would be treated confidentially without mentioning their names in the presentation and the discussion of the results. Each participating laboratories has therefore been assigned a code number which has only been disclosed to the concerned participant

REPEATABILITY AND REPRODUCIBILITY

The statistical analysis for the repeatability and reproducibility of this International Interlaboratory Comparison follows the ISO 5725-2 (2002) procedure. The data treatment is explained in Annex 2.

Hill model ErC50 Macro Regtox

[2] ISO 5725-2:1994/Cor 1:2002. Accuracy (trueness and precision) of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

The ErC50 data calculated with the Macro Regtox for the 11 labs have been summarized in Figure 1, as histograms ordered from the lowest to the highest average of each laboratory, and the data on repeatability and reproducibility are reported in table 1.

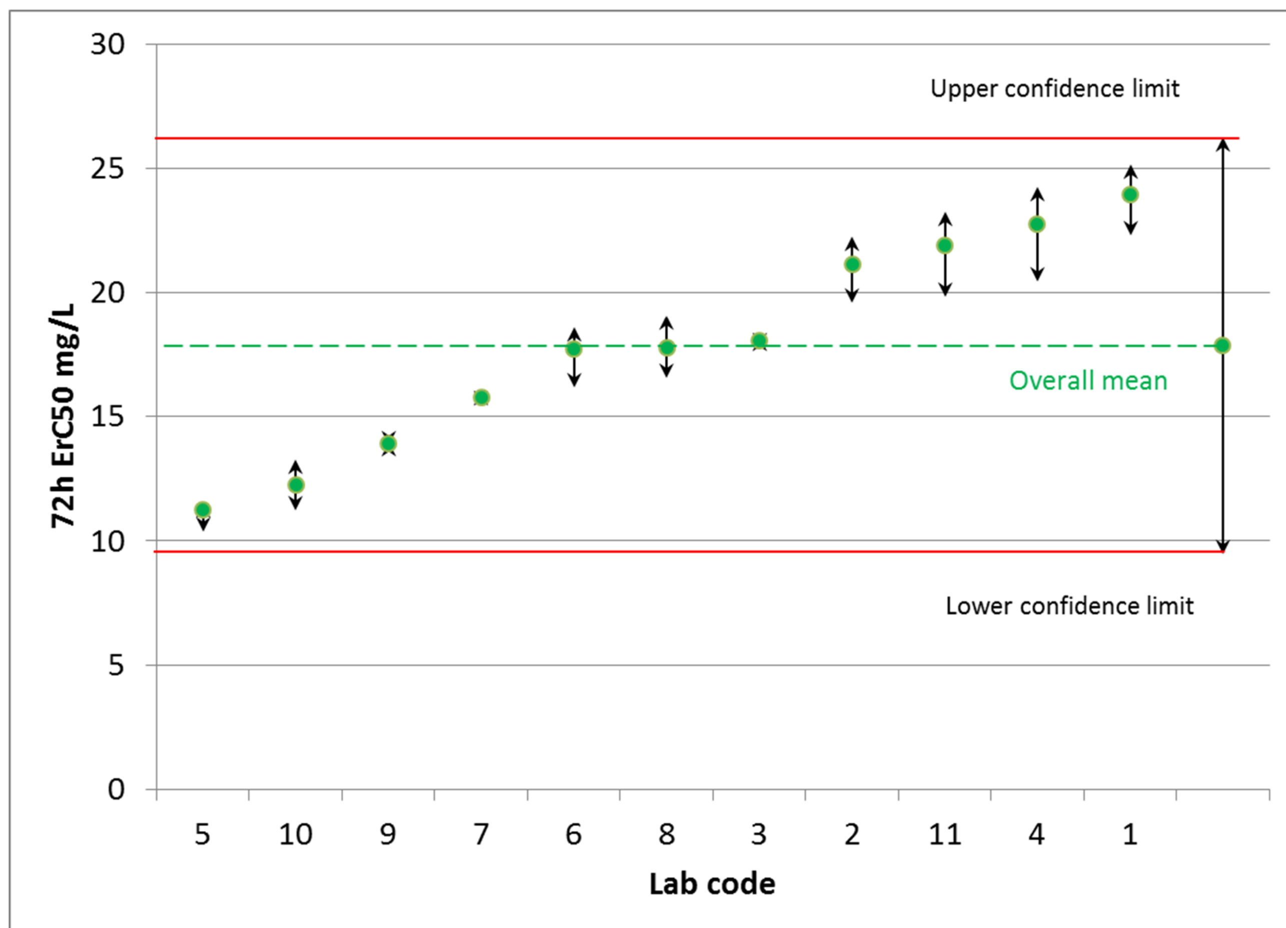


Fig. 1 - ErC50 (Hill model Macro Regtox) from 11 labs, in increasing order, along with their respective 95 % confidence limits.

Tab. 1 – Mean EC50 values estimated by the Hill model Macro Regtox) and their 95 % upper (UCL) and lower confidence limits (LCL).

n lab	n replicates	Mean	s_r	CV%	s_R	CV%	UCL	LCL
11	3	17.87	1.11	6.22	4.38	24.53	26.46	9.28

The Macro Regtox produced remarkably comparable data, with no outliers and no stragglers.

Conclusions

This interlaboratory comparison points out that 11 out of 12 participating labs found consistent results and that no outliers or stragglers were detected in this ringtest.

The intra-laboratory variability (repeatability) is very low (CV % = 6.22 %), and the inter-laboratory variability (reproducibility) is acceptable (CV % = 24.53 %) for an ecotoxicological test, taking into account the biological variability of living organisms.

