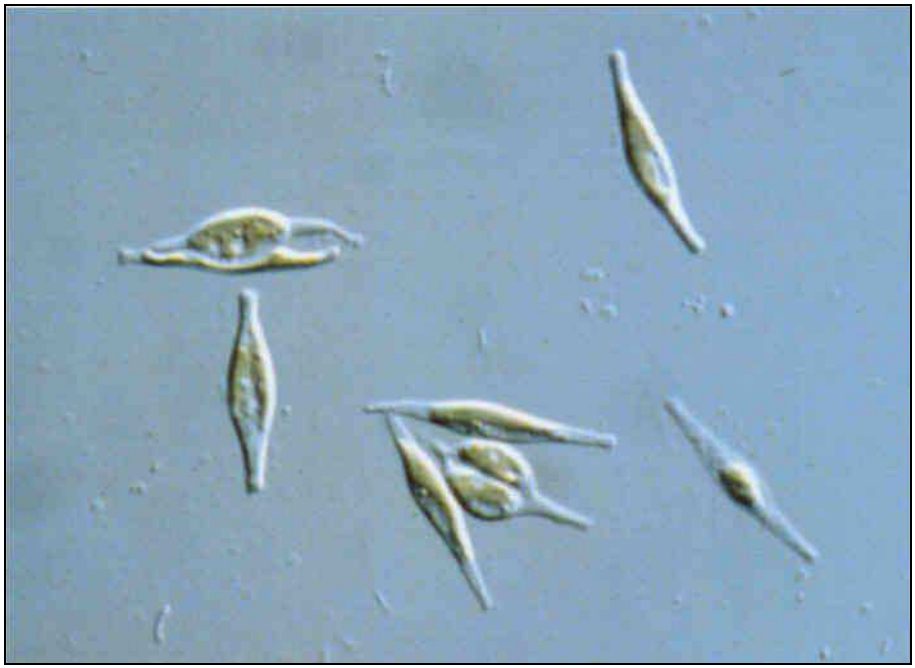
MARINE ALGALTOXKIT

Marine Toxicity Test

with Microalgae



STANDARD OPERATIONAL

PROCEDURE

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INTRODUCTION TO THE MARINE ALGALTOXKIT

Origin :
This new marine algal growth inhibition bioassay has been developed by the research team of Prof. Dr. G. Persoone at MicroBioTests Inc. in Nazareth, 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 testing 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 marine Algaltoxkit test

- No culturing

The Marine Algaltoxkit makes use of microalgae inoculum that can be stored for several months without losing its viability. Transfer of the algal inoculum in an adequate growth medium reactivates the microalgae leading - within 3 days - to a culture in the exponential growth phase ready for the bioassay.

- Rapid scoring

Like the freshwater Algaltoxkit, the Marine Algaltoxkit makes use of a unique type of test vials, namely disposable spectrophotometric cells of 10 cm length in polystyrene. The so called "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. optical density measurements of the algal suspensions at 670 nm wavelength in 10 cm long cells correlate very well with algal numbers and are hence in accordance with the prescription of ISO Guideline 10253 and other standard methods for determination of algal densities.

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 which 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 Marine Algaltoxkit test :

A 72h algal growth inhibition test is performed in long cell test vials, with the marine diatom *Phaeodactylum tricornutum*. The Marine Algaltoxkit test has been modelled on and follows the prescriptions of the ISO guideline "Water Quality - Marine Algal Growth Inhibition Tests with *Skeletonema costatum* and *Phaeodactylum tricornutum*" (Guideline ISO/CD 10253).

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 20 °C (+/- 2 °C) provided with light,

b) a spectrophotometer for OD measurements at 670 nm and a holder for cells of 10 cm path-length.

Sensitivity :
The sensitivity of the Marine Algaltoxkit test has been determined for a variety of inorganic and organic chemicals in parallel to the conventional algal growth inhibition test. The data generated by MicroBioTests Inc. indicates that the sensitivity of the new algal microbiotest is the same as that of the conventional algal assay.

Precision :
An extensive comparative study between the conventional marine algal test and the Marine Algaltoxkit assay performed in MicroBioTests Inc. also revealed that the precision of the Algaltoxkit assays is equal to (and often better than) that of conventional algal tests for both chemicals and natural samples.

Shelf life :
The concentrated algal growth medium can be stored for months in the refrigerator; storage of the inactive algal inoculum for several months in the refrigerator does not affect the viability of the microalgae either.
The inoculum can hence be used any time prior to the indicated expiry date to perform algal assays after 3 days of pre-culturing.

CONTENTS OF THE ALGALTOXKIT

Tubes with microalgae inoculum

Two tubes containing *Phaeodactylum tricornutum* microalgae inoculum in a storage medium. The tubes must be kept in the refrigerator in darkness at 5 °C (+/- 2 °C) until use.

Concentrated algal growth medium

One plastic vial containing sodium chloride (NaCl), six glass bottles containing concentrated solutions of different chemicals (to make up synthetic seawater) and 4 vials with nutrient stock solutions to make up 2 litres of algal culturing medium with deionized water, according to the formula of the ISO/CD Guideline 10253 "Water Quality - Marine Algal Growth Inhibition Tests with *Skeletonema costatum* and *Phaeodactylum tricornutum*". The vials with nutrient stock solutions must be stored in the refrigerator in darkness at 5 °C (+/- 2 °C) until use.

Composition of the concentrated solutions :

Vial 1 : NaCl (52.8 g - dissolved in 2 l. = 26.4 g/l)
Vial 2 : KCl (1680 mg - dissolved in 2 l. = 840 mg/l)
Vial 3 : $CaCl_2$ (3340 mg - dissolved in 2 l. = 1670 mg/l)
Vial 4 : $MgCl_2$ (9200 mg - dissolved in 2 l. = 4600 mg/l)
Vial 5 : $MgSO_4$ (11160 mg - dissolved in 2 l. = 5580 mg/l)
Vial 6 : $NaHCO_3$ (340 mg - dissolved in 2 l. = 170 mg/l)
Vial 7 : H_3BO_3 (60 mg - dissolved in 2 l. = 30 mg/l)

Long cells and holding trays

Two sets of 18 disposable 10 cm 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 pre-culturing cells

Three long cells with lid, one for zero calibration of the spectrophotometer and two for the pre-culturing of the microalgae and scoring of the OD of the concentrated algal suspension.

