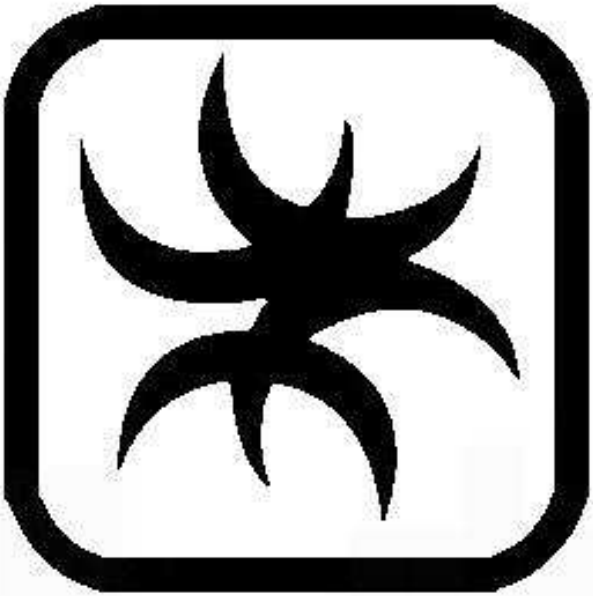
ALGALTOXKIT F

Freshwater Toxicity Test

with Microalgae



STANDARD OPERATING

PROCEDURE

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INTRODUCTION TO THE ALGALTOXKIT

Origin :
This new algal growth inhibition bioassay has been developed by the research teams of Prof. Dr. G. Persoone at the Laboratory for Biological Research in Aquatic Pollution (LABRAP) at the University of Ghent in Belgium.

Scope :
TOXKITS are microbiotests in kits containing all the necessary materials including the test organisms to perform simple, rapid, sensitive and reproducible tests at low cost. Toxkit tests are suited for toxicity of all chemicals and wastes released in aquatic as well as terrestrial environments.

Advantages of Toxkit tests :
The major advantage of Toxkits, in comparison to "conventional" bioassays, is that the test organisms are incorporated in the kits in a "resting" or "immobilized" form, from which they can be activated "on demand" prior to performance of the toxicity test.
This eliminates the need for continuous recruitment and/or stock culturing of test organisms, and hence the major cost factor.
Furthermore, all Toxkits have been "miniaturized" into low cost microbiotests which can be performed with conventional lab materials and equipment, on little bench space.

Advantages of the Algaltoxkit test

- No culturing

The Algaltoxkit makes use of microalgae which are immobilized in a special matrix in which they survive for several months without losing their viability. After de-immobilization and transfer into an adequate algal culturing medium, the microalgae indeed resume their growth immediately; the good physiological status is shown by the fact that the rate of division is analogous to that of algae from stock cultures.

- Rapid scoring

The Algaltoxkit makes use of a new type of test vials, namely disposable long cells in polystyrene. The "long cells" can be used with any type of spectrophotometer equipped with a holder for cells of 10 cm path-length.
Each cell is provided with a special lid in a chemically inert thermoplastic elastomer.

- Long cells as test vials

a. allow for the determination of the growth of the algae by optical density measurement directly into the test vials. This new technology leads to a substantial gain in time for the daily measurement of algal growth, in comparison to conventional counting techniques.

b. the use of cells with a 10 cm path-length allows the Algaltoxkit test to be performed in accordance with the conditions prescribed by national and international Guidelines (e.g. ISO, OECD, USEPA, ASTM). Optical density measurements can indeed by used as a rapid and very convenient technique for determining algal growth with good accuracy.

c. the long cells can contain 25 ml algae-toxicant test volume, yet take but a base surface of 12 cm^2. The holding tray containing the 18 test vials only takes 13 x 25 cm bench space. In practice this means that 3 Algaltoxkit tests can be performed on the space needed for one conventional algal assay.

d. since the long cells are low cost and disposable there is no need for costly and time consuming washing and cleaning of the test vials.

Principle of the Algaltoxkit test :

A 72h algal growth inhibition test is performed in long cell test vials, with *Selenastrum capricornutum* (first renamed as *Raphidocelis subcapitata* and presently as *Pseudokirchneriella subcapitata*) de-immobilized from algal beads. The Algaltoxkit test has been modelled on and follows the prescriptions of the OECD "Algal growth inhibition test" (Guideline 201) and the ISO "Water Quality - Freshwater Algal Growth Inhibition Tests with Unicellular Green Algae" (ISO Standard 8692).

Features :

Each Algaltoxkit contains all the (disposable) materials to perform two complete 72h assays (range finding or definitive tests). Besides conventional laboratory glassware, the only equipment needed is

a) an incubator (or a temperature controlled room at 23°C $\pm$ 2 °C) provided with light,
b) a spectrophotometer and a holder for cells of 10 cm path-length.

Sensitivity :
The sensitivity of the Algaltoxkit test has been determined for a variety of inorganic and organic chemicals and environmental samples in parallel to the conventional algal growth inhibition test. The data generated by various laboratories in different countries as well as by interlaboratory exercises indicate that the sensitivity of the algal microbiotest is the same as that of the conventional algal assay.

Precision :
A comparative study between the conventional algal test and the Algaltoxkit assay also revealed that the precision of the Algaltoxkit assays for both chemicals and natural samples is equal to (and even often better than) that of conventional algal tests.

Shelf life :
Since the growth rate of the microalgae stays constant even after storage for months in the refrigerator in darkness, the Algaltoxkit assays can be carried out within a time frame of several months, as indicated by the expiry date.

CONTENTS OF THE ALGALTOXKIT

Tubes with algal beads
Two tubes containing small beads with *Selenastrum capricornutum* microalgae immobilized in an inert matrix. The tubes with the storage medium and the algal beads should be kept in the refrigerator in darkness at 5 °C (+/- 2 °C) until use.

Matrix dissolving medium
One glass bottle containing the special medium to dissolve the matrix in which the microalgae are immobilized. The matrix dissolving medium must be stored in the refrigerator in darkness at 5°C (+/- 2° C) until use.

Concentrated algal growth medium
Five glass bottles containing concentrated solutions of various chemicals to make up 2 litres of algal culturing medium with deionized water, according to the formula of the OECD Guideline 201 "Alga, Growth Inhibition Test" and the ISO/DIS Guideline 8692 "Water Quality - Freshwater Algal Growth Inhibition Tests with Unicellular Green Algae". The vials should be stored in the refrigerator at 5°C (+/- 2° C) in darkness until use.

Long cells and holding trays
Two sets of 18 disposable 10cm path-length cells with lid, each in a transparent holding tray provided with two plastic strips. The long cells serve as test vials and allow for direct measurement of the OD in the test containers.

Calibration cell and Algal Stock cell
Two long cells with lid, for zero calibration of the spectrophotometer and scoring of the OD of the concentrated algal suspension.

Bench protocol
An abbreviated version of the detailed Standard Operational Procedure manual.

Standard Operational Procedure manual
A detailed brochure with all instructions for performance of range finding and/or definitive assays on pure chemicals and effluents. The SOP manual also contains an annex with the ISO instructions for data processing and calculation of the 72hEC50.

Results sheets
Sheets for data scoring of the daily OD readings in the long cell test vials.

Specification sheet
A sheet indicating the batch number of the algal beads, the matrix dissolving medium, the algal culturing medium, the expiry date of the Toxkit and the 72hEC50 for the reference chemical potassium dichromate.