The robustness of the marine algal test in long cells is clearly shown when comparing the outcome of this International Interlaboratory Comparison with the data of the ringtest performed in 1989/1990 and reported in the table on the latter ringtest in ISO standard 10253. For the tests with *Phaeodactylum tricornutum* on potassium dichromate (with 10 participants and 3 outliers) the mean ErC50 is 20.1 mg/L potassium dichromate with a variation coefficient of 26%. The outcome of the present ringtest with the long cells (mean ErC50 = 17.87 mg/L and CV% = 24%) is hence "virtually the same" as that of the very first ringtest performed 25 years ago in culture flasks.

Annex 1
PROCEDURE FOR THE PERFORMANCE OF A REFERENCE TEST WITH $K_2Cr_2O_7$

OPERATIONAL PROCEDURE

1. PREPARATION OF ALGAL CULTURING MEDIUM (see Figure).

Preparation of synthetic Seawater

1. Fill a **2 liter** volumetric flask with approximately 1500 ml deionized water.

2. Take vial number 1 (NaCl) and pour the contents in the flask. Shake until all the salt is dissolved.

3. Uncap the vial with concentrated salt solution labelled number 2 (KCl), and pour the contents into the volumetric flask.

4. Repeat step 3 for the other vials with concentrated salt solutions i.e. vial number 3 ($CaCl_2$), vial number 4 ($MgCl_2$), vial number 5 ($MgSO_4$), vial number 6 ($NaHCO_3$) and vial number 7 (H_3BO_3), respecting this sequence.

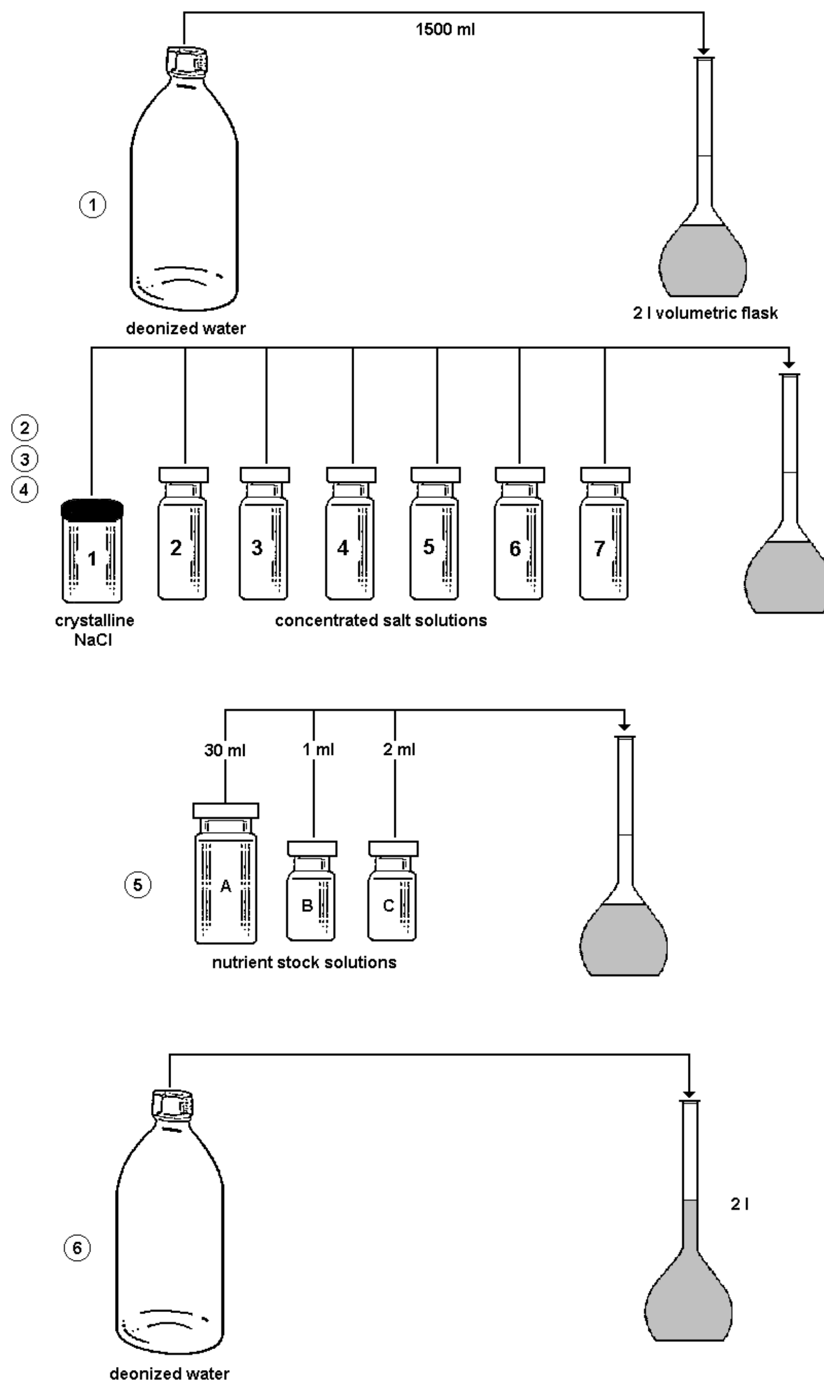
Addition of Nutrient Stock Solutions

5. Add 30 ml (2 x 15 ml) of Stock Solution A, 1 ml of Stock Solution B and 2 ml of Stock Solution C to the volumetric flask.

