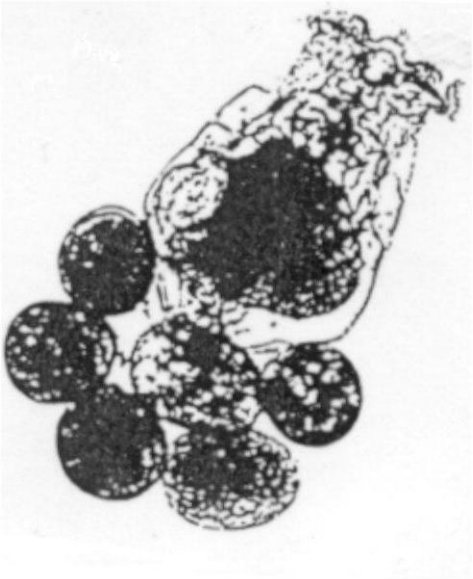
ROTOXKIT M

Rotifer Toxicity Screening Test

for Estuarine and Marine waters



STANDARD OPERATING

PROCEDURE

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INTRODUCTION TO THE ROTOXKIT M

A Screening Rotifer Toxicity Test for Marine and Estuarine waters

Origin : This screening bioassay was developed by the research teams of Prof. em. Dr. Guido Persoone at the State University of Ghent, Belgium and Prof. Dr. Terry W. Snell at the University of Tampa, Florida, USA.

Scope : TOXKITS are aquatic toxicity tests in kits containing all materials as well as the test animals to perform simple, rapid, sensitive and cost-effective acute toxicity tests for chemicals or effluents released in freshwater, estuarine or marine environments.

The major advantage and asset of the TOXKITS, in comparison to conventional bioassays, is that each kit contains the test animals in the form of dormant eggs (cysts) from which larvae can be hatched "on demand", within 24 hours. This eliminates one of the major bottle-necks in aquatic toxicology : the need for continuous stock culturing of test animals.

Principle : A 24h and 48h LC50 bioassay is performed in a multiwell test plate using larvae of the estuarine rotifer *Brachionus plicatilis*, which are hatched from cysts.

Features : Each ROTOXKIT M contains all the (disposable) materials to perform 6 complete screening toxicity tests (range finding of definitive 24h/48h LC50) or 5 bioassays and one quality control test with a reference toxicant. Using juveniles of the rotifer *Brachionus plicatilis* hatched from cysts, an acute toxicity test is executed in 24/48 hours. The only equipment needed is an incubator (25 °C), a dissection microscope (magnification 10-12x) and conventional laboratory glassware.

Sensitivity : The sensitivity of *Brachionus plicatilis* compares favourably with that of many invertebrates currently used in aquatic toxicology.

Precision : Since each ROTOXKIT contains standard test materials, concentrated salt solutions and reference cysts, the repeatability of the toxicity test is very high.

Cyst viability : Optimal viability of the cysts is maintained by storing the vials in the refrigerator (4°C) in darkness. The hatching success of cysts kept in such conditions is guaranteed for at least six months.

Representativity : Rotifers are ecologically important members of many aquatic communities (see Appendix). Rotifers of the genus *Brachionus* have a cosmopolitan distribution and are found in diverse aquatic habitats on all continents.

CONTENTS OF THE ROTOXKIT M

Vials with rotifer cysts
Six 1 ml plastic vials containing reference cysts of the rotifer *Brachionus plicatilis*, preferably to be stored in a refrigerator (5°C (± 2°C)) in darkness to maintain maximum viability. If the hatching procedure is followed properly, the number of neonates obtained will exceed by far the number of test organisms needed for the toxicity test.

Multiwell Test Plates
Six specially designed PVC test plates (13.5 x 9.5 cm), provided with one hatching well, 6 rinsing wells and 36 test wells, which serve both as hatching and test containers.