Bench protocol

An abbreviated version of the 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 instructions for data processing and calculation of the 72hIC_{50} according to the ISO procedure.

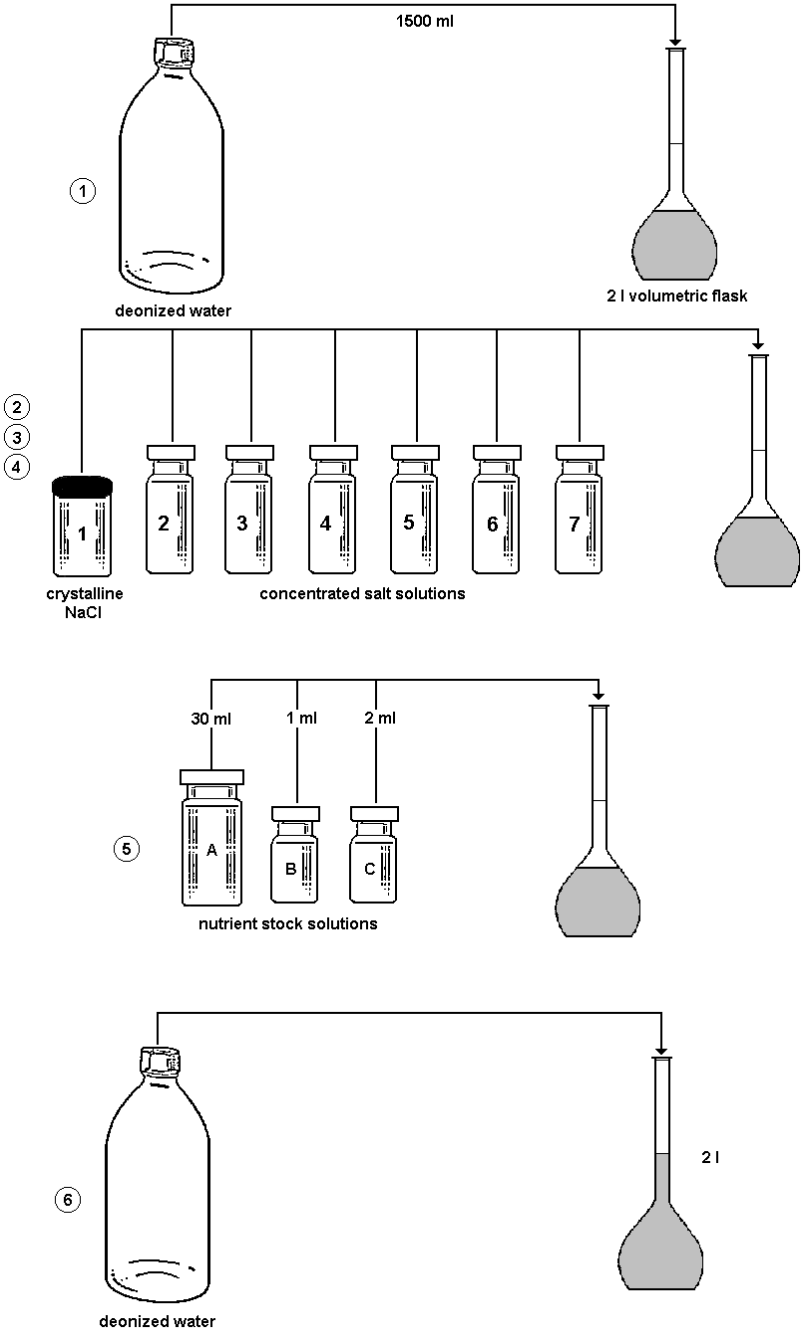
Results sheets

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 inoculum, the matrix dissolving medium, the algal culturing medium, the expiry date of the Toxkit and the 72h IC_{50} for the reference chemical potassium dichromate.

PREPARATION OF ALGAL CULTURING MEDIUM



1. PREPARATION OF ALGAL CULTURING MEDIUM

Procedure (see Figure)

Preparation of synthetic Seawater

1. Fill a 2 litre 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

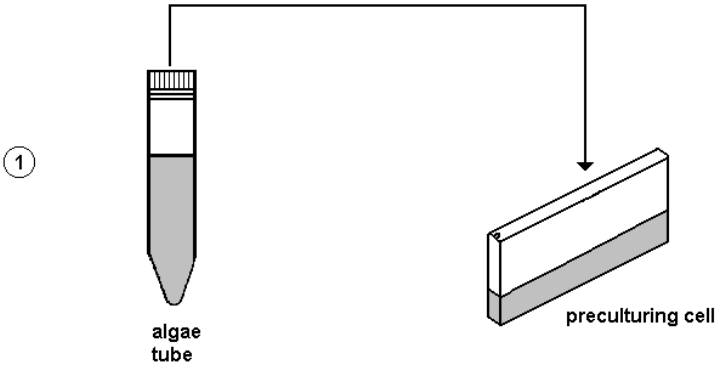
5. Add 30 ml (2 x 15 ml) of Stock Solution A, 1 ml of Stock Solution B and 2 ml of Stock Solution C to the volumetric flask.