COMPLIANCE WITH ISO STANDARD 8692 AND OECD GUIDELINE 201

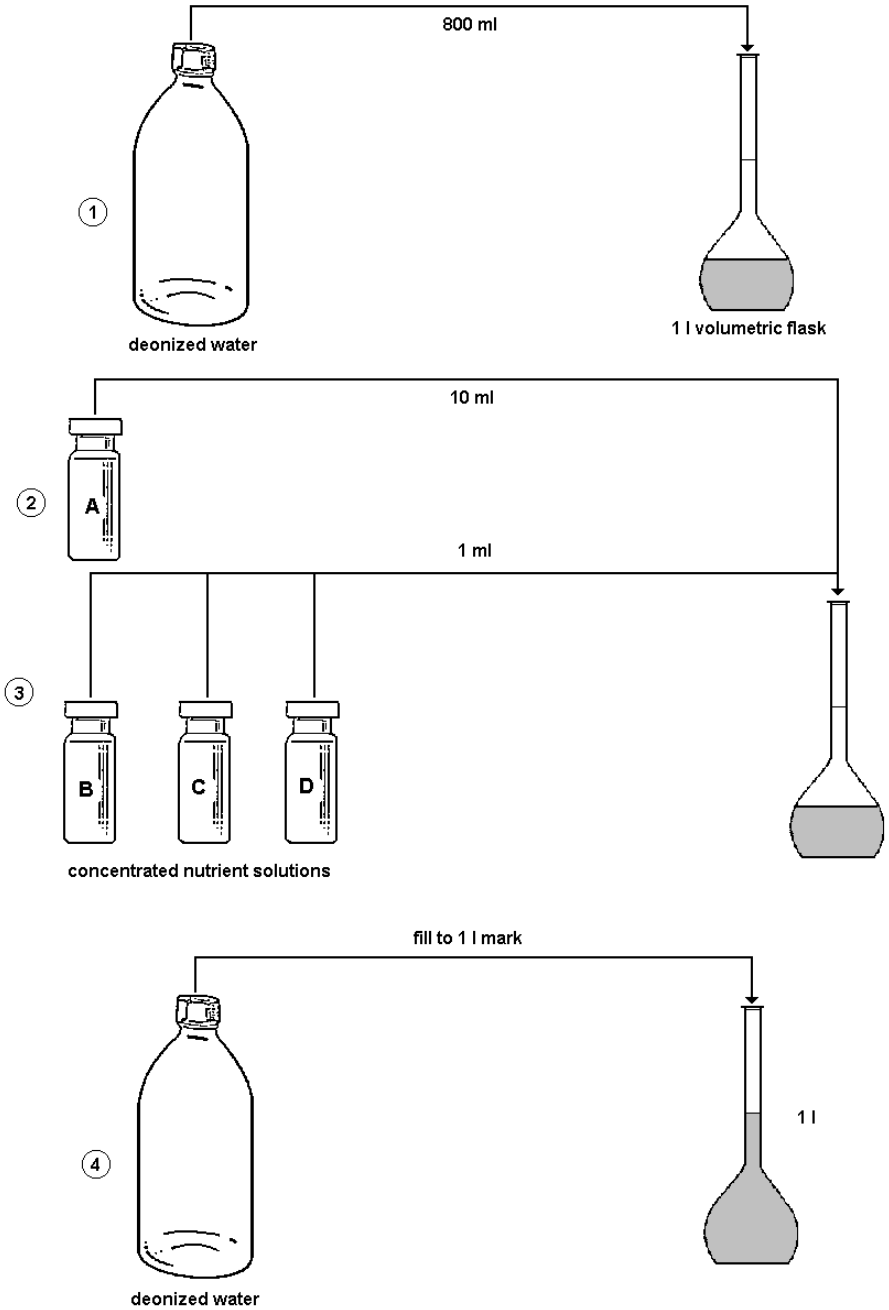
In order to comply with the specifications of the (recently modified) ISO standard 8692 and OECD Guideline 201 for algal growth inhibition tests, the following modifications have been incorporated in the present version of the Standard Operational Procedure of the Algaltoxkit microbiotest :

1. The measurement of the algal growth in the long cells is carried out after 24h, 48h and 72h incubation and must not be determined anymore at the start of the assay (i.e. at t0). ISO standard 8692 indeed specifies in section "7.7. Measurements" : "*the nominal cell density can be used as the initial cell density and no initial cell density measurement is then required".*

2. Calculation of the growth inhibition in the test concentrations versus the growth in the control is based on the determination of the average growth rates μ, after transformation of the OD values into cell numbers. A computer programme has been worked out for the Algaltoxkit data treatment, which calculates the 72hErC50, the 72hErC10 and the 72hErC20, with the corresponding 95% confidence limits. This computer programme is available free of charge to Algaltoxkit users.

N.B : For the convenience of Algaltoxkit users who are familiar with the (former) test procedure based on calculation of the 72hEbC50, two additional Results Sheets are added to each Algaltoxkit, in which the t0 optical density values can be noted.

PREPARATION OF ALGAL CULTURING MEDIUM



1. PREPARATION OF ALGAL CULTURING MEDIUM

Procedure (see Figure)

1. Transfer 800 ml deionized water into a 1000 ml volumetric flask (cf. ISO Standard 8692 - Section 5.3 '*Water*').

2. Uncap one of the two vials labelled "Nutrient Stock A" and transfer 10 ml into the flask.

3. Uncap vials labelled "Nutrient Stock B, C and D", and transfer 1 ml of each respective solution into the flask. Recap the vials and store them again in the refrigerator in darkness at 5°C (+/- 2°C) (cf. ISO Standard 8692 - Section 7.1 '*Preparation of growth medium*').

4. Fill the flask up to the 1 litre mark with deionized water, stopper the flask and shake thoroughly to homogenize the algal culturing medium.

5. Before use, equilibrate the solution by leaving it overnight in contact with air, or by bubbling with filtered air for 30 min. After equilibration, adjust the pH if necessary to 8.1 $\pm$ 0.2, with either 1 mol/l HCl or 1 mol/l NaOH.

*N.B. In THE ISO Standard 8692 - Section 5.3 '*Nutrients*' are named Stock solution(s) 1, 2, 3 and 4 (corresponding with Nutrient Stock(s) A, B, C and D of the Algaltoxkit).*

2. DE-IMMOBILIZATION OF THE ALGAE

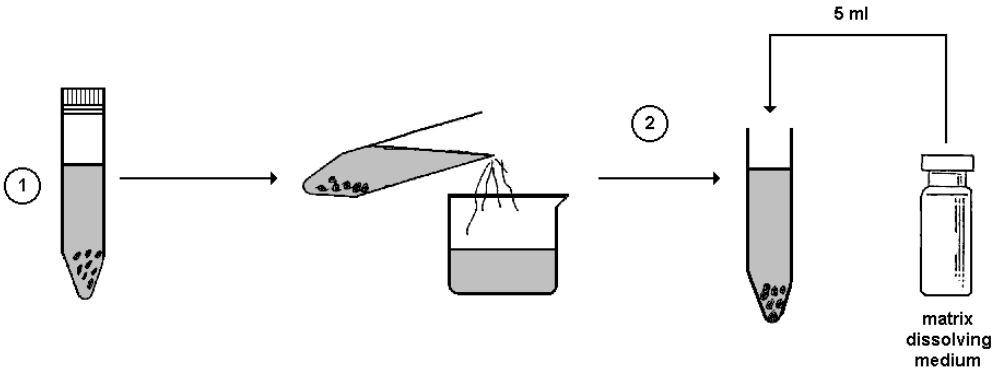
*According to ISO Standard 8692 - Section 5.1 '*Test organism*', the algae beads supplied by MicroBioTests Inc. are an example of a suitable commercially available product.*

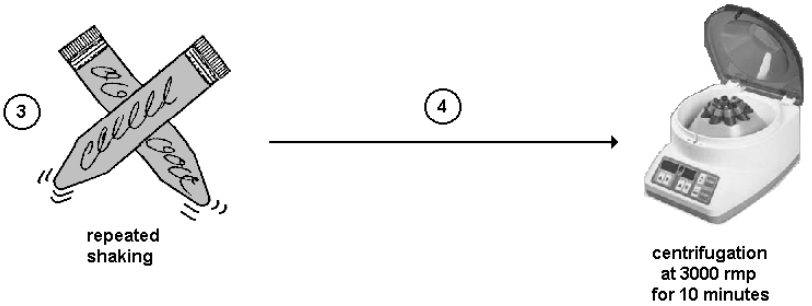
Procedure (see Figure : Preparation of algal inoculum)

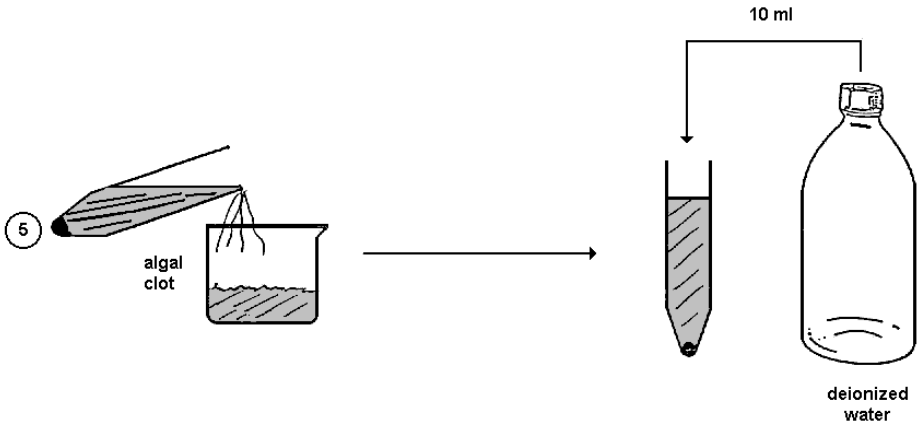
1. Take one of the two tubes containing algal beads and pour out the liquid; take care not to eliminate any of the algal beads during the process.