Concentrated salt solutions
One glass vial with sodium chloride (NaCl) and six 15 ml glass bottles, each containing a concentrated solution of one specific salt, to make up one liter Standard Seawater with deionized water, for preparation of the hatching and the toxicant dilution media.
Composition :
Vial 1 : NaCl (26.4 g - dissolved in 1 l. = 26.4 g/l)
Vial 2 : KCl (840 mg - dissolved in 1 l. = 840 mg/l)
Vial 3 : $CaCl_2.2H_2O$ (1670 mg - dissolved in 1 l. = 1670 mg/l)
Vial 4 : $MgCl_2.6H_2O$ (4600 mg - dissolved in 1 l. = 4600 mg/l)
Vial 5 : $MgSO_4.7H_2O$ (5580 mg - dissolved in 1 l. = 5580 mg/l)
Vial 6 : $NaHCO_3$ (170 mg - dissolved in 1 l. = 170 mg/l)
Vial 7 : H_3BO_3 (30 mg - dissolved in 1 l. = 30 mg/l)

Micropipettes
Six polyethylene micropipettes for transferring the rotifers.

Parafilm strips
Six 10x15 cm strips of Parafilm for sealing the multiwell plate to minimize evaporation during the hatching and incubation periods.

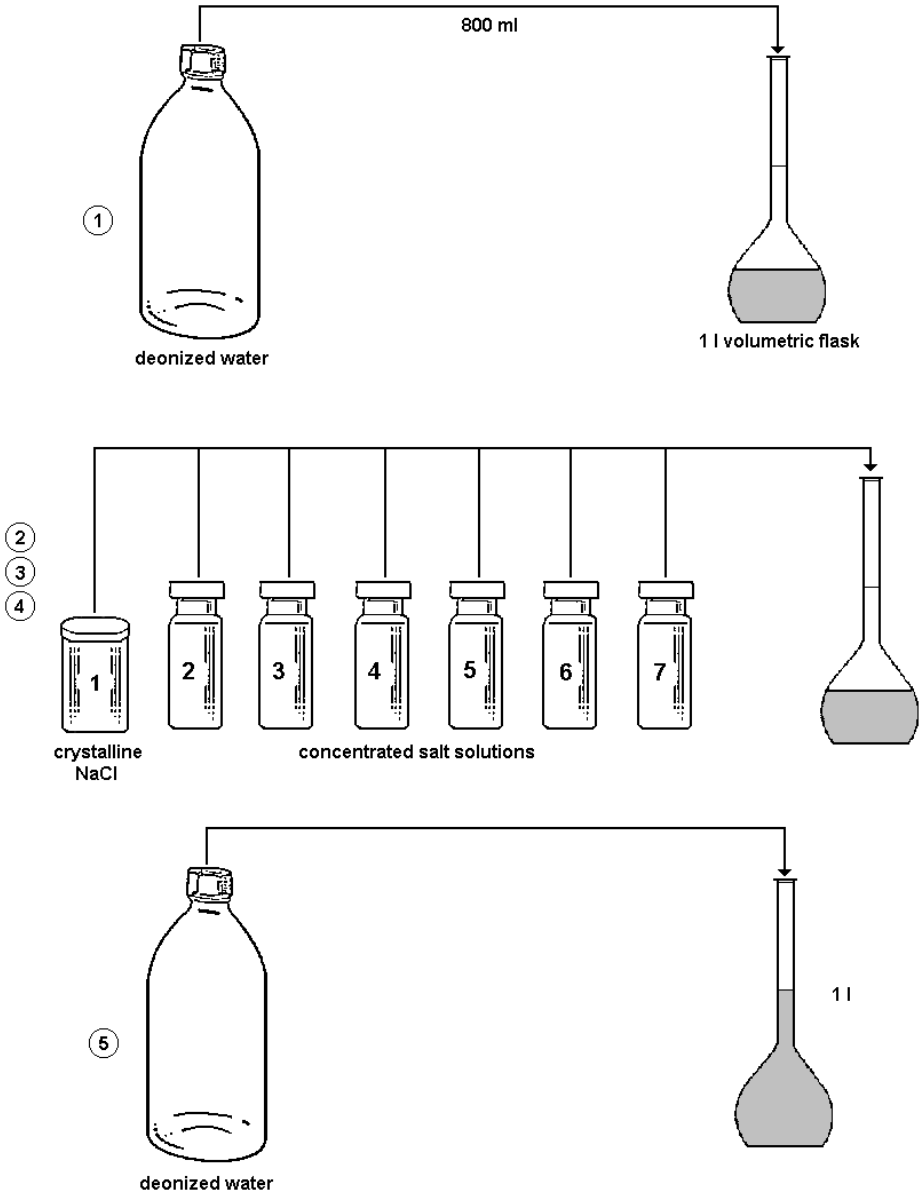
Bench protocol
An abbreviated version of the extended Standard Operational Procedure manual, for those who are familiar with aquatic toxicity tests.