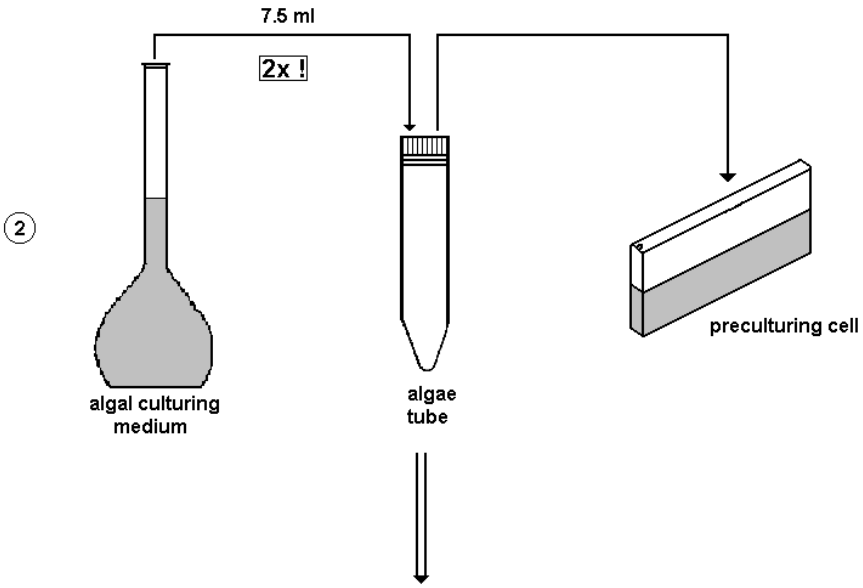
6. Add deionized water up to the 2000 ml mark and shake to homogenize the medium.

2. STORAGE OF THE ALGAL CULTURING MEDIUM

Two litres algal culturing medium suffice for performance of 2 complete algal bioassays.
If the 2 tests are not carried out concurrently, the culturing medium should be stored in the refrigerator in darkness.

PRECULTURING OF THE ALGAE





INCUBATION
- 3 days
- 20 °C (+/- 2 °C)
- 10000 lux (sideway illumination) or
- 3000-4000 lux (bottom illumination)

3. PRECULTURING OF THE ALGAE

Procedure (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 10000 lux in case of sideway illumination of the long cell or 3000-4000 lux for bottom illumination.

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 special (10 cm) "long cells".

(*Procedures are given in section 8 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 nanometer filter, and equipped with a holder for 10 cm cells.

Optical densities can be converted very easily into algal numbers with the aid of the "Optical Density/Algal Number" (OD/N) sheet 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 given in the OD/N sheet included in each Algaltoxkit has been determined with a "Long Cell 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.