6. Add pure water (deionized or distilled) up to the 2000 ml mark and shake to homogenize the medium.

NOTE : Two liters algal culturing medium are normally prepared for performance of 2 complete algal bioassays, but only half of it will be needed for the performance of the reference test.
The culturing medium should *be stored in the refrigerator in darkness.*

PREPARATION OF ALGAL CULTURING MEDIUM

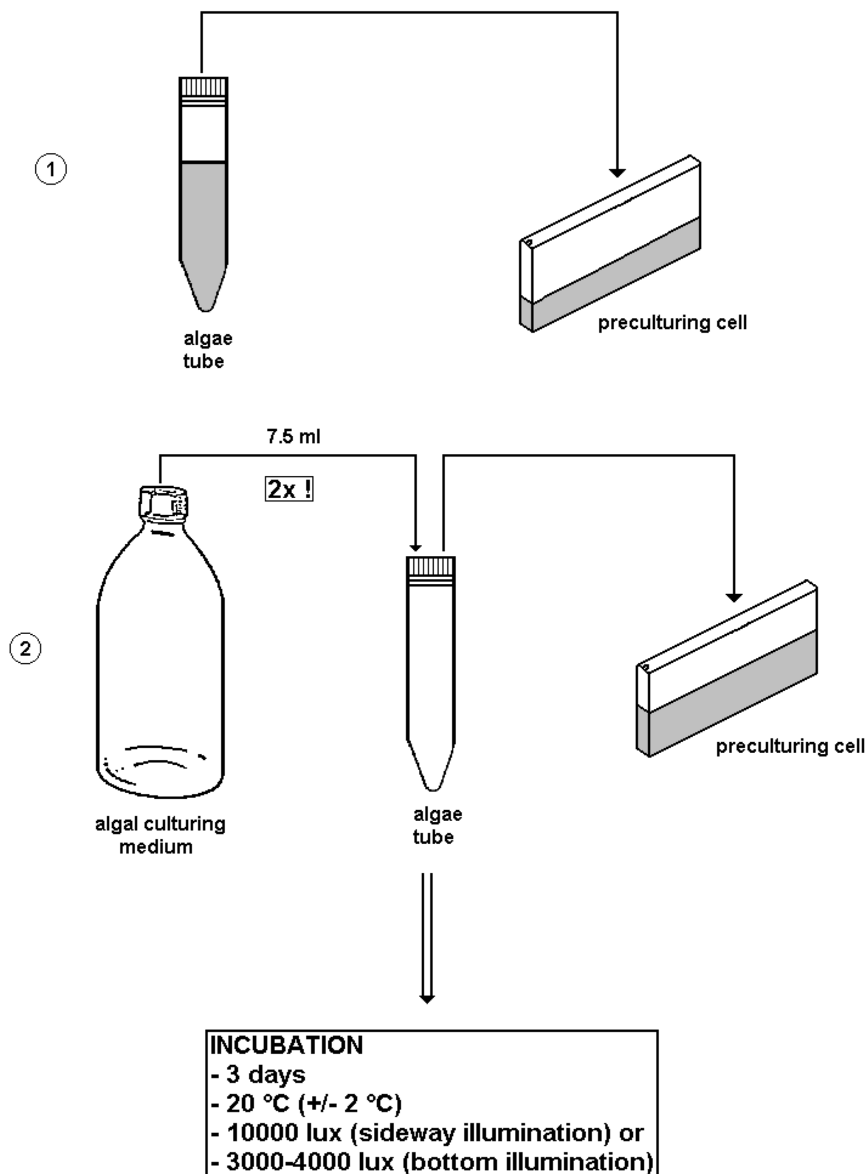


2. PRECULTURING OF THE ALGAE (see Figure)

1. Take one of the two tubes containing the microalgae inoculum, handshake it vigorously and pour out the contents into one of the pre-culturing cells.

2. Rinse the (same) tube **twice** with 7.5 ml algal culturing medium and transfer the contents into the pre-culturing cell to ensure the total transfer of the microalgae inoculum.

3. Close the pre-culturing cell with the lid and incubate the long cell **for 3 days** in an incubator or a temperature controlled room at **20 °C** (+/- 2 °C), with a constant uniform illumination supplied by cool white fluorescent lamps. The illumination should be **10 000 lux** in case of sideway illumination of the long cell or **3 000-4 000 lux** for bottom illumination.