2. Open the vial labelled "Matrix dissolving medium" and transfer 5 ml to the tube.

PREPARATION OF ALGAL INOCULUM





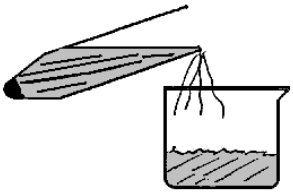


6 7

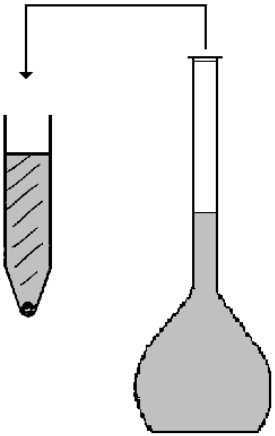
resuspension of algae
by shaking

centrifugation
at 3000 rmp
for 10 minutes

7 8



10 ml



algal culturing
medium

8

resuspension of algae
by shaking

algal suspension

3. Cap the tube and handshake vigorously. Repeat the shaking every two minutes until the matrix immobilizing the algae is totally dissolved. The algae should be entirely freed within 5 to 10 minutes. A Vortex shaker may be used to speed up the process.

4. Centrifuge the tube for 10 minutes at 3000 rpm in a conventional lab centrifuge.

5. Pour out the supernatant and replace it by 10 ml deionized water.

6. Cap the tube and shake it vigorously to re-suspend the algae homogenously.

7. Centrifuge the tube again at 3000 rpm for 10 minutes and decant the supernatant.

8. Re-suspend the algae in 10 ml algal culturing medium.

GENERAL RULES FOR RELIABLE OPTICAL DENSITY MEASUREMENTS OF ALGAL SUSPENSIONS

The Algaltoxkit technology is based on the (rapid) measurement of the optical density (OD) of algal cell suspensions in disposable spectrophotometric cells of 10 cm path-length, called "long cells".

(*Procedures are given in section 7 to cope with the interference problem of turbid or coloured samples.)*

Measurement of the OD can be performed with any spectrophotometer provided with a 670 nanometre filter, and equipped with a holder for 10 cm cells.

Optical densities can easily be converted into algal numbers with the aid of the regression formula, given on the OD/N-sheet which is included in each Algaltoxkit.

In order to maximize the reproducibility of the OD readings, the following precautions **must, however, be abided by very strictly** :

- The long cells must always be placed in the spectrophotometer in the same direction, namely with the arrows imprinted on both lateral sides of the cells pointing to the left.

- The spectrophotometer must be zero-calibrated prior to OD measurements of algal suspensions. Zero-calibration is performed with the calibration long cell filled with algal growth medium.
  It is advised to zero-calibrate the equipment at regular intervals during the daily OD measurements of algal suspensions.

- The algal suspensions in the long cells must be shaken for 10 seconds, immediately prior to their introduction in the spectrophotometer, to ensure a homogenous distribution of the algae.

- OD readings must be made within 10 seconds after shaking the cells, i.e. before the algae start to settle.

IMPORTANT REMARK :

The OD/N regression has been determined with a Jenway 6300 spectrophotometer (manufacturer : Jenway Ltd, England), and is specific for this type of equipment.
In case a different type of spectrophotometer is used, the OD values measured may not correspond exactly with the algal numbers of the regression provided. In such case it is advised to first control the OD readings with own algal counting, and if necessary to work out a new OD/N graph.

3. PREPARATION OF CONCENTRATED ALGAL INOCULUM

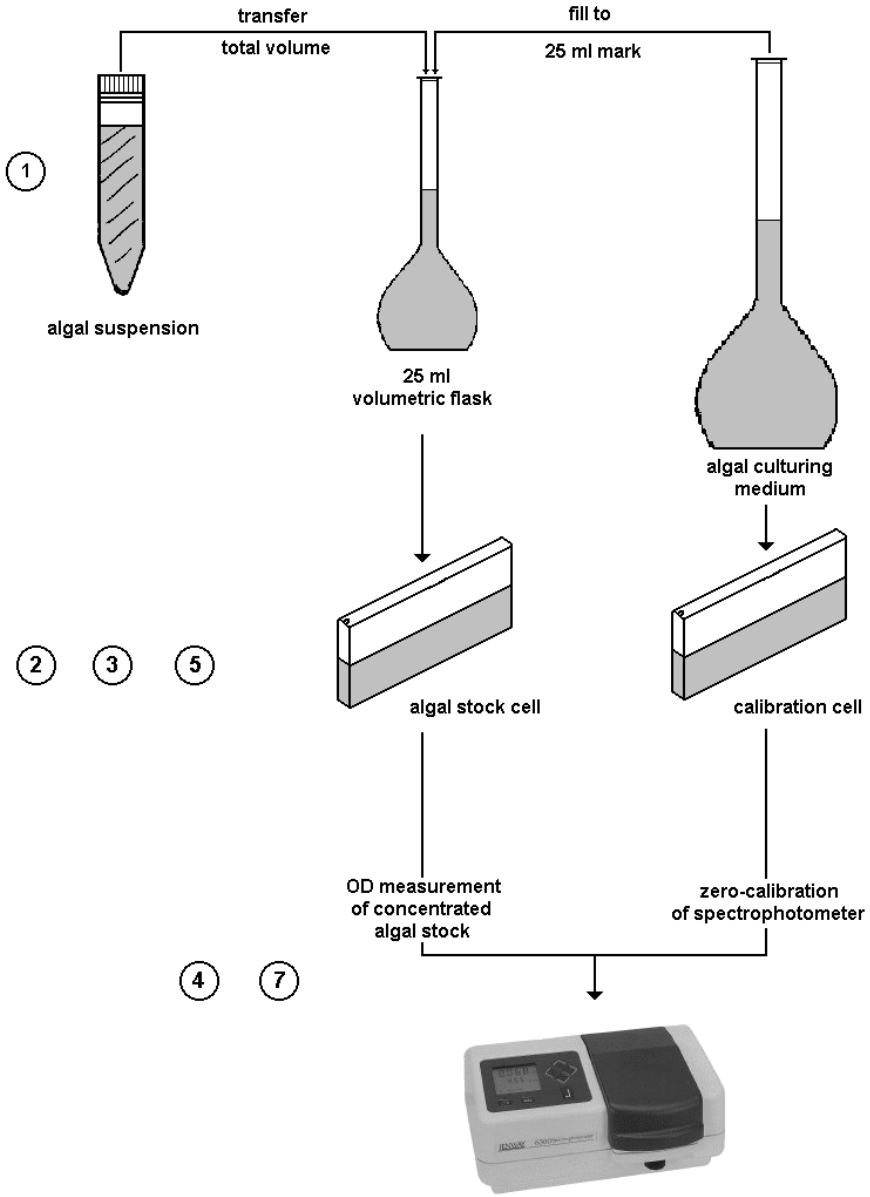
Procedure (see Figure)

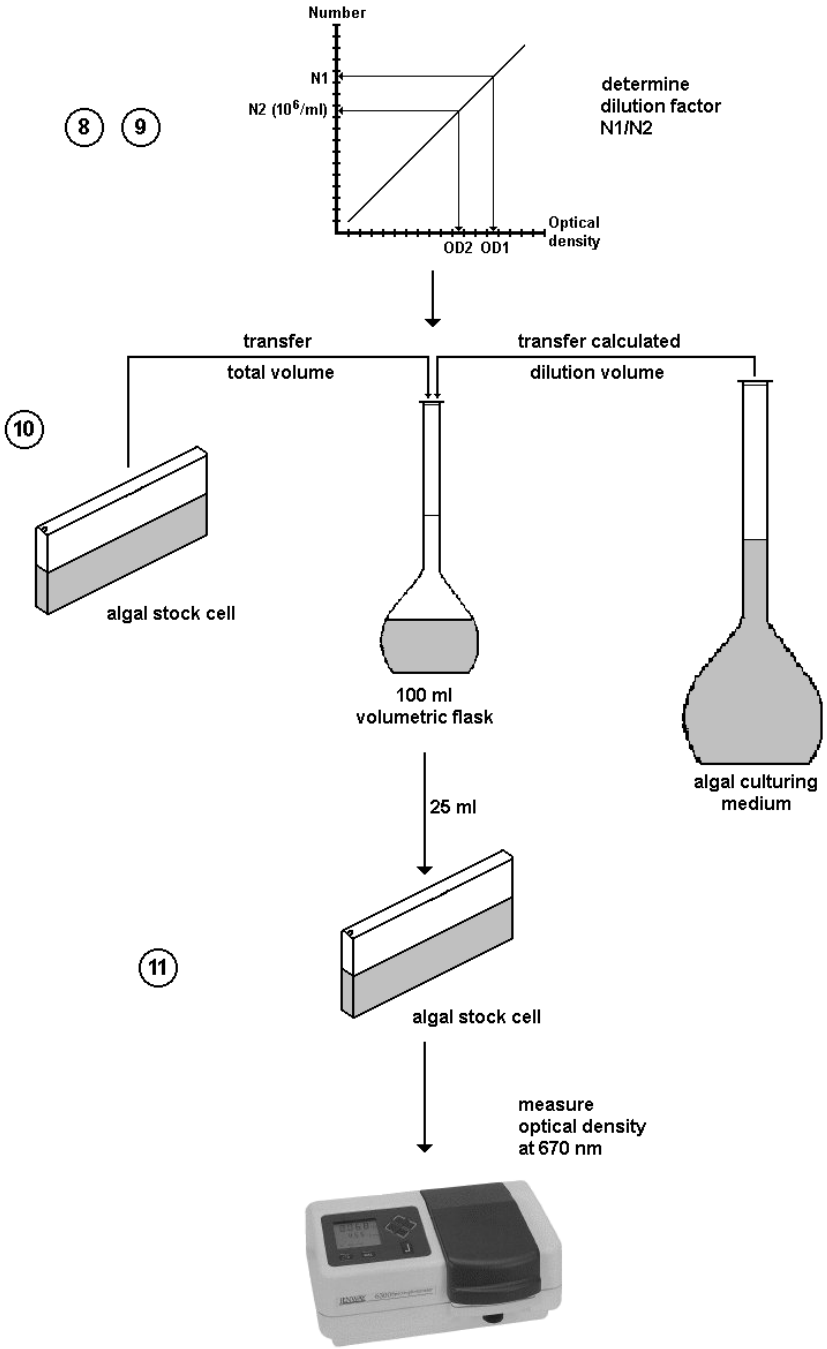
1. Pour the algal suspension from the tube into a 25 ml calibrated flask and add algal culturing medium to the 25 ml mark. Stopper and shake to homogenize the algal suspension.