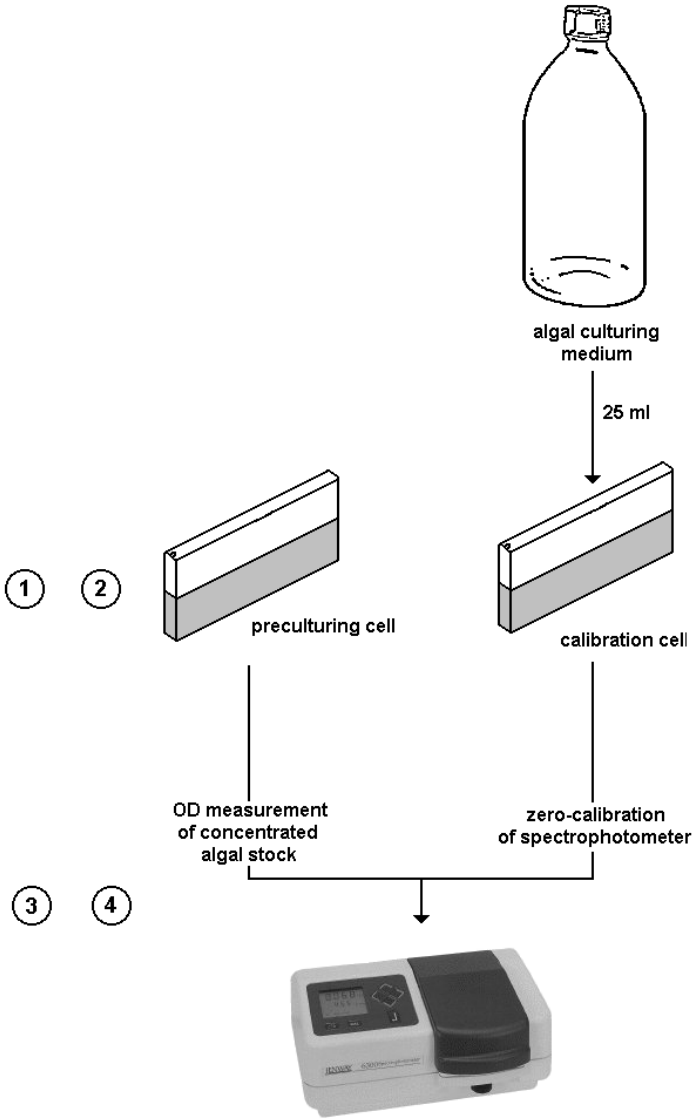
4. PREPARATION OF CONCENTRATED ALGAL INOCULUM

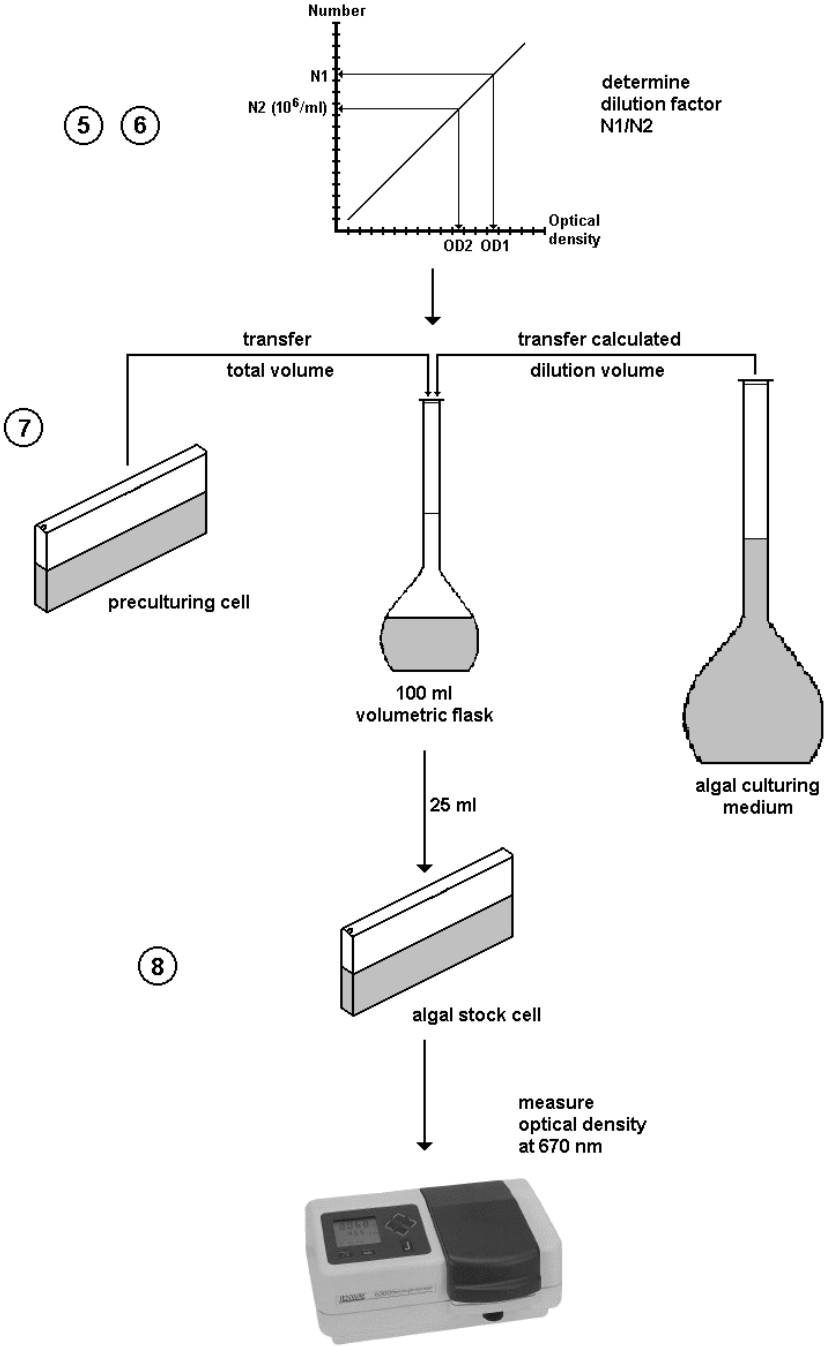
Procedure (see Figure)

1. After 3 days of 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.

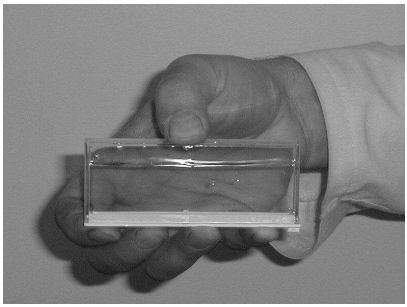
PREPARATION OF CONCENTRATED ALGAL INOCULUM




Number
N1
N2 (10^6/ml)
Optical density
OD2 OD1
5
6
determine dilution factor N1/N2
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

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.*

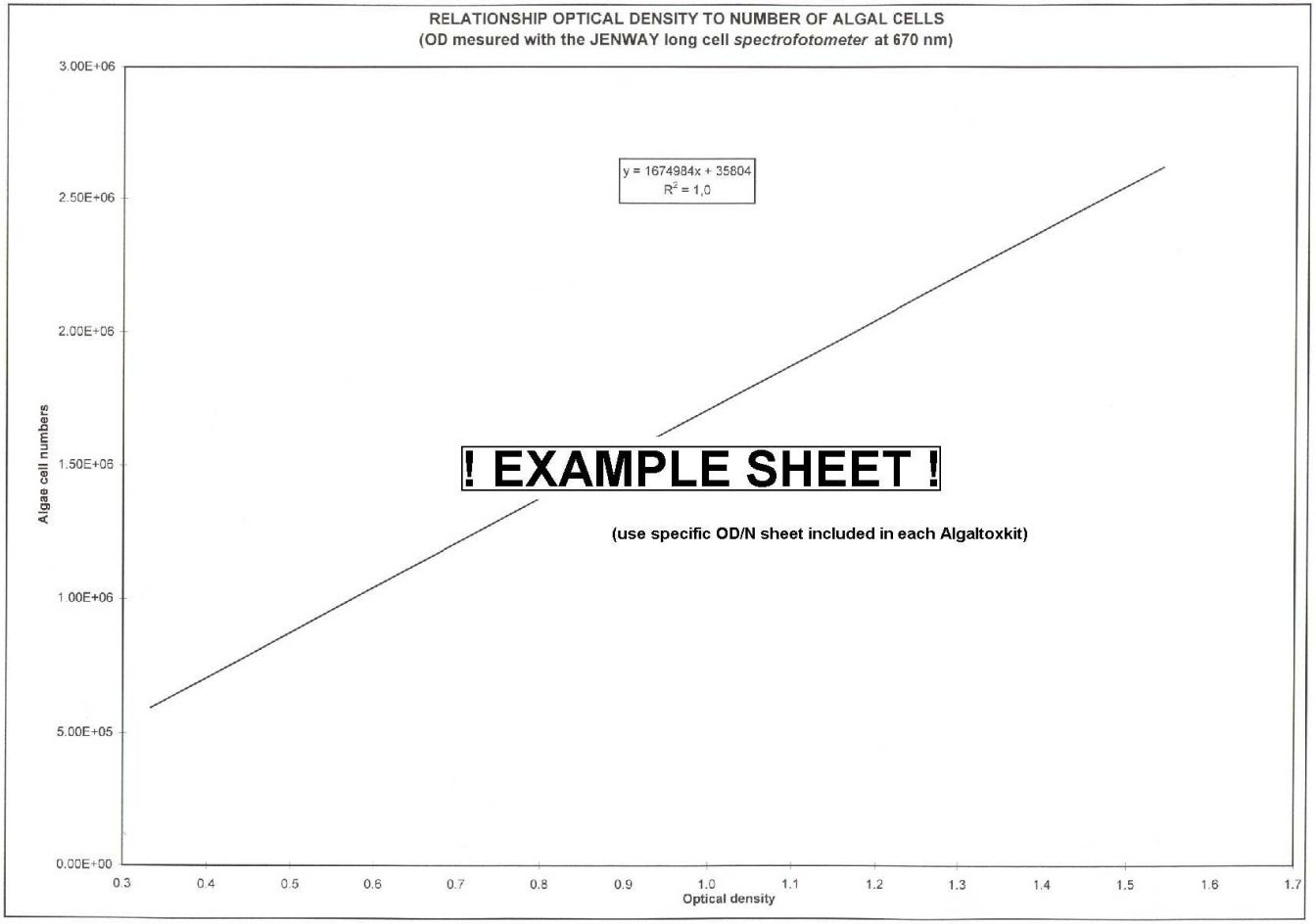
2. Take the long cell with the label "Calibration long cell", fill it with 25 ml algal culturing medium and close the cell with the lid.