3. PREPARATION OF CONCENTRATED ALGAL INOCULUM (see Figure)

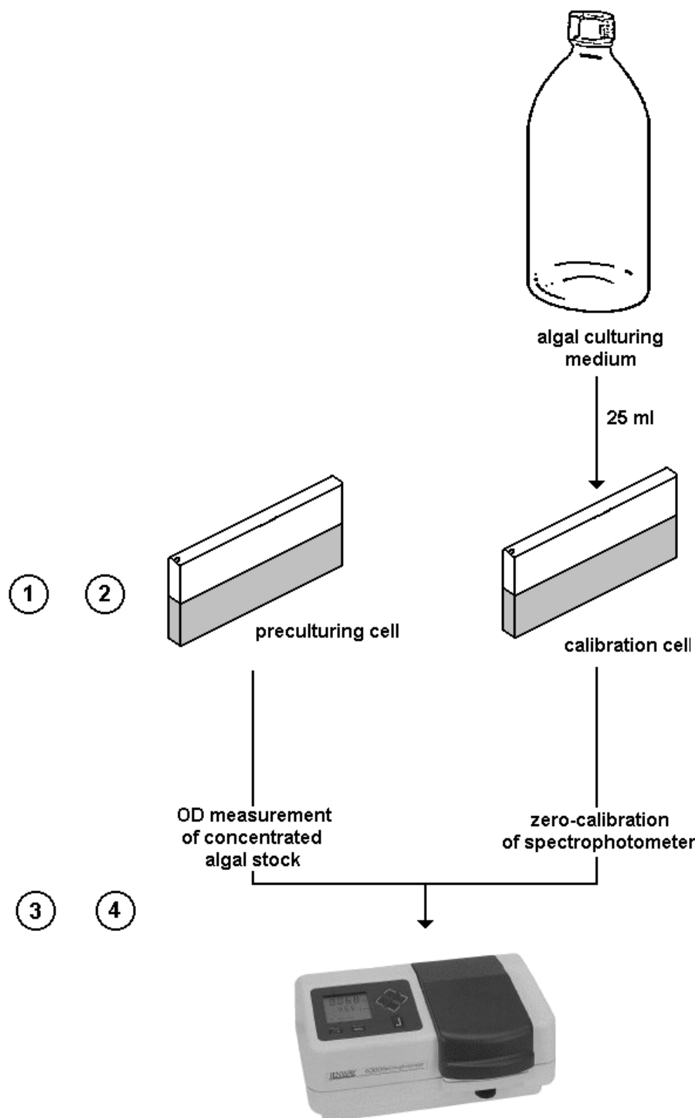
1. After 3 days incubation, take the pre-culturing cell and shake it to homogenize the algal suspension.

To ensure maximum reproducibility, this operation - which will subsequently be applied to all OD measurements of algal suspensions in long cells - should be performed in a standard way.

2. Take the long cell with the label "Calibration long cell", fill it with 25 ml algal culturing medium and close the cell with the lid.

3. Put this cell in the spectrophotometer and zero-calibrate the instrument.

PREPARATION OF CONCENTRATED ALGAL INOCULUM



4. Put the pre-culturing cell in the spectrophotometer and read the optical density (OD1) after 10 seconds.

5. Take the optical density/algal number (OD/N) sheet and look up the number of algae (N1) corresponding with OD1.

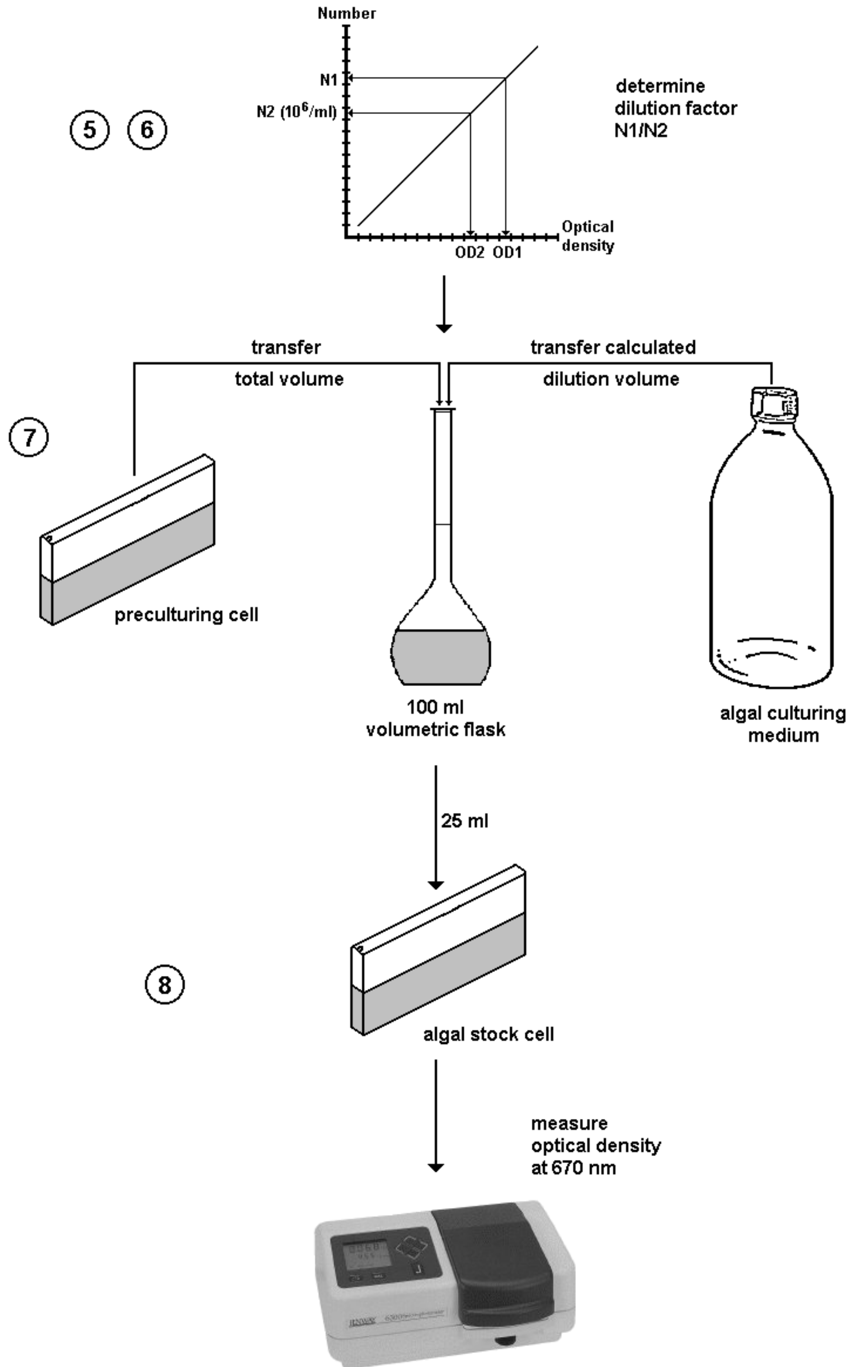
6. With N2 equal to 1.10^6 algae/ml, calculate from the N1/N2 ratio the dilution factor needed to reach an optical density equal to OD2, corresponding to an algal density of 1.10^6 cells/ml.