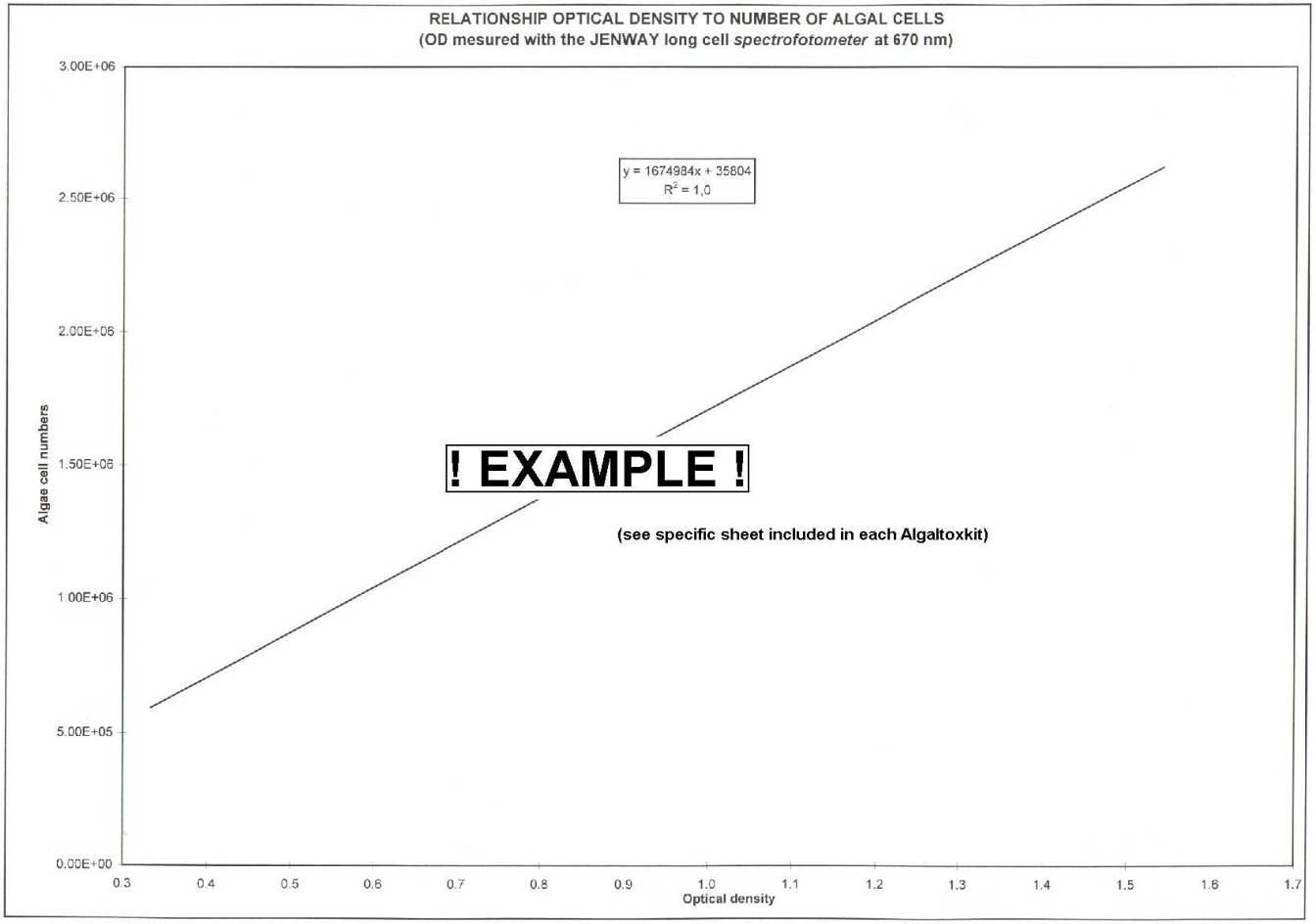
2. Take the two long cells with the labels "Calibration long cell" and "Algal Stock cell".

3. Fill the calibration cell with 25 ml algal culturing medium and close the cell with the lid.

PREPARATION OF CONCENTRATED ALGAL INOCULUM




Number
N1
N2 (10^6/ml)
Optical density
OD2 OD1
8
9
determine dilution factor N1/N2
transfer total volume
transfer calculated dilution volume
10
algal stock cell
100 ml volumetric flask
algal culturing medium
25 ml
11
algal stock cell
measure optical density at 670 nm


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
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
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
Optical density

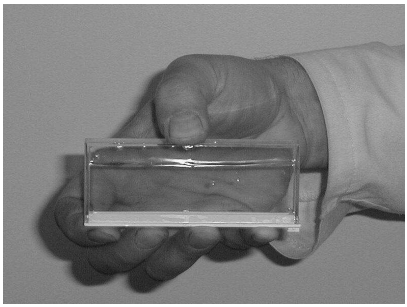
4. Put this cell in the spectrophotometer and zero-calibrate the instrument.

5. Transfer the algal suspension into the Algal Stock cell and tightly close the cell with the lid.

6. Shake this cell thoroughly to distribute the algal suspension evenly.

SHAKING PROCEDURE OF LONG CELLS WITH ALGAL SUSPENSIONS

1



2

- *Place thumb on the bottom of the cell, in the middle, and all other fingers on the lid, at equal distance from each other (see picture 1)*
- *Press firmly to ensure a tight closing of the cell*
- *Turn the cell upside down and gently shake for approximately 10 seconds (see picture 2)*
- *Turn the cell upwards again*
- *Put the cell in the spectrophotometer and read the OD after 10 seconds.*

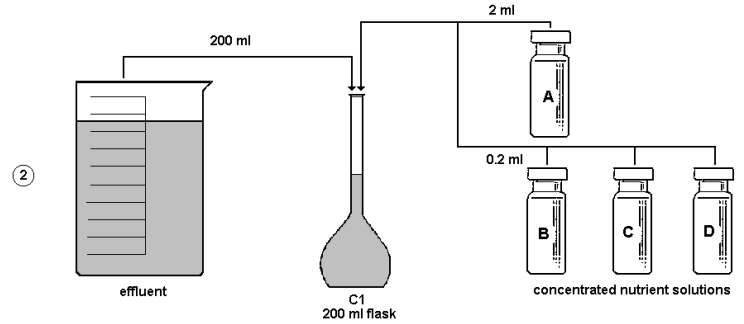
To ensure maximum reproducibility, this operation, which will subsequently be applied to all OD measurements of algal suspensions in long cells, should be performed in a standard way.

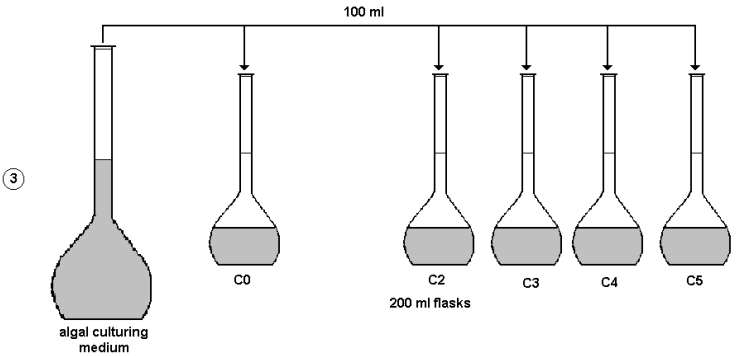
7. Put the Algal Stock cell in the spectrophotometer and read the optical density (OD1) after 10 seconds.