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

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.


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 SHEET !
(use specific OD/N 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

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.
   Stopper and shake the flask thoroughly to distribute the algae evenly.

8. 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.

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

5. PREPARATION OF THE TOXICANT DILUTION SERIES

A. EFFLUENTS

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 pre-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

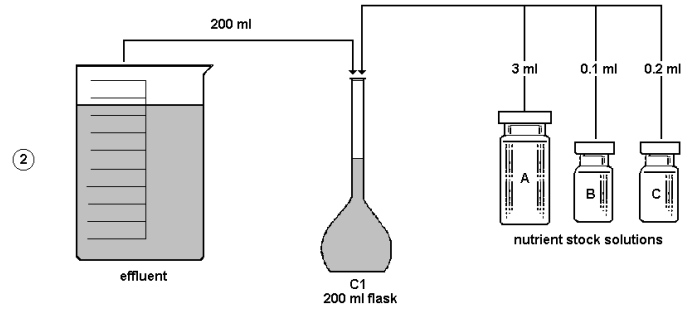
Procedure (see Figure)

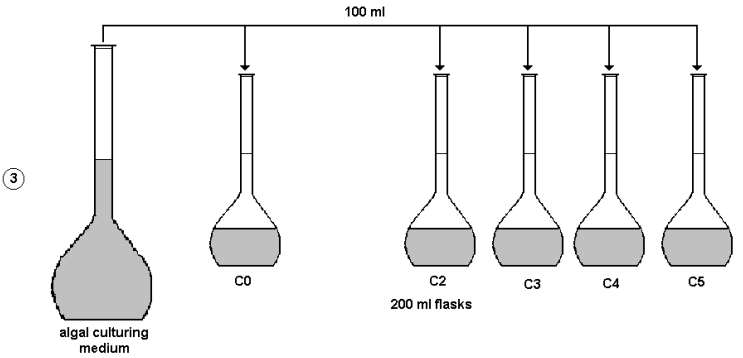
1. Take six 200 ml calibrated flasks and label them from C0 to C5. The C0 flask is the control, C1 the non-diluted effluent and C5 the highest dilution (see Table 1).

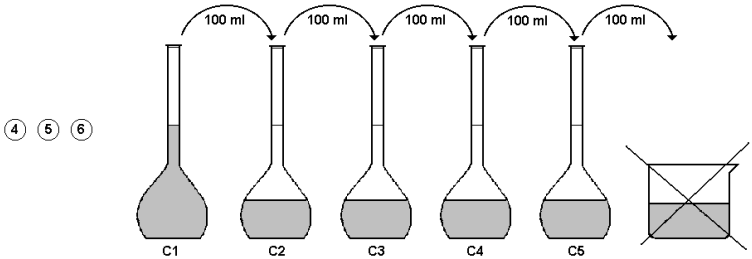
2. Fill flask C1 to the mark with the filtered effluent and add 3 ml nutrient stock solution A, 0.1 ml of solution B and 0.2 ml of solution C. Stopper the flask and shake thoroughly to mix the contents.

I - TEST ON EFFLUENTS

preparation of 1 : 1 dilution series







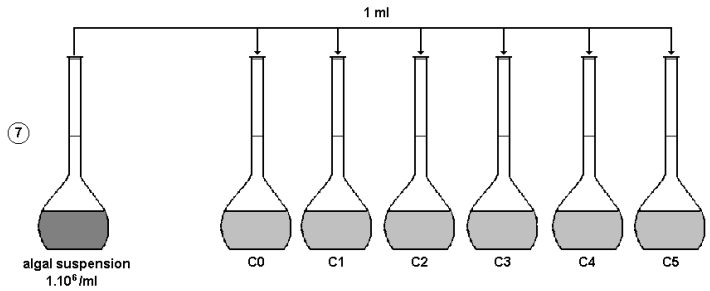


Table 1 : Dilution series of the effluent

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

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.