7. Transfer the algal suspension from the culturing cell into a 100 ml flask and add the volume of algal culturing medium needed to make up a 1.10^6 cells/ml suspension.
8. Stopper and shake the flask thoroughly to distribute the algae evenly.

9. Rinse the pre-culturing cell, transfer 25 ml of the 1.10^6 algae/ml into this cell, put the lid on the cell, shake gently and read the OD after 10 seconds.

10. Check on the OD/N graph whether the OD corresponds with the desired OD2 value (1.10^6 algal cells/ml).

NOTE : An example for calculation of the dilution factor and its application (based on an OD/N "Example Sheet"), is given in Annex A of this Operational Procedure for the reference test.



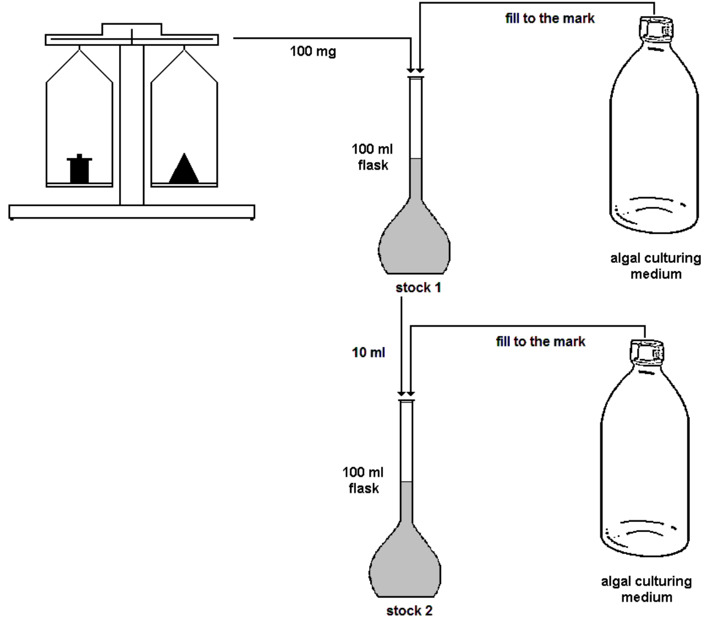
Number
N1
N2 (10⁶/ml)
Optical density
OD2 OD1
determine dilution factor N1/N2
5
6
transfer
total volume
transfer calculated
dilution volume
7
preculturing cell
100 ml volumetric flask
algal culturing medium
25 ml
8
algal stock cell
measure optical density at 670 nm

4. PREPARATION OF THE TOXICANT DILUTION SERIES

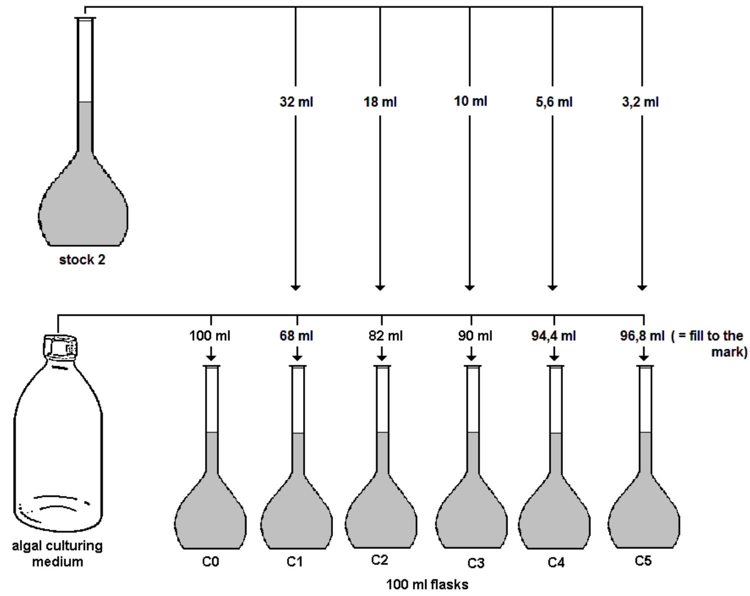
(see Figure)

The test will be performed in the following toxicant concentration range :
32 mg/l – 18 mg/l – 10 mg/l – 5,6 mg/l – 3,2 mg/l potassium dichromate.