Test protocol
The Standard Operational Procedure manual for the rotifer bioassay, with detailed instructions for performance of range finding tests and/or definitive tests, on pure chemicals or effluents.

Results sheets
Six sheets for scoring the results and calculation of the mean effect percentages.

Specification sheet
A sheet with specifications proper to each individual Rotoxkit, such as the batch number of the cysts, the concentrated salt solutions and the reference toxicant, and with the figure of the average 24h/48h LC50 (with 95% confidence limits) of the reference test.

PREPARATION OF STANDARD SEAWATER



1. PREPARATION OF THE STANDARD SEAWATER

The vials with salt and concentrated salt solutions provided in the kit, are used to prepare one liter standard (artificial) seawater of normal seawater salinity (35 ppt). The Standard Seawater solution is used to prepare the hatching medium for the cysts, and as dilution medium for the toxicant dilution series.

Procedure (see figure) :

1. Fill a 1 liter volumetric flask with approximately 800 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 ($CaCl_2.2H_2O$), vial number 4 ($MgCl_2.6H_2O$), vial number 5 ($MgSO_4.7H_2O$), vial number 6 (NaHCO3) and vial number 7 (H3BO3), respecting this sequence.
5. Add deionized water up to the 1000 ml mark and shake to homogenize the medium.

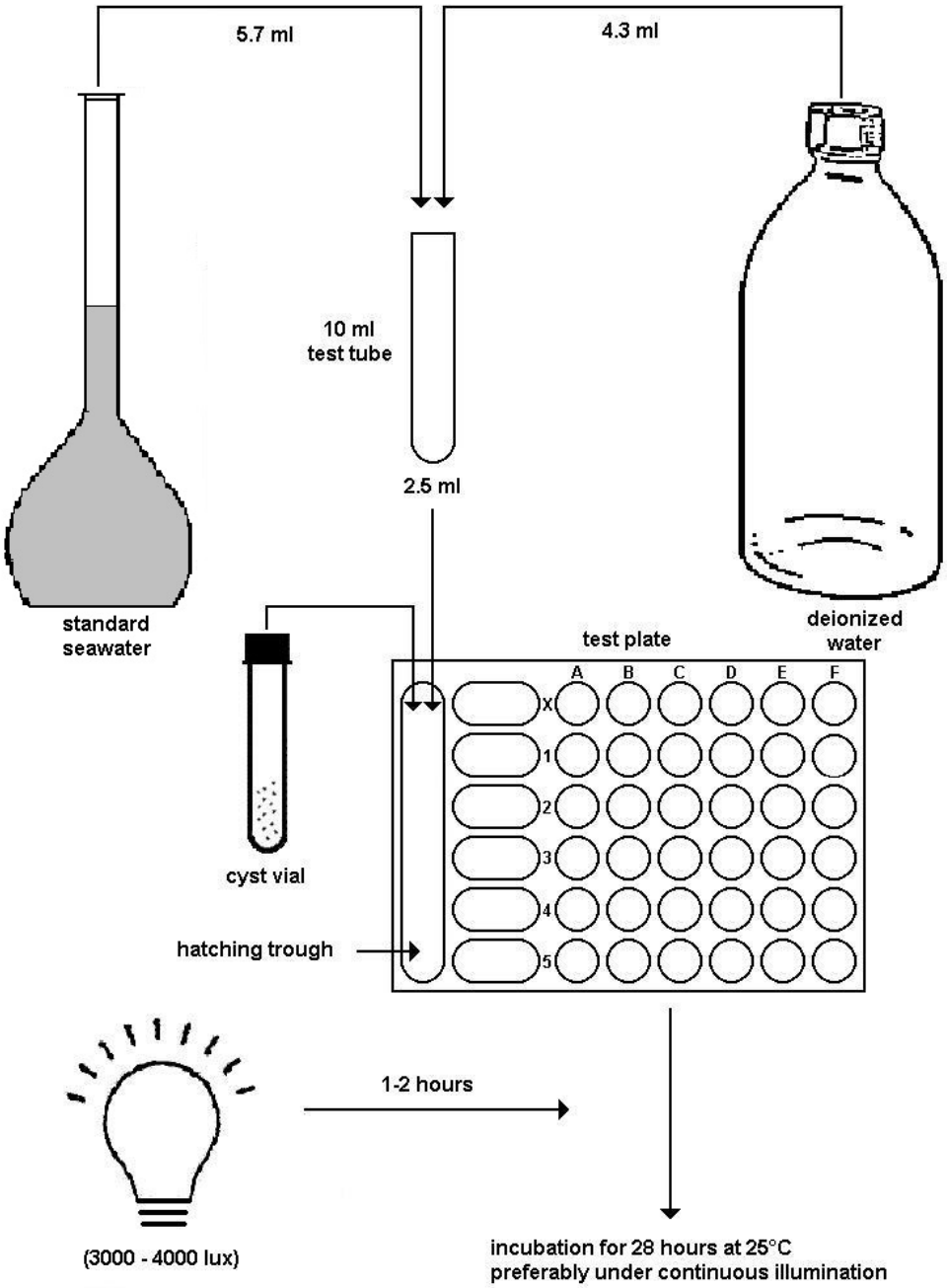
<u>Important remark</u> : For estuarine water bioassays, seawater of reduced salinity can be prepared by diluting the Standard Seawater with deionized water. This "brackish" water has to be used (instead of the 35 ppt Standard Seawater) as dilution medium for preparation of the toxicant dilution series.