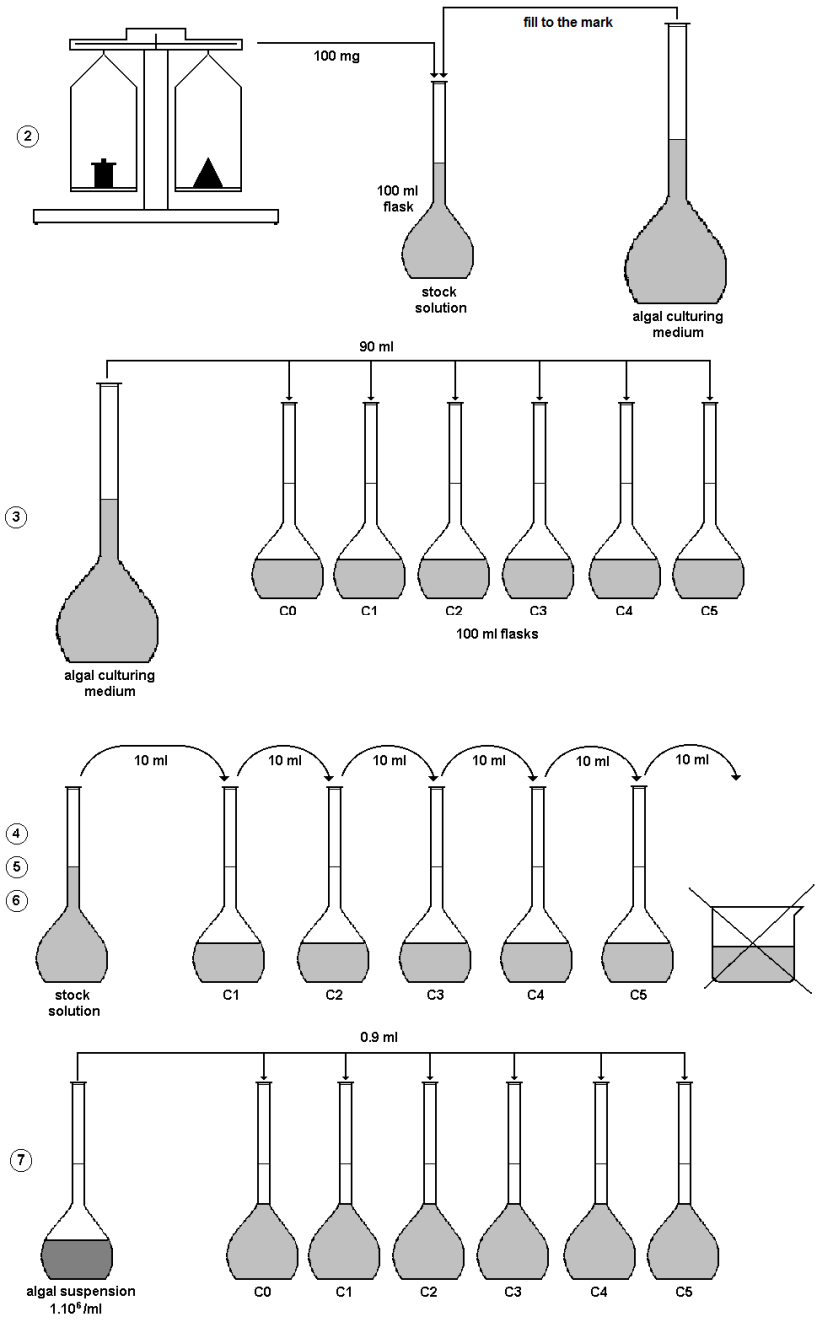
The addition of 3.3 ml nutrient stock to the 200 ml non-diluted effluent in the C1 flask means a decrease of 1.65 % of the effluent concentration.

The addition of 1 ml concentrated algal suspension to the (100 ml) C1-C5 dilution series also results in a 1 % error in the respective effluent concentration.
These small errors do not have any significant impact on the outcome of the assays whereas in turn they allow a simple procedure for the preparation of the toxicant dilutions with 1.10^4 algal cells/ml.

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

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 e.g. 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	15
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 in the tube 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, there will also be a very small error (negligible) as mentioned earlier for effluent tests.

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

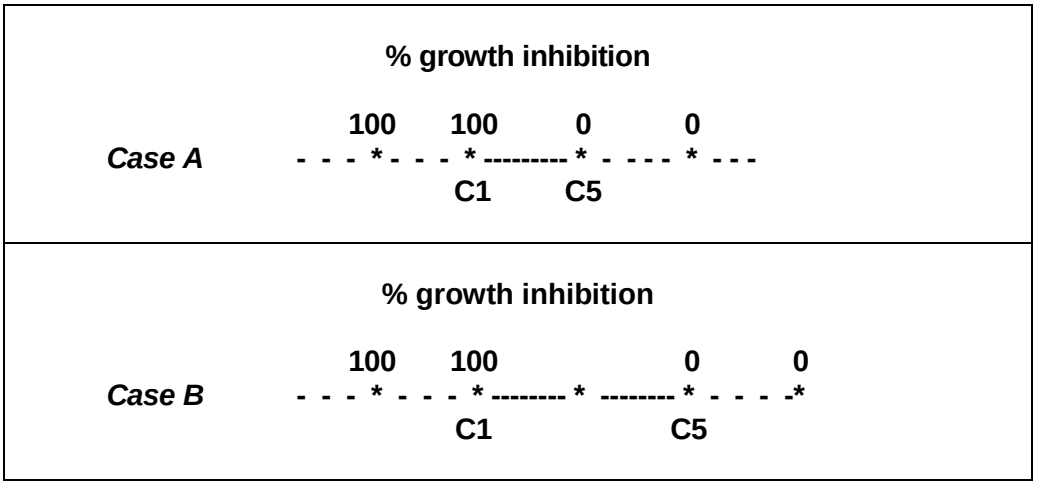
B.2. <u>Definitive test</u>

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.

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



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.