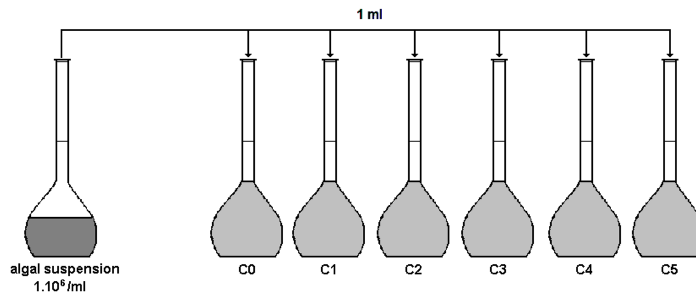
1. PREPARATION OF STOCK SOLUTION



2. PREPARATION OF TOXICANT DILUTIONS



3. ADDITION OF ALGAE



1. Take 8 calibrated flasks of 100 ml contents, label two of them as 'Stock 1' and 'Stock 2' and the others C0, C1 to C5.

2. Weigh 100 mg potassium dichromate on an analytical balance and transfer it into 'Stock 1' flask. Add algal culturing medium to the mark and shake to dissolve the chemical and to obtain a 1 g/l ('Stock 1') concentration.

3. Transfer 10 ml from 'Stock 1' into 'Stock 2' flask and fill to the mark with algal culturing medium. Shake to homogenize the contents and make a 100 mg/l ('Stock 2') toxicant concentration.

4. Transfer the following volumes of toxicant solution from 'Stock 2' into the following flasks :
   - 32 ml to flask C1
   - 18 ml to flask C2
   - 10 ml to flask C3
   - 5,6 ml to flask C4
   - 3,2 ml to flask C5

5. Add algal culturing medium up to the 100 ml mark in the C0, C1, C2, C3, C4 and C5 flasks.

6. Add 1 ml algal suspension to flasks C0, C1, C2, C3, C4 and C5 in order to obtain an algal density of 1.10^4/ml in each flask. Stopper and shake the flasks thoroughly to distribute the algal suspensions evenly.

NOTE : the addition of 1 ml concentrated algal suspension to the flasks containing 100 ml toxicant solution leads to a "1% decrease" of the toxicant concentrations.

5. TRANSFER OF THE ALGAE-TOXICANT DILUTIONS IN THE LONG CELLS

1. Take one tray with its 18 long cells and mark the cells on their lid from C0 to C5, in 3 replicates (i.e. 3 long cells for each test concentration).

2. Open all the long cells by lifting up their lid on one end.

3. Pour the contents of the C0 flask (= "the control) in a beaker and measure the pH.

4. After thorough shaking, pour 25 ml of the algae-toxicant dilutions from each 100 ml flask (and from the beaker) into the corresponding 3 long cells.

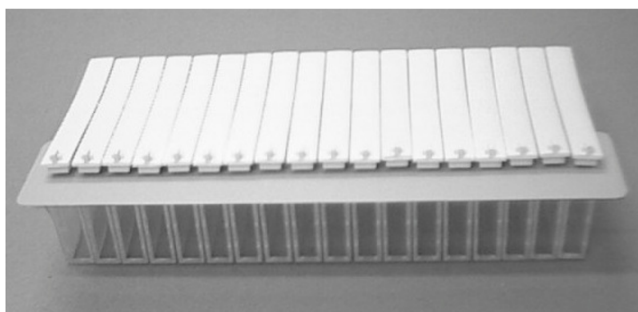
4. Close all the long cells with their lid, shake them to ensure a homogenous algal suspension, and measure the OD of each long cell in the spectrophotometer.

5. Score all the t0h OD values on the Result Sheet (see copy of the Results Sheet in Annex B)

6. INCUBATION OF THE LONG CELLS

1. Put all the long cells back into their holding tray, lift up slightly all the lids on the same side and slide the plastic strip over the open part of the long cells, taking care to leave an opening near the middle of the long cells for gas exchange (see photo below).

NOTE : In order to compensate for possible small "site to site" differences during incubation, the long cells must be placed in the holding tray <u>in a random way</u> (i.e. not in the sequence CO to C5, and not all three parallels from each toxicant concentration next to each other).



2. Put the holding tray in the incubator or in a temperature controlled room at 20°C (± 2°C) with a constant uniform illumination of 10000 lux for sideway illumination, or 3000-4000 lux for bottom illumination.

7. SCORING OF THE RESULTS

After 24h, 48h and 72h incubation, measure the OD in each long cell and score the results on the Results Sheet, a copy of which is given in Annex B.

The OD measurements shall be made after zero-calibration of the spectrophotometer and thorough shaking of the long cell (to re-suspend the algae).

Put the long cells back in the holding tray in a random way after the daily measurement.

At the end of the 3 days incubation, pour the contents of the 3 control long cells in a beaker and measure the mean pH of the 3 control long cells.

8. DATA TREATMENT

Transfer all the OD data noted in the Results Sheet to the Excel Sheet of the "Algaltoxkit Data Treatment programme – Calculation of the 72h ErC50".

Follow the instructions given in the "Instructions Sheet" for Algaltoxkit data treatment ErC50, for the calculation of the 72h ErC50 and the 72h ErC10 and 72h ErC20.

NOTE : a copy of the Instructions Sheet for the Algaltoxkit data treatment ErC50 programme is given in Annex C.

9. VALIDITY CRITERIA FOR THE REFERENCE TEST

For the reference test to be valid, the following 3 conditions must be met :

1. The mean algal density in the control replicates must have increased by a factor of at least 16 after 3 days incubation. This increase corresponds to a specific growth rate of 0,9 per day.
2. The variation coefficient of the specific growth rates in the controls should not be higher than 7%.
3. The mean pH value in the control replicates should not have increased by more than 1 pH unit by the end of the test.