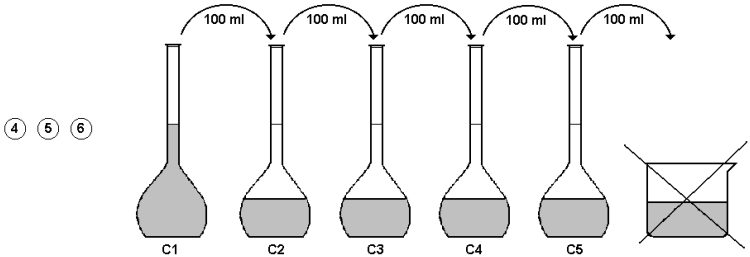
8. Take the optical density/algal number (OD/N) sheet and look up the number of algae (N1) corresponding with OD1.

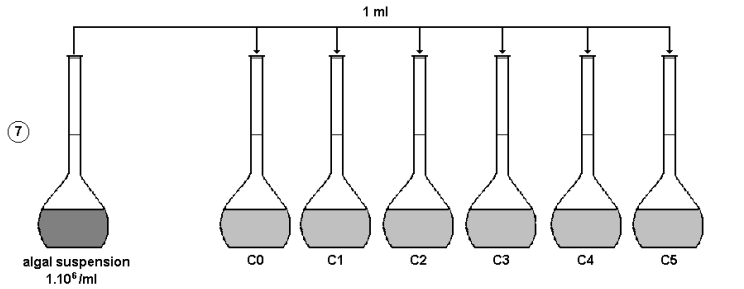
I - TEST ON EFFLUENTS

preparation of 1 : 1 dilution series









9. 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.

10. Transfer the algal suspension from the Algal Stock 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.
    Stopper and shake the flask thoroughly to distribute the algae evenly.

4. PREPARATION OF THE TOXICANT DILUTION SERIES

A. EFFLUENTS

As indicated in ISO Standard 8692 - Section 7.4 '*Preparation of test sample and stock solutions*' and/or Annex A (informative) - '*Rapid screening of wastewater algal growth inhibition*' a 1:1 dilution series 100% - 50% - 25% - 12.5% and 6.25% of the effluent sample is prepared by serial dilution, i.e. by successive dilutions of the original effluent by half (cf. US EPA/600/4-85/013, 1985).

A.1. Sample treatment

To eliminate turbidity, samples must be vacuum-filtered (e.g. over a membrane filter of 0.45 µm porosity), before testing.

A.2. Preparation of the 1:1 effluent dilution series

Procedure (see Figure)

1. Take six 200 ml calibrated flasks and label them from C0 to C5. The C0 flask is the control, C1 the non-diluted effluent and C5 the highest dilution (see Table 1).

Table 1 : Dilution series of the effluent

Flask	Effluent concentration (in %)
C0	0
C1	100
C2	50
C3	25
C4	12.5
C5	6.25

2. Fill flask C1 to the mark with the filtered effluent and add 2 ml of nutrient stock solution A and 0.2 ml of solutions B, C and D. Stopper the flask and shake thoroughly to mix the contents.

3. Put 100 ml algal culturing medium in flasks C0, C2, C3, C4 and C5.

4. Transfer half of the contents of flask C1 (i.e. 100 ml) into flask C2 (up to the mark of C2) to make up the first 1:1 dilution (50% effluent); stopper flask C2 and shake thoroughly to mix the contents.

5. Repeat the operation indicated in step 4, for flasks C3, C4 and C5, i.e.
   - 100 ml from C2 to C3 (= 25% effluent)
   - 100 ml from C3 to C4 (= 12.5% effluent)
   - 100 ml from C4 to C5 (= 6.25% effluent).

6. Remove and discard 100 ml solution from flask C5.

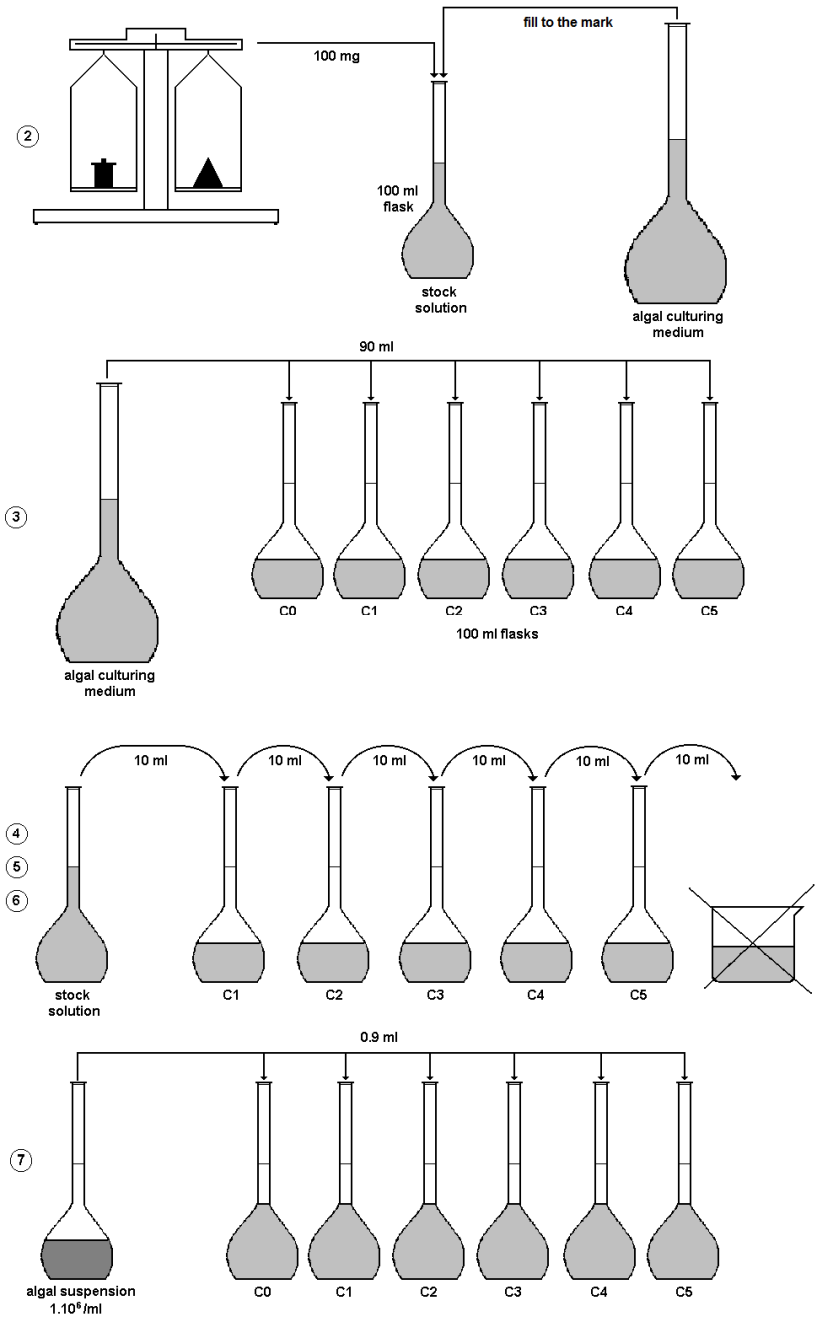
7. Add 1 ml of the 1.10^6/ml algal suspension to each flask, in order to obtain an initial algal concentration of 1.10^4/ml in each effluent flask. Stopper the flask and shake thoroughly to distribute the algae evenly.

The addition of 1 ml concentrated algal suspension to each flask and of 2.6 ml nutrient stock solution to C1 (100% effluent) leads to an 1% error in the respective effluent dilutions.
This small error has been permitted to allow for a less complicated procedure in the preparation of the toxicant dilution series.

8. Proceed to section : **Transfer of the algae-toxicant dilutions into the test vials.**

II - TEST ON CHEMICALS

range finding test



If at the end of the 3 day test period the lowest effluent concentration (6.25%) inhibits the algal growth relative to the control substantially (i.e. by a figure close to or over 50% inhibition), a second test has to be performed with a new (lower) dilution series. The highest effluent concentration of this new dilution series is the lowest one that produced 90-100% algal growth inhibition in the first test.

B. CHEMICAL COMPOUNDS

As indicated in ISO Standard 8692 - Section 7.3 '*Choice of test sample concentrations*', and if the approximate toxicity of the chemical compound to microalgae is known, a **definitive test** can be performed immediately. However, if no information is available on the toxicity of the chemical, two consecutive assays must be performed :

a) a **range finding test** to determine the 0-100% tolerance range of the algae to the toxicant,
b) a **definitive test** to determine with more precision the 50% inhibition threshold.

B.1. Range finding test

A "tenfold" dilution series must be prepared, starting at 100 mg/l as the highest toxicant concentration.