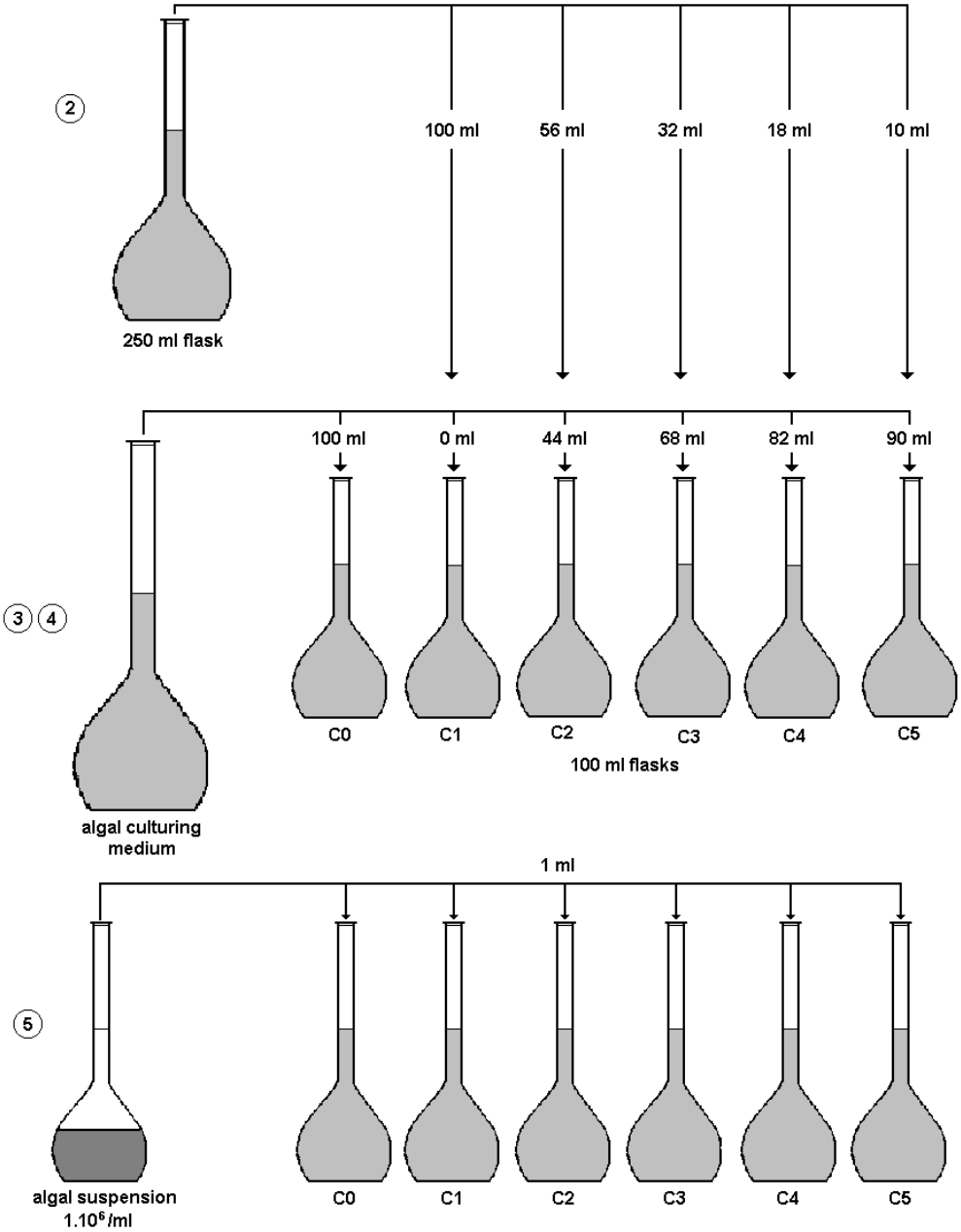
2. Take one 250 ml calibrated flask to make up 250 ml toxicant concentration, according to the instructions given for the range finding test (in this case 25 ml of the (1 g/l) Stock Solution should be transferred into the 250 ml flask).

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

a) C1-C5 spans one order 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, resulting from the addition of the concentrated algal suspensions.

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 IC_{50} 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. C1-C5 spans two orders of magnitude

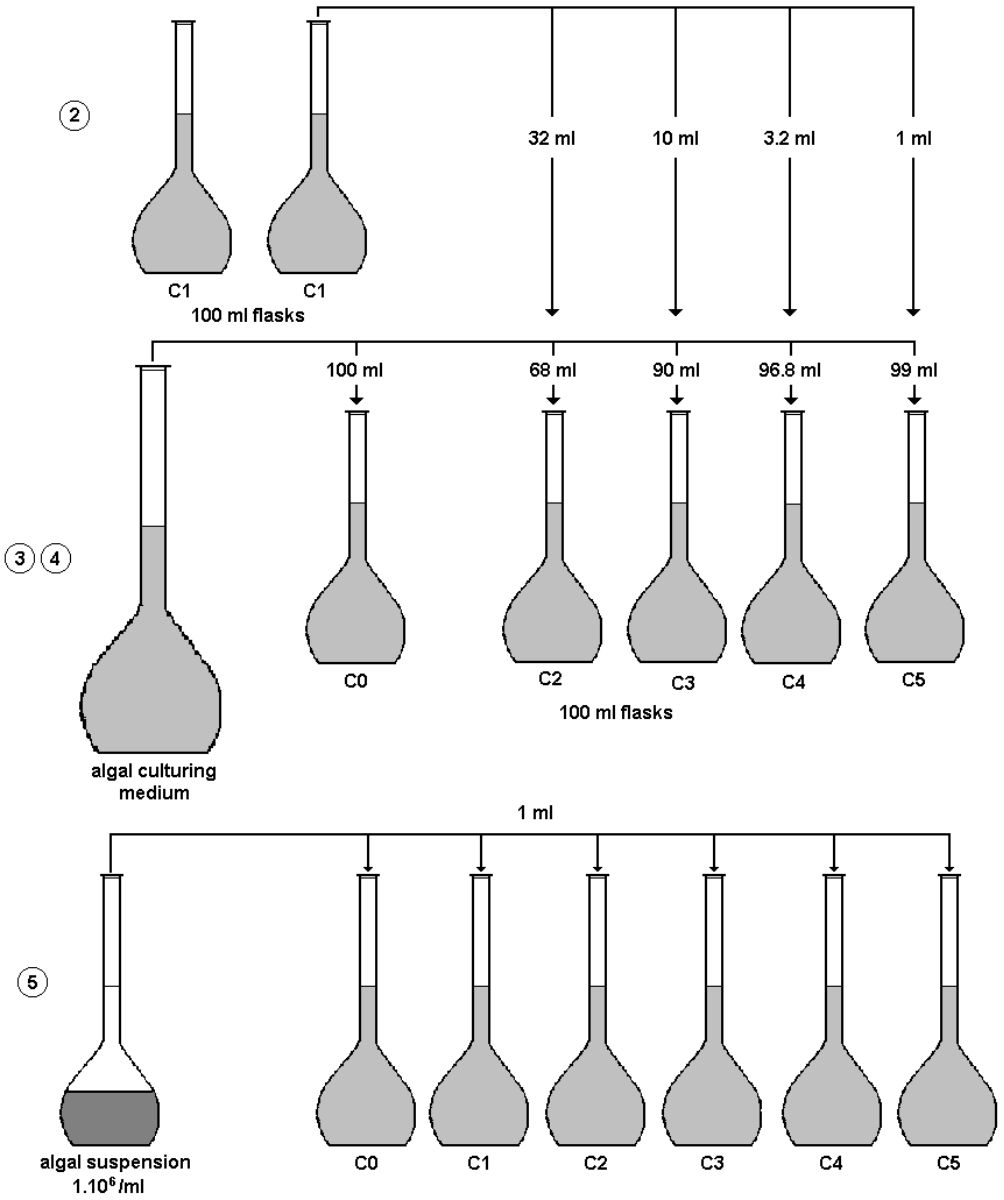
Procedure (see Figure)

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

II - TEST ON CHEMICALS

definitive test

b) C1-C5 spans two orders of magnitude



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 <u>two</u> 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.