Table 1 gives the ratios of Standard Seawater to deionized water to prepare 100 ml dilution medium of various salinities.

Table 1 : Preparation of seawater of reduced salinity

Salinity (ppt)	Standard seawater (ml)	Deionized water (ml)
5	14	86
10	29	71
15	43	57
20	57	43
25	71	29
30	86	14

HATCHING OF THE ROTIFER CYSTS



2. STORAGE OF THE MEDIUM

The 1 liter solution of Standard Seawater suffices for the 6 bioassays of each Toxkit. If all 6 tests are not carried out within a few days after preparation of the medium, store the Standard Seawater in the refrigerator in darkness.
Take care to bring the cooled medium (gradually) back to room temperature prior to use.

3. HATCHING OF THE ROTIFER CYSTS

Rotifer cyst hatching should be initiated **24-26 hours** prior to the start of the toxicity test, and has to be carried out in hatching medium, consisting of seawater of reduced salinity (20 ppt).

Procedure (see figure) :

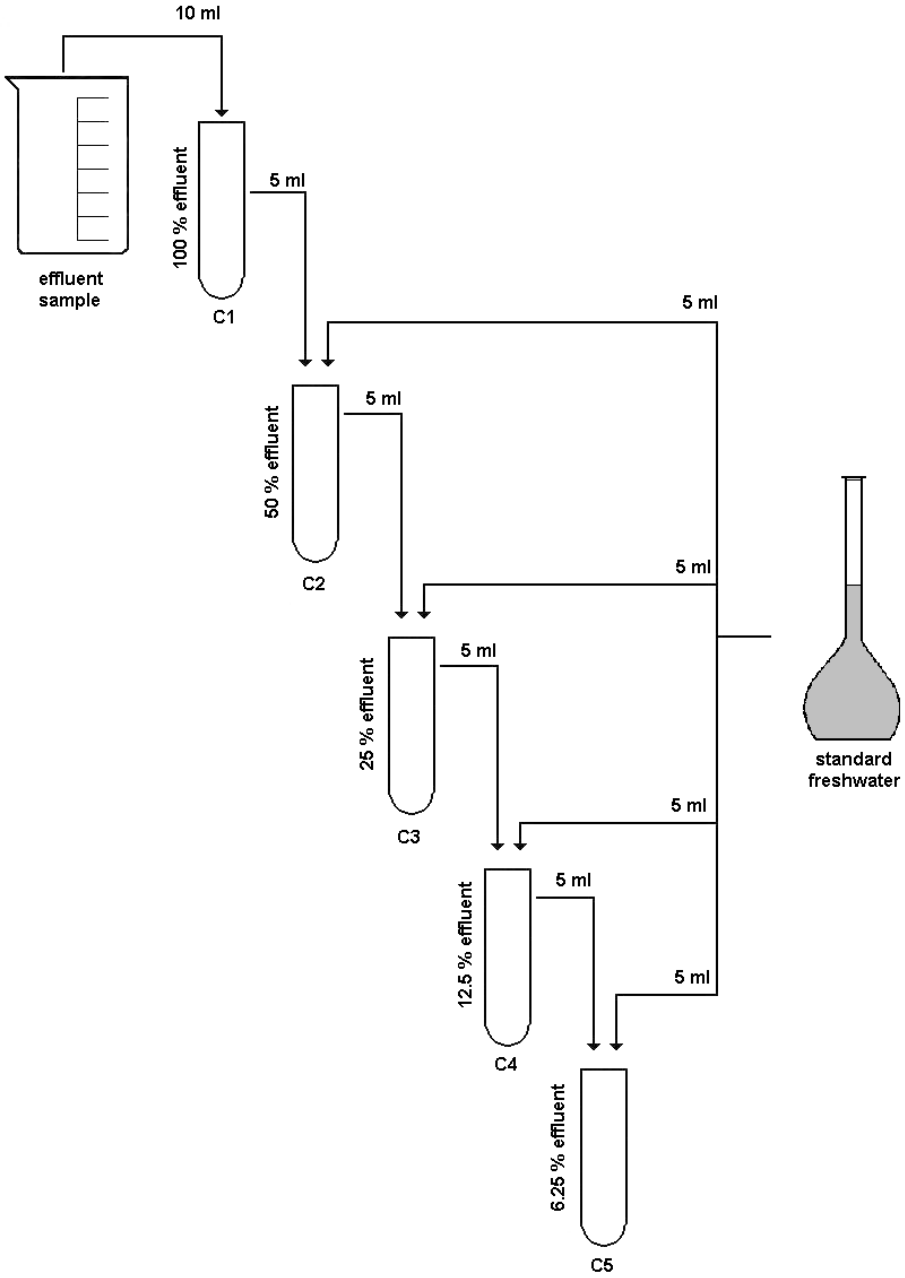
1. Prepare 10 ml hatching medium of 20 ppt salinity by mixing 5.7 ml Standard Seawater with 4.3 ml deionized water in a test tube.
2. Add 2 ml hatching medium to the hatching well.
   One can also use a 5 cm petri dish (with 10 ml hatching medium) to perform the hatching.
3. Empty the contents of one vial with cysts into the hatching well of the test plate; make sure most of the cysts are removed from the vial.
   To secure complete transfer of the cysts, the vial should be rinsed with 0.5 ml hatching medium.
4. Put a strip of Parafilm on the test plate, cover the multiwell and incubate the plate **at 25 °C for 24-28 hours, with continuous illumination (light source of min. 3000-4000 lux)**.

The hatching time for different cyst batches may vary slightly and is indicated on the specification sheet.

<u>If, after 28 hours the number of neonates is too small to start a test, incubate the test plate for another 2 hours.</u>

PREPARATION OF THE TOXICANT DILUTIONS

A. EFFLUENTS



4. PREPARATION OF THE TOXICANT DILUTION SERIES

The TOXKIT bioassays have been designed primarily for cost-effective acute toxicity screening; consequently this section of the Standard Operational Procedure proposes a simple and rapid way to make toxicant dilution series with the aid of disposable 10 ml plastic tubes and disposable 1 ml and 10 ml plastic graduated pipettes.

Clearly greater precision may be gained by using conventional laboratory glassware.

A. Effluents

A dilution series 100% - 50% - 25% - 12.5% and 6.25% of the effluent sample is prepared by the serial dilution procedure; each dilution is made by diluting the previous concentration by half with dilution medium (cf. US-EPA/600/4-85/013, 1985).