Procedure (see Figure)

1. Take seven 100 ml calibrated flasks and label them as follows :
   Stock Solution - C0 - C1 - C2 - C3 - C4 - C5 (see Table 2).

Table 2 : Dilution series of the chemical compound

Flask	Chemical concentration (in mg/l)
Stock solution	1000
C0	0
C1	100
C2	10
C3	1
C4	0.1
C5	0.01

2. Weigh 100 mg of the compound to be tested on an analytical balance. Transfer the chemical to the stock solution flask, fill this flask to the 100 ml mark with algal culturing medium, stopper and shake to dissolve the chemical.

3. Transfer 90 ml algal culturing medium into all the other flasks.

4. Transfer 10 ml of the (1 g/l) Stock Solution into flask C1, in order to prepare the first (100 mg/l) toxicant concentration. Stopper flask C1 and shake thoroughly to homogenize the contents.

5. Repeat the operation indicated in step 4 for flasks C2 to C5, i.e.
   - 10 ml from C1 to C2 (10mg/l)
   - 10 ml from C2 to C3 (1 mg/l)
   - 10 ml from C3 to C4 (0.1 mg/l)
   - 10 ml from C4 to C5 (0.01 mg/l)

6. Remove (and discard) 10 ml solution from flask C5.

7. Add 0.9 ml of the 1.10^6/ml algal stock suspension to each flask (except the stock flask), in order to obtain an initial concentration of 1.10^4 algae/ml in each toxicant concentration. Stopper the flasks and shake them thoroughly to distribute the algal suspension evenly.

Due to the addition of the (small volume of) concentrated algal suspension, the same 1% error as mentioned previously also applies here.

8. Proceed to section : **Transfer of the algae-toxicant dilutions into the test vials.**

B.2. <u>Definitive test</u>

The dilution series to be prepared spans the range of the <u>lowest</u> concentration producing 90-100 % inhibition and the <u>highest</u> one producing 0-10% growth inhibition relative to the control in the range finding test.

As shown in Table 3 this range can span one order of magnitude (case A) or two orders of magnitude (case B).

The new concentration range to be tested out will again be called C1-C5.

II - TEST ON CHEMICALS

definitive test

a) C1-C5 spans one order of magnitude

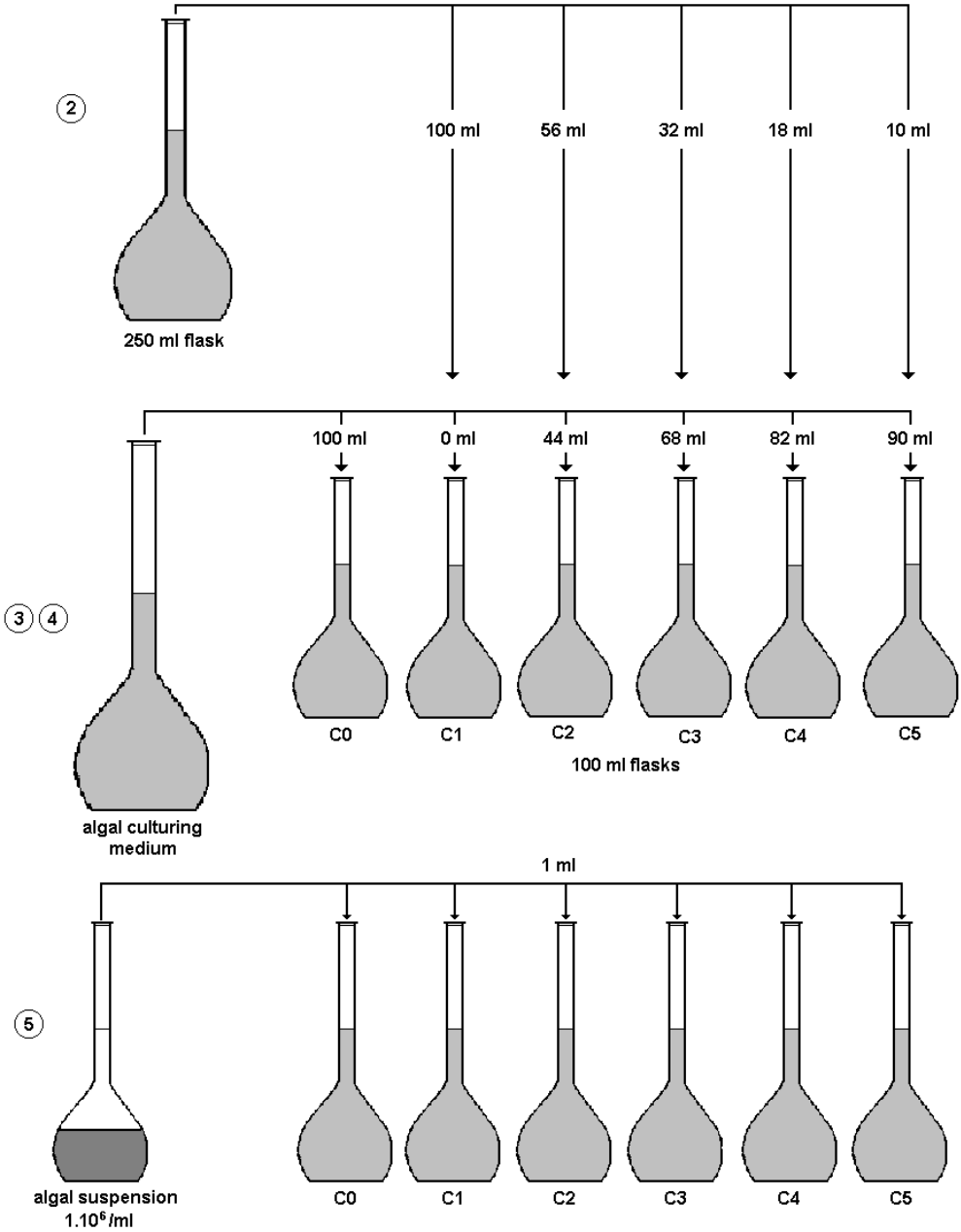


Table 3 : Schematic presentation of the 100-0% effect concentration range determined in the range finding test

	% growth inhibition
Case A	**100 100 0 0** **- - - * - - - * --------- * - - - - * - - -** **C1 C5**
	% growth inhibition
Case B	**100 100 0 0** **- - - * - - - - * -------- * -------- * - - - -* C1 C5**

A. C1-C5 spans one order of magnitude

Procedure (see Figure)

1. Take six 100 ml calibrated flasks and label them as follows :
   C0 - C1 - C2 - C3 - C4 - C5.
   C0 is the control, C1 the lowest concentration that produced 90-100% growth inhibition and C5 the highest that gave 0-10% growth inhibition in the range finding test.

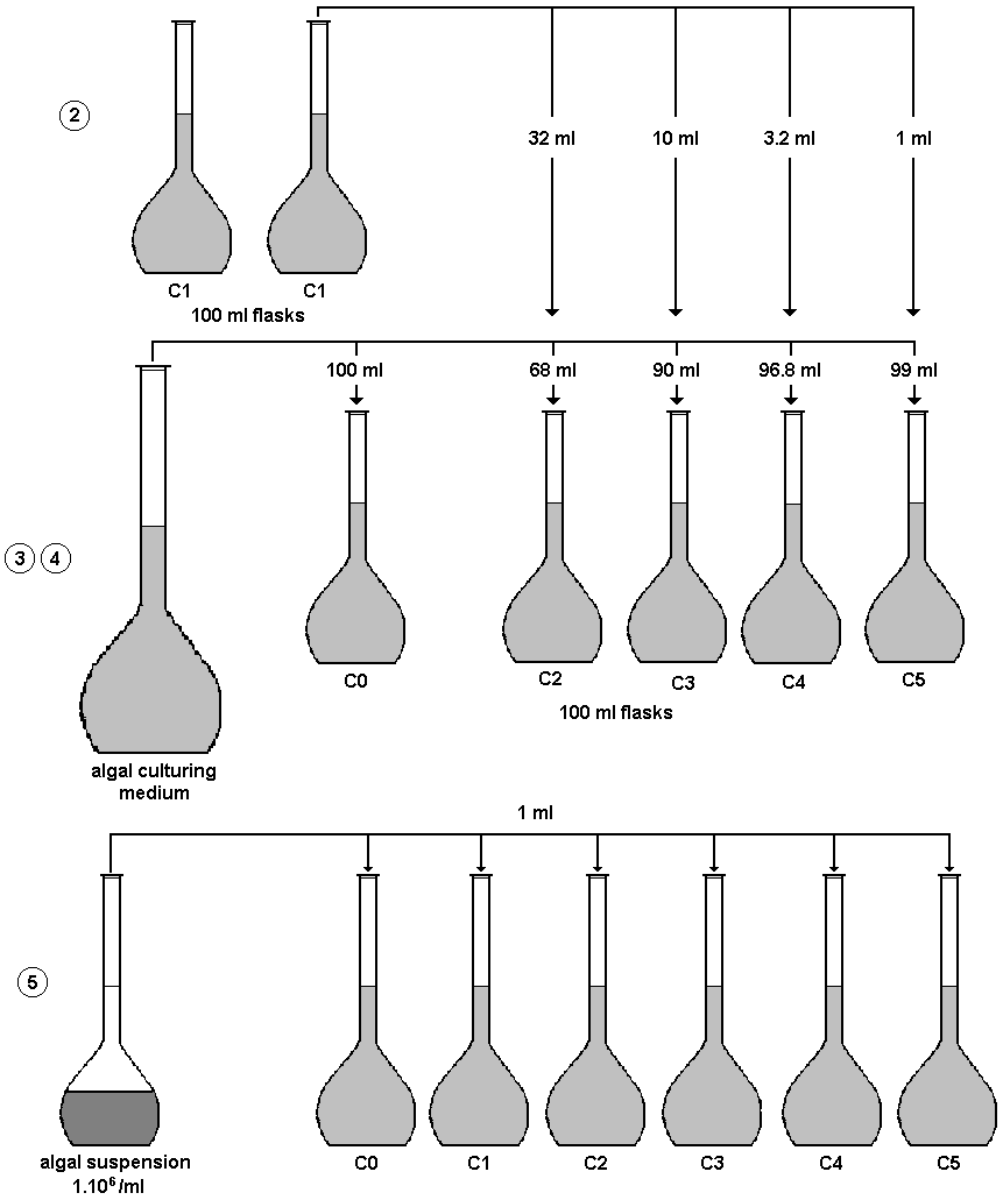
2. Take one 250 ml calibrated flask to make up 250 ml toxicant concentration, according to the instructions given for the range finding test (in this case 25 ml of the (1 g/l) Stock Solution should be transferred into the 250 ml flask).