<u>Table 5</u> : 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 IC_{50} 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.**

6. 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, 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 by lifting up one end of the lids.

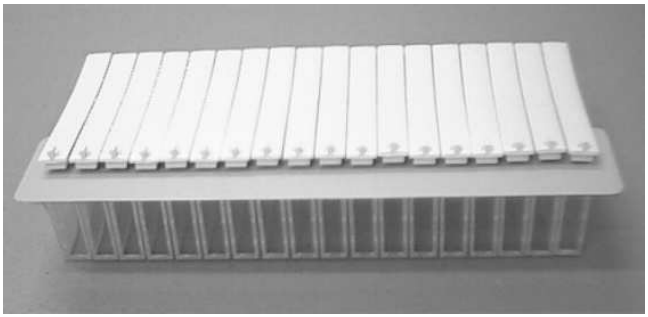
3. After thorough shaking, transfer 25 ml of the algae-toxicant dilutions from each flask into the corresponding 3 long cells.

4. Close all cells and shake them as indicated in the section "Shaking procedure of long cells with algal suspensions". Determine the OD of each long cell in the spectrophotometer.

5. Record the T_0 data on the Results Sheet

7. INCUBATION OF THE TEST VIALS

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 figure below).
   The long cells shall be placed in the holding tray in a random way (i.e. not in the sequence CO 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. Put the holding tray for 3 days in an incubator or in a temperature controlled room, with a constant uniform illumination supplied by cool white fluorescent lamps; make sure that the illumination is 10000 lux for sideway illumination 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 20°C (+/- 2 °C).

Standard protocols for toxicity tests with algae usually prescribe that the algae should 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.
Consequently, the continuous shaking of the test vials is not mandatory for the Algaltoxkit assay.

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						
	Mean						
	CV%						
24h	**1**						
	2						
	3						
	Mean						
	CV%						
48h	**1**						
	2						
	3						
	Mean						
	CV%						
72h	**1**						
	2						
	3						
	Mean						
	CV%						

8. SCORING OF THE RESULTS

Inhibition of the algal growth relative to the control is determined by daily measurement of the OD of the algal suspensions in the long cells during the 3 days of the test, i.e. after 24h, 48h and 72h exposure to the toxicant.
Put the long cells back in the holding tray 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.

In order to cope with this interference, one extra long cell is needed for each toxicant dilution, which will not be inoculated with algae.

N.B. The 5 extra long cells are not included in the Algaltoxkit, but can be obtained separately

Procedure

1. Prepare the toxicant dilution series as indicated in the section on effluents, and transfer 25 ml of each toxicant dilution into the extra long cell.

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 concentrated algal suspension to each flask, instead of 1 ml.

N.B. Since there is only 75 ml toxicant dilution left in each flask, only 0.75 ml concentrated algal suspension needs to be added to obtain 1.10^4 cells/ml.

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.

The long cells containing the coloured toxicant dilutions shall preferably 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.

9. DATA TREATMENT

1. Calculate the mean daily OD values for the 3 replicate long cells of each toxicant dilution.

2. Calculate the algal growth inhibition from these data by integrating the mean values from t_0 to t_{72} hrs (termed as the "area under the curve") for each concentration tested, including the control.

These calculations can be made by hand, with determination of the EbC_{50} or ErC_{50}, according to the procedures outlined in the ISO guideline "Water Quality - Marine Algal Growth Inhibition Tests with *Skeletonema costatum* and *Phaeodactylum tricornutum*" (Guideline ISO/CD 10253).

The ISO calculation procedure is provided in annex in this manual.

A computer programme for the automatic data treatment can be obtained from the distributors of the Algaltoxkit.

VALIDITY OF THE TEST

For the toxicity test to be acceptable, the number of algae (or the algal biomass) in the control test vials must have increased by at least a factor 16 during the 72h test period. Furthermore, the control pH shall not have increased by more than 1.0 relative to the pH of the growth medium.

10. 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 (preferably concurrently with the bioassays on the toxic samples).

Quality control tests can e.g. be carried out with the reference chemical potassium dichromate ($K_2Cr_2O_7$).

Procedure :

A dilution series ranging from 32 mg/l to 3.2 mg/l is 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 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

Same remark as made earlier for the small (2%) 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 inoculum. The average of all 72h EC_{50} values of the batches of algal inoculum produced by MicroBioTests Inc. during the last years is also mentioned on the Specification Sheet.

The mean ErC_{50} value on $K_2Cr_2O_7$ obtained from the International Interlaboratory Exercise on our Marine Algaltoxkit which has been performed in 2012 (12 results of 12 laboratories in 6 countries) is 17.87 mg/l.

FROM : WATER QUALITY - MARINE ALGAL GROWTH INHIBITION TEST WITH *SKELETONEMA COSTATUM* AND *PHAEODACTYLUM TRICORNUTUM* (International Organization for Standardization - ISO)

9 Expression of results

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 substance 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.

9.2 Calculation of percentage inhibition

Calculate first the average specific growth rate, μ, for each test culture, using the following equation :

$$\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0}$$

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 (9.1.) ;

N_0 is the nominal initial cell density ;

N_L is the measured cell density at time t_L.

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

Calculate mean values of μ for each test concentration and control. From these values, calculate the percentage inhibition for each test concentration, from the following equation :

$$I_{\mu i} = \frac{\overline{\mu}_c - \overline{\mu}_i}{\overline{\mu}_c} \times 100$$

where

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

$\overline{\mu}_i$ is the mean growth rate for test concentration i ;

$\overline{\mu}_c$ is the mean growth rate for the control.

9.3 Determination of E_rC_x

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 references [3] and [4] of Bibliography) 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.

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 short 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.

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

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.

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

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

SPIRODELA DUCKWEED TOXKIT : 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.

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

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