Procedure (see figure) :

1. Add 5 ml dilution water to test tubes 2, 3, 4, and 5.

2. Add 10 ml effluent sample to test tube 1 and rinse the pipette.

3. Using the same pipette, transfer 5 ml of test tube 1 to test tube 2 and rinse the pipette; cap and shake test tube 2.

4. Repeat this procedure (step 3) for the next dilutions (see Table 2)

   * 5 ml form test tube 2 to test tube 3
   * 5 ml from test tube 3 to test tube 4
   * 5 ml from test tube 4 to test tube 5

5. Proceed to section 5 : **Filling of the Test Plate**.

PREPARATION OF THE TOXICANT DILUTIONS

B. CHEMICAL COMPOUNDS

Range finding test

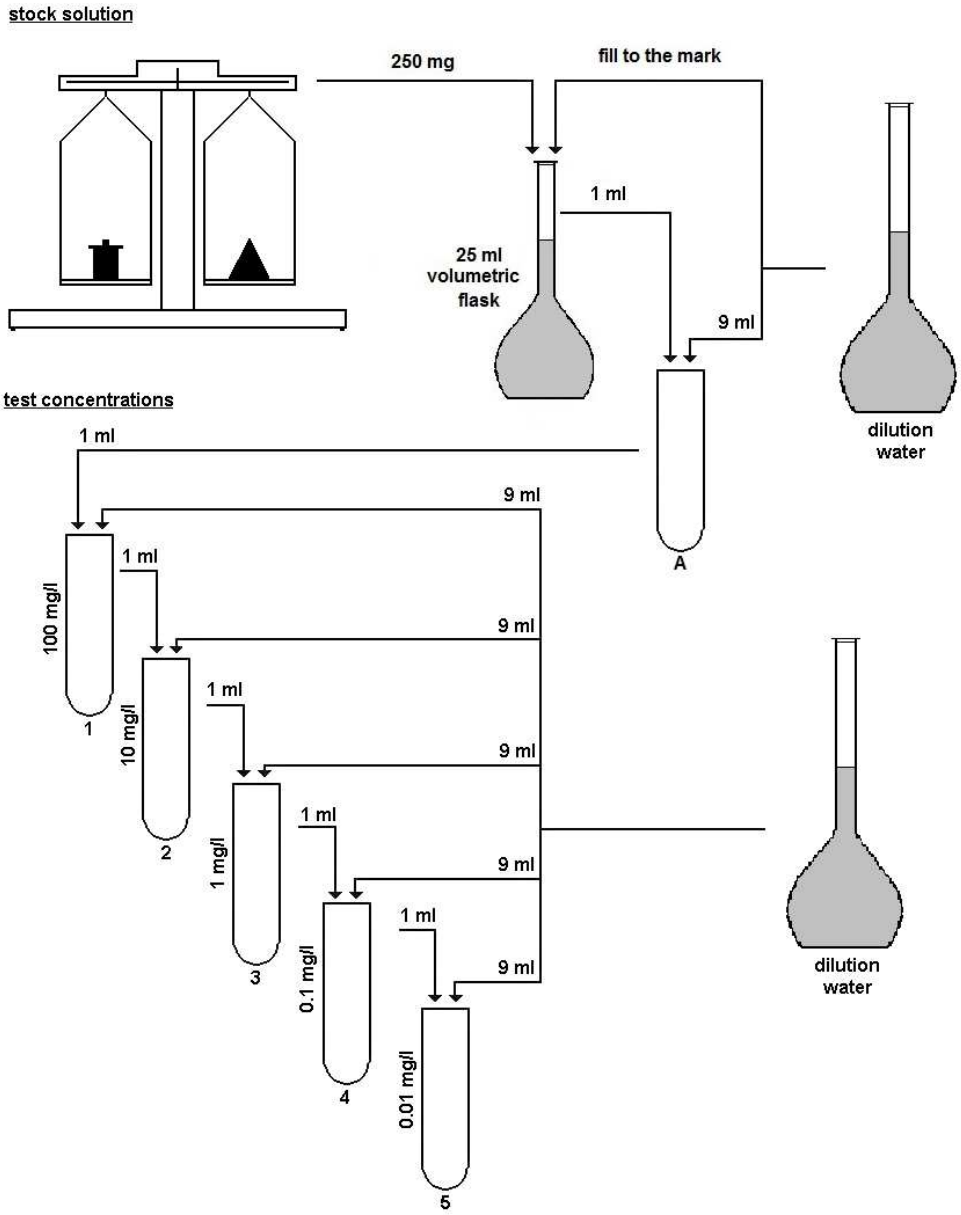


Table 2 : Dilution series of the effluent

Test tube	Effluent concentration (%)
1	100
2	50
3	25
4	12.5
5	6.25

B. Chemical compounds

If the approximate toxicity of the chemical compound is known, one may proceed directly to the **definitive test**.
If the approximate toxicity of the chemical is not known, a **range finding** test must be carried out.

Range finding test