NOTE : For the validity criteria 1 and 2, the validity conditions of the reference test can be checked on the Excel data sheet "Algaltoxkit Data Treatment – Calculation of the 72h ErC50.
1. The increase factor of the number of algae can be checked in "Table 2 – OD to N conversion" which shows the mean number of algae in the control at t72h.
Calculate from the t72h and t0h values the t72h/t0h ratio. This ratio must be higher than 16.
2. The variation coefficient of the specific growth rates in the controls can be found in "Table 4 – Average specific growth rate (µ) after 72h.
Check if the CV% in the controls is < 7%.

IMPORTANT NOTE : if any of the 3 stipulated validity criteria is not fulfilled, a second test must be performed (with the second set of materials provided in the kit).

The Excel sheet of the Algaltoxkit Data Treatment Programme, with the calculation of the 72h ErC50, has to be sent to Dr Renato Baudo (r.baudo@ise.cnr.it) who will perform the statistical analysis of the results of all the participants in this ringtest.

ANNEX A

Example of calculation for the dilution to be applied on the concentrated algal inoculum, to obtain an algal density of 1.10^6 cells/ml

Take a look at the OD/N sheet shown below, which also contains the OD/N regression formula specific for this OD/N sheet.

NB : each Algaltoxkit has its own OD/N sheet which is specific for this Algaltoxkit.

The regression formula in the OD/N sheet shown below is **y = 1674984x + 35804**

Suppose a measured OD1 value (x) in the pre-culturing cell of 1.2

The corresponding N1 value (y) is (1674984 x 1.2) + 35804 = 2.045784 algal cells/ml.

The N2 value to be reached is 1.10^6 cells/ml (= 1.000 000)

The N1/N2 ratio is 2,045, which means that a dilution factor of 2,045 must be applied to the concentrated algal suspension to reach an algal density of 1.10^6 cells/ml.

Transfer the contents of the pre-culturing cell in a graduated 100 ml cylinder and measure the total volume of the algal suspension.

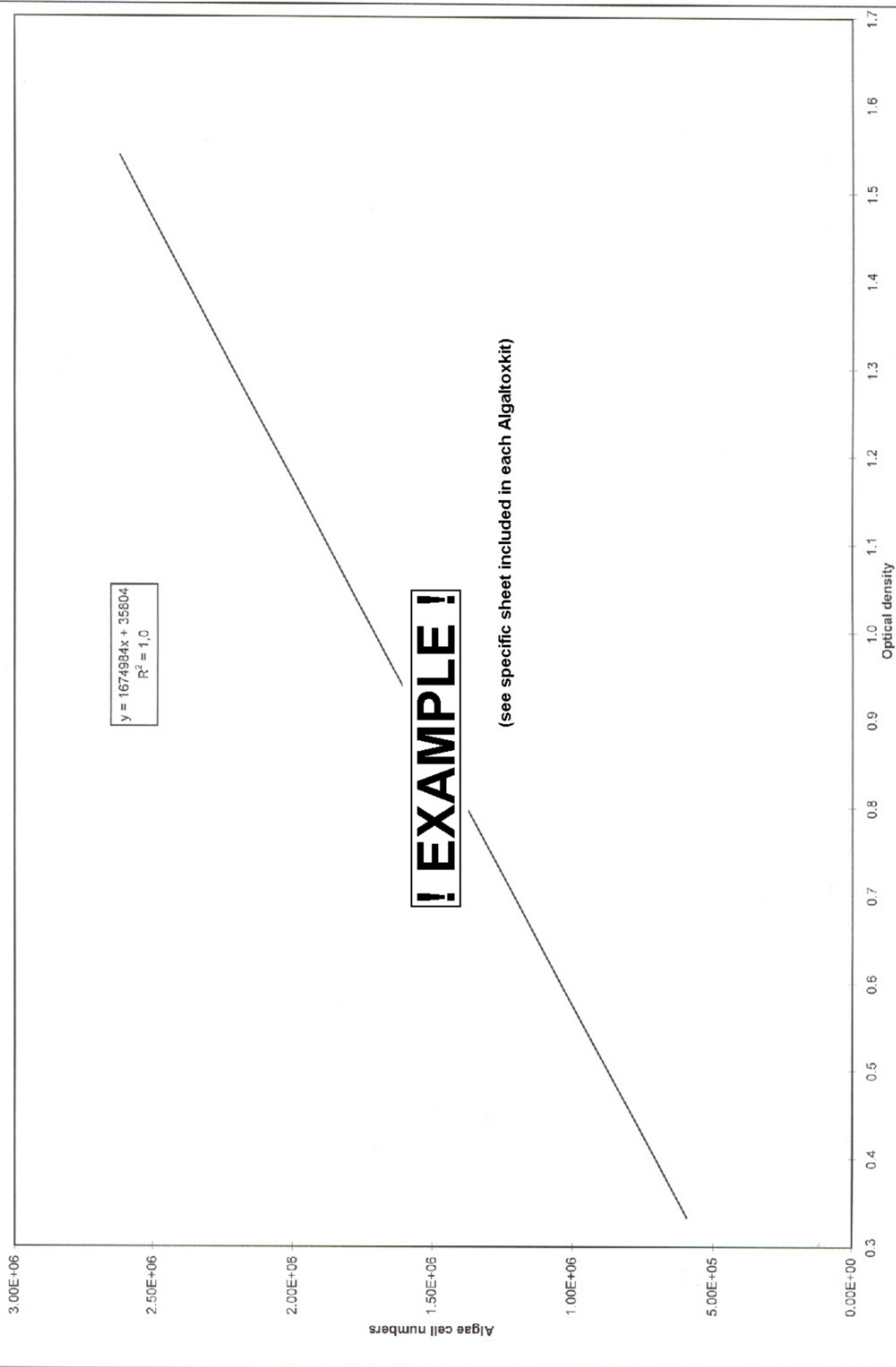
Let’s suppose that this total volume is 25 ml.