3. Transfer the following volumes of toxicant solution from the 250 ml flask into the other flasks :
   - 100 ml in flask C1
   - 56 ml to flask C2
   - 32 ml to flask C3
   - 18 ml to flask C4
   - 10 ml to flask C5

II - TEST ON CHEMICALS

definitive test

b) C1-C5 spans two orders of magnitude



4. Add algal culturing medium up to the 100 ml mark in the C0, C2, C3, C4 and C5 flasks (see Table 4).

Table 4 : Dilution series C1-C5

Flask	C1 (in ml)	Algal culturing medium (in ml)
C0	0	100
C1	100	0
C2	56	44
C3	32	68
C4	18	82
C5	10	90

5. 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.

Same remark as made earlier for the small (1%) error in the dilutions.

6. Starting from the toxicant concentration in flask C1, calculate the actual concentration of toxicant in each flask (these figures will be needed for the IC50 estimation) :

   C1 = ...mg/l
   C2 = 0.56 x C1 = ...mg/l
   C3 = 0.32 x C1 = ...mg/l
   C4 = 0.18 x C1 = ...mg/l
   C5 = 0.10 x C1 = ...mg/l

7. Proceed to section : **Transfer of the algae-toxicant dilutions into the test vials.**

B. <u>C1-C5 spans two orders of magnitude</u>

Procedure (see Figure)

1. Take seven 100 ml calibrated flasks and label them as follows :
   C0 - C1 - C1 - C2 - C3 - C4 - C5.

C0 is the control, C1 the lowest concentration that produced 90-100% growth inhibition and C5 the highest that gave 0-10% growth inhibition in the range finding test.

2. Make up two C1 flasks with 100 ml toxicant concentration C1, according to the instructions given for the range finding test.

3. Transfer the following volumes of toxicant solution from one of the two C1 flasks to the other flasks :
   - 32 ml to flask C2
   - 10 ml to flask C3
   - 3.2 ml to flask C4
   - 1 ml to flask C5

   Discard the half empty C1 flask.

4. Add algal culturing medium up to the 100 ml mark in the C0, C2, C3, C4 and C5 flasks (see Table 5).

5. Add 1 ml of the 1.10^6/ml algal stock to all flasks, 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.

Same remark as made earlier for the small (1%) error in the dilutions, resulting from the addition of the concentrated algal suspension.

Table 5 : Dilution series C1-C5

Flask	C1 (in ml)	Algal culturing medium (in ml)
C0	0	100
C1	100	0
C2	32	68
C3	10	90
C4	3.2	96.8
C5	1	99

6. Starting from the toxicant concentration in flask C1, calculate the actual concentration of toxicant in each flask (these figures will be needed for the IC50 estimation).

C1 = ...mg/l
C2 = 0.32 x C1 = ...mg/l
C3 = 0.10 x C1 = ...mg/l
C4 = 0.03 x C1 = ...mg/l
C5 = 0.01 x C1 = ...mg/l

7. Proceed to section : **Transfer of the algae-toxicant dilutions into the test vials.**

5. TRANSFER OF THE ALGAE-TOXICANT DILUTIONS INTO THE TEST VIALS

For a statistically acceptable evaluation of algal growth inhibition, each test concentration as well as the control have to be assayed in three replicates. Each Algaltoxkit contains two sets of 18 long cells in a transparent holding tray provided with 2 plastic strips and kept in position by 2 rubber bands.

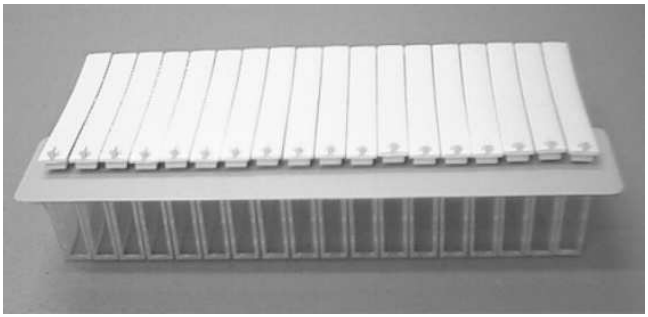
1. Take one of the two trays, remove the rubber bands and the plastic strips, and mark the cells in sets of 3 (a, b, c) for each concentration (from CO to C5).

2. Open all the cells and after thorough shaking, transfer 25 ml of the algae-toxicant dilutions from each flask into the corresponding 3 long cells.

6. INCUBATION OF THE TEST VIALS

1. Close all the cells and put them back into their holding tray. Then slightly lift up the lid on one side of the cells and slide the plastic strip over the open part in order to leave some opening near the middle of the cells for gas exchange (see figure).

The long cells shall be placed in the holding tray in a random way (i.e. not in the sequence C0 to C5, and not all three parallels next to each other), in order to compensate for possible small "site to site" differences during incubation.



2. Following the prescription of ISO Standard 8692, section 7.6 '*Incubation*', the holding tray with the cells shall be incubated for 72h in an incubator or in temperature controlled room, with a constant uniform illumination supplied by cool white fluorescent lamps. An illumination of 10000 lux is needed for sideway illumination of the holding tray or 3000-4000 lux for bottom illumination.

To obtain a satisfactory algal growth during the 3 day test period the temperature in the incubator or temperature controlled room should be 23°C ± 2 °C.

Standard protocols for toxicity tests with algae usually prescribe that the algae be kept in continuous suspension to facilitate CO_2 transfer and reduce pH variation.
Detailed investigations with the Algaltoxkit have, however, revealed that the re-suspension 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 divisions prescribed in standard procedures.
Continuous shaking of the test vials is therefore not mandatory for the Algaltoxkit assay.

7. SCORING OF THE RESULTS

As indicated in ISO Standard 8692, section 7.7 '*Measurements*' inhibition of the algal growth relative to the control has to be determined every 24 hours. Measurement of the OD at 670 nm of the algal suspensions in the long cells shall be performed during the 3 days of the test, i.e. after 24h, 48h and 72h exposure to the toxicant. Make sure to put the long cells back in the incubator in a random way after the daily measurement.

Daily results for each long cell are recorded on the "Results Sheets"

Interference of OD readings by coloured samples

Coloured natural samples may interfere with OD readings of the algal suspensions, especially when the colour shows absorption at the (670 nm) wavelength which is used to measure algal density.

Anyhow, it has to be reminded that the determination of the toxicity of highly coloured samples to microalgae is automatically biased by interference of the colour with light penetration in the medium containing the algae. In addition, optical density measurements in the long cells, are anyhow only possible up to a certain intensity of colouration.

For coloured samples a dilution series without algae should be made up. This technique requires 5 extra long cells, which are not included in the 2 sets of 18 long cells included in each Algaltoxkit, but which can be obtained separately.

1. After preparing the toxicant dilution series in the calibrated flasks, but prior to the addition of the concentrated algal stock, fill 5 long cells with 25 ml of the corresponding dilution.