The dilution series: 100 mg/l, 10 mg/l, 1 mg/l, 0.1 mg/l, and 0.01 mg/l will be prepared for testing.

Procedure (see figure) :

Stock solution

1. Weigh 250 mg of the chemical on an analytical balance and transfer it into a 25 ml volumetric flask.

2. Fill to the mark with dilution water and shake vigorously.

3. Transfer 1 ml of the 25 ml volumetric flask to tube A and rinse the toxicant pipet.

4. Add 9 ml dilution water, cap and shake the test tube.

Test concentrations (see Table 3)

1. Add 9 ml dilution water to test tubes 1, 2, 3, 4, and 5.

2. Add 1 ml of test tube A to test tube 1 and rinse the toxicant pipette; cap and shake test tube 1.

3. Add 1 ml of test tube 1 to test tube 2 and rinse the toxicant pipette; cap and shake test tube 2.

4. Repeat this procedure (step 3) for the next dilutions:

   * 1 ml from test tube 2 to test tube 3.
   * 1 ml from test tube 3 to test tube 4.
   * 1 ml from test tube 4 to test tube 5.

5. Proceed to section 5: **Filling of the Test Plate**.

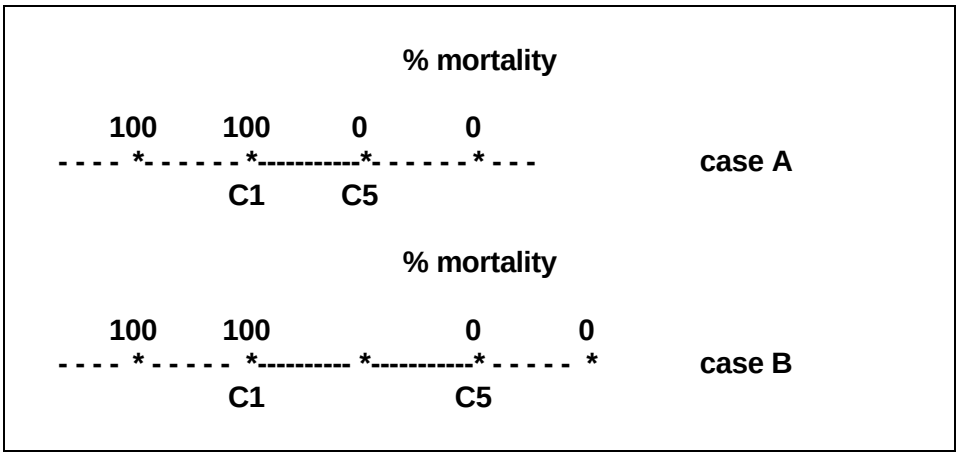
Table 3 : Dilution series of chemical compound

Test tube	Chemical concentration (mg/l)
1	100
2	10
3	1
4	0.1
5	0.01

Definitive test

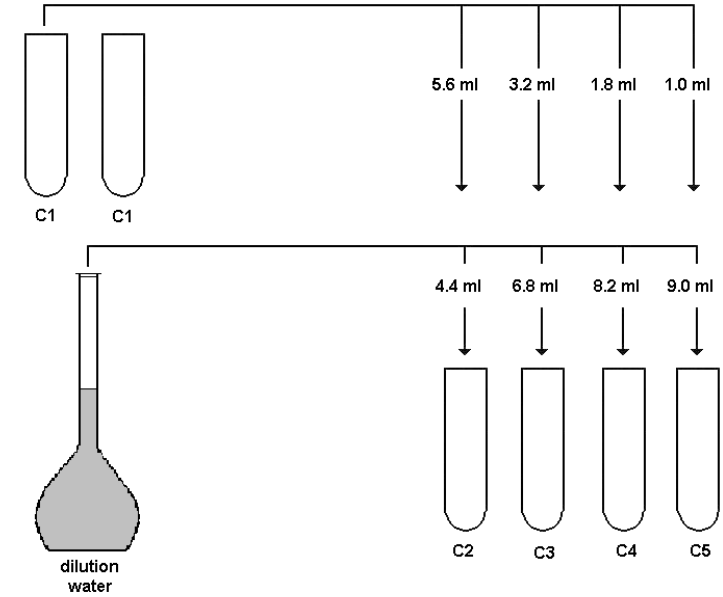
The dilution series to be tested in the definitive test spans the range of the lowest concentration producing 100 % mortality and the highest concentration producing 0 % mortality in the range finding test. This range can span one order of magnitude (case A) or two orders of magnitude (case B) as indicated in Table 4. The concentration range used will be called C1 - C5.

Table 4 : Diagrammatic representation of the 100 % and 0 % mortality concentration range, as determined in the range finding test

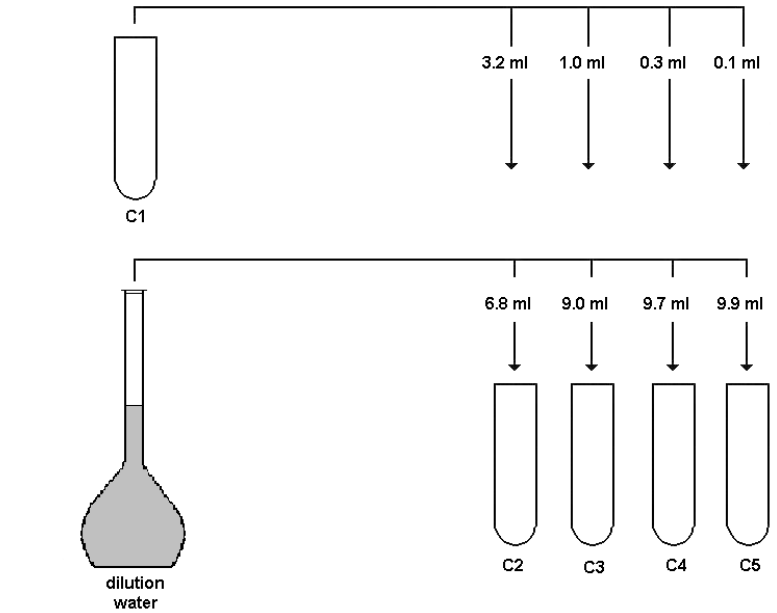


Definitive test

A : C1- C5 spans one order of magnitude



B : C1 - C5 spans two orders of magnitude



Procedure (see figure) :

A dilution series ranging from C1 (100 % mortality) to C5 (0 % mortality) is prepared.
C1 is prepared according to the dilution instructions given in Table 3.

A. C1 - C5 spans one order of magnitude

***Important remark* : in this case concentration C1 must be prepared in duplicate (two test tubes).**

1. Add the volumes of dilution water as indicated in Table 5 to the respective test tubes.

2. Add the volumes of toxicant concentration C1 as indicated in Table 5.

3. Cap and shake the test tubes.

Table 5 : Dilution series C1 - C5

Test tube	dilution water (ml)	C1 (ml)
C1	0	10
C2	4.4	5.6
C3	6.8	3.2
C4	8.2	1.8
C5	9.0	1.0

4. Calculate the actual concentrations of C1, C2, C3, C4 and C5 (these figures are needed for the LC50 estimation).

C1 = ...mg/l

C2 = 0.56 × C1 = ...mg/l

C3 = 0.32 × C1 = ...mg/l

C4 = 0.18 × C1 = ...mg/l

C5 = 0.10 × C1 = ...mg/l

5. Proceed to section