Transfer the concentrated algal suspension from the graduated cylinder in a 100 ml flask.

The original volume of concentrated algal suspension must be diluted by a factor 2,045 to reach the (requested) 1.10^6 algal cells/ml concentration.

This means that the final volume of algae in the graduated cylinder has to be 25 ml x 2,045 = 51 ml.

This is achieved by adding 26 ml algal culturing medium to the graduated cylinder to obtain an 1.10^6 ml algal cell concentration (i.e. 25 ml of the original concentrated algal suspension + 26 ml algal culturing medium = 51 ml).


RELATIONSHIP OPTICAL DENSITY TO NUMBER OF ALGAL CELLS
(OD mesured with the JENWAY long cell spectrofotometer at 670 nm)
y = 1674984x + 35804
R² = 1,0
! EXAMPLE !
(see specific sheet included in each Algaltoxkit)
Algae cell numbers
0.00E+00
5.00E+05
1.00E+06
1.50E+06
2.00E+06
2.50E+06
3.00E+06
Optical density
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7

ANNEX B

Marine Algaltoxkit – Results Sheet

MARINE ALGALTOXKIT - RESULTS SHEET

Name of operator : …………………………………………

Date of performance of test : ………………………………

Test species : ……………………………………………..

Toxicant tested : ……………………………………………

Dilution series tested : concentration 1 : ……………………………

concentration 2 : ……………………………

concentration 3 : ……………………………

concentration 4 : ……………………………

concentration 5 : ……………………………

OPTICAL DENSITY AT 670 nm							
Exposure time	**Replicate**	**Control**	**C5**	**C4**	**C3**	**C2**	**C1**
0h	**1**						
	2						
	3						
24h	**1**						
	2						
	3						
48h	**1**						
	2						
	3						
72h	**1**						
	2						
	3						

ANNEX C

Instructions Sheet for Algaltoxkit data treatment ErC50 programme

*** Start up Windows**
*** Open Microsoft Excel**
*** Open Al*galtoxkit Data Treatment ErC50.XLS*"**
*** Click on "switch on macro's"**
*** Click on "Enter Data" in box MicroBioTests Inc.**

<u>Fill in all the boxes which are "in blue colour"</u>

- **Specifics of the test** (*name of operator, etc.*)
- **Test concentrations** (C5 to C1)
- **OD values** for all the test concentrations and the 3 replicates, at 24h, 48h and 72h
- **Coefficients of the OD/N regression** : coefficient a
  coefficient b

N.B. The OD/N regression is specific for each batch of algae, and is given in the OD/N sheet of each Algaltoxkit

*<u>Important</u> : for coefficient b, <u>the **– sign** has also to be typed in !!</u>*

*<u>Example</u> : y = 1700481x – 68237 : **coefficient a** = 1700481 **; coefficient b** = **-** 68237*

<u>Calculations</u>

Go to the last line of the programme and click on "Calculate"

The programme will then start making the calculations and will show the data for the ErC50, the ErC10 and the ErC20, with the corresponding 95% confidence limits.

<u>Print or save</u>

The results can be printed and/or saved in a computer file.

Annex 2
STATISTICAL ANALYSIS

It was agreed with the participants that their results would be treated confidentially without mentioning names of the participating laboratories, organisations, institutes and companies in the presentation and discussion of the results. Therefore, in the following each laboratory is identified only by a randomly given code (the same for all data set).

The repeatability and reproducibility of the interlaboratory comparison have been calculated according to the ISO 5725-2 (2002)[3] procedure, providing the following results:

s_L^2 the estimate of the between-laboratory variance;

s_W^2 the estimate of the within-laboratory variance;

s_r^2 the arithmetic mean of the within-laboratory variances (after outliers have been excluded);

s_R^2 the estimate of the reproducibility variance: $s_R^2 = s_L^2 + s_r^2$.

To check the consistency of the data, the Mandel's h and k statistics have been used: the first (h) provides the between-laboratory consistency statistic, and the second (k) the within-laboratory consistency statistic.

The Grubb's test has then been applied to identify stragglers (if the test statistic is greater than its 5 % critical value and less than or equal to its 1 % critical value, the item tested is called a straggler and is indicated by a single asterisk), and outliers (if the test statistic is greater than its 1 % critical value, the item is called a statistical outlier and is indicated by a double asterisk).

If some straggler and/or outlier can be explained by a technical error, for example in transcribing a test result, after the proper correction the data is retained and the Grubb's test repeated. If it proves impossible to replace the suspect test result, then it should be discarded as a "genuine" outlier, while stragglers are retained as correct items. Therefore, the final statistics of this interlaboratory comparison, to be used in conclusions, include stragglers, but not outliers.

The tables show the statistical analysis, for all data and the same without h or k outliers (data higher than overall mean + 3 times the interlaboratory standard deviation s_R, or lower than overall mean - 3 times the interlaboratory standard deviation s_R), and without h or k stragglers (data higher than overall mean + 2 times the interlaboratory standard deviation s_R, or lower than overall mean - 2 times the interlaboratory standard deviation s_R).

[3] ISO 5725-2:1994/Cor 1:2002. Accuracy (trueness and precision) of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method