2. Keep the 5 long cells with the (coloured) dilutions separately and proceed further with step 7 of Procedure A2 (Preparation of the 1:1 dilution series), adding, however, only 0.75 ml of the 1.10^6/ml algae suspension to each flask of the toxicant dilution series (which now contain 75 ml solution each).

3. Prior to the daily measurement of the OD of the long cells containing the algae-toxicant dilutions, zero-calibrate the spectrophotometer with the long cells containing the corresponding coloured toxicant dilutions.

ALGALTOXKIT F - RESULTS SHEET

Name of operator : ……

Date of performance of test : ……

Dilution series tested : concentration 1 : ……
concentration 2 : ……
concentration 3 : ……
concentration 4 : ……
concentration 5 : ……

Test species : ……

Toxicant tested : ……

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

The long cells containing the coloured toxicant dilutions must also be incubated in the same light and temperature conditions as the long cells in the holding tray. This way, changes in colour during the 3 days of exposure will automatically be taken into account by the daily zero-calibration with the corresponding long cells.

In case of interference of OD readings by turbidity this method can also be used.

8. DATA TREATMENT

1. Calculate the mean daily OD values for the 3 replicate long cells of the control and the toxicant dilutions.

2. Calculate the algal growth inhibition in each toxicant concentration and the resulting 72h E_rC_{50} according to internationally accepted data treatment procedures (e.g. ISO Standard 8692 or OECD Guideline 201).

The detailed data treatment method for algal growth inhibition tests of ISO Standard 8692 - Section 9 '*Calculation*' is given in annexe to this manual.

N.B. A computer programme for automatic data treatment of Algaltoxkit results is available for Algaltoxkit users.

VALIDITY OF THE TEST

National and international guidelines for algal toxicity tests each have their own validity criteria.
ISO Standard 8692 - Section 8 'Validity criteria*' e.g. stipulates that the average growth rate in the control shall be at least 1.4 per day, which corresponds to an increase in cell density by a factor 67 in 72h.*

OECD 201 in turn indicates that the control growth rate must be at least 0.92 per day, i.e. a multiplication factor of only 16.

Both organisations furthermore indicate that the pH in the controls shall not have increased by more than 1.5 units relative to the initial pH in the growth medium.

9. REFERENCE TEST

In order to check the correct execution of the test procedure and the sensitivity of the test, it is advised to perform a reference test from time to time (e.g. every 5 to 10 assays).

Such a quality control test can e.g. be carried out with the reference chemical potassium dichromate ($K_2Cr_2O_7$).

Procedure :

A dilution series ranging from 1.8 mg/l to 0.18 mg/l has to be prepared following the guidelines given in section B.2. Definitive test - A. C1-C5 spans one order of magnitude.

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 1 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 10 mg/l ('Stock 2') toxicant concentration.

4. Transfer the following volumes of toxicant solution from 'Stock 2' into the following flasks :
   - 18 ml to flask C1
   - 10 ml to flask C2
   - 5,6 ml to flask C3
   - 3,2 ml to flask C4
   - 1,8 ml to flask C5

Same remark as made earlier for the small (1%) error in the dilutions, resulting from the addition of the concentrated algal suspension.

5. Add algal culturing medium up to the 100 ml mark in the C0, C1, C2, C3, C4 and C5 flasks (see Table 4).

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.

7. Proceed further as of section **5. Transfer of the algae-toxicant dilutions into the test vials** and the following sections of this manual.

The 72h EC_{50} values mentioned on the corresponding Specification Sheet are the values of MicroBioTests Inc.'s internal quality control test performed at the production of the specific batch of algal beads. The average of all 72h EC_{50} values of the batches of algal beads produced by MicroBioTests Inc. during the last years is also mentioned on the Specification Sheet.

In the table below you can find the mean EC_{50} values on $K_2Cr_2O_7$ obtained from the International Interlaboratory Exercise on our Algaltoxkit F Selenastrum which has been performed in 2006 (42 results of 28 laboratories in 14 countries).

42 tests Performed	Mean Value	Standard Deviation
EbC_{50}	0.52 mg/l	0.21 mg/l
ErC_{50}	0.84 mg/l	0.27 mg/l

FROM : WATER QUALITY - FRESHWATER ALGAL GROWTH INHIBITION TESTS WITH UNICELLULAR GREEN ALGAE (ISO Standard 8692)

9 Interpretation of data

9.1 Plotting growth curves

Tabulate the cell density measurements or other parameters correlated with cell density in the test media according to the concentration of test sample and the time of measurement.

Plot a growth curve for each test concentration and control, as a graph of the logarithm of the mean cell density against time. A linear growth curve indicates exponential growth, whereas a levelling off indicates that cultures entered the stationary phase.

In case the control cultures show declining growth rate towards the end of the exposure period, inhibited cultures may tend to catch up with the controls, falsely indicating a decreased growth inhibiting effect. In this case, perform the calculations of growth rate and growth inhibition based on the last measurement within the exponential growth period in the control cultures.

9.2 Calculation of percentage inhibition

Calculate first the average specific growth rates, μ, for each test culture, using equation (1).

$$\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0} \quad (1)$$

Where :

t_0 is the time of test start ;

t_L is the time of test termination [or the time of the last measurement within the exponential growth period in the control cultures (9.1)] ;

N_0 is the nominal initial cell density ;

N_L is the measured cell density at time t_L.

Alternatively determine the average growth rate from the slope of the regression line in a plot of the natural logarithm of the mean cell density against time (9.1).

Calculate the mean value of μ for each test and control batch replicate. From these values, calculate the percentage inhibition for each test batch replicate, from equation (2).

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100 \quad (2)$$

Where :

$I_{\mu i}$ is the percentage inhibition (growth rate) for test concentration i ;

μ_i is the mean growth rate for test concentration i ;

μ_c is the mean growth rate for the control.

9.3 Determination of E_rC_x (e.g. E_rC_{10} and E_rC_{50})

Tabulate and plot for each individual flask the normalised inhibition ($I_{\mu i}$) against the test concentration on a logarithmic scale. If the scatter of data points is large, plot means of replicates with corresponding standard deviations.

Fit a suited non-linear model to the experimental data points by regression analysis (for example see ISO/TS 20281, [9] and [10]) in order to determine E_rC_x values, preferably with their confidence intervals.

If data are too few or uncertain for regression analysis, or if inhibitions appear not to follow a regular concentration response relation (e.g. stimulation), then a graphical method might be applied. In this case draw a smooth eye fitted curve of the concentration response relationship and read E_rC_x values from this graph. If extreme stimulation at intermediate concentrations of the test substance is observed, use of a hormesis model should be considered [8].

LIST OF TOXKIT MICROBIOTESTS

Tests for freshwater and soils

PROTOXKIT F : 24h reproduction inhibition test based on the ciliate protozoan *Tetrahymena thermophila*. This assay is under consideration as an OECD Guideline.

ROTOXKIT F : 24h mortality test, based on the rotifer *Brachionus calyciflorus*. This assay adheres to ASTM Standard Guide E1440-91.

ROTOXKIT F chronic : 48h reproduction inhibition test based on the rotifer *Brachionus calyciflorus*. This assay adheres to ISO norm 20666 and AFNOR norm T90-377.

THAMNOTOXKIT F : 24h mortality test, based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

CERIODAPHTOXKIT F : 24h mortality test, based on the cladoceran crustacean *Ceriodaphnia dubia*. This assay is in current practice in the USA as an EPA Method.

DAPHTOXKIT F: 24h-48h mobility inhibition test, based on the cladoceran crustacean *Daphnia magna*. This assay adheres to ISO norm 6341 and OECD Guideline 202.

OSTRACODTOXKIT F : 6 days chronic mortality and growth inhibition test with the ostracod crustacean *Heterocypris incongruens*. This assay adheres to ISO norm 14370.

RAPIDTOXKIT F Thamno: 30-60 min particle ingestion inhibition test based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

ALGALTOXKIT F : 72h growth inhibition test, based on the green alga *Selenastrum capricornutum* (presently named *Pseudokirchneriella subcapitata*). This assay adheres to ISO norm 8692 and OECD Guideline 201.

PHYTOTOXKIT solid samples: 3 days germination and root growth inhibition test with seeds of 3 higher plants.

PHYTOTOXKIT liquid samples: A short germination and root/shoot growth inhibition microbiotest for determination of the direct effect of chemicals on higher plants.

DUCKWEED TOXKIT F : 72h growth inhibition test with the duckweed species *Spirodela polyrhiza*.

Tests for estuarine/marine environments

ROTOXKIT M : 24h mortality test based on the rotifer *Brachionus plicatilis*. This assay adheres to ASTM Standard Guide E1440-91.

ARTOXKIT M : 24h mortality test based on the anostracan crustacean *Artemia salina* (renamed *Artemia franciscana*). This assay adheres to ASTM Standard Guide E1440-91.

ALGALTOXKIT M : 72h growth inhibition test based on the marine diatom *Phaeodactylum tricornutum*. This test adheres to ISO norm 10253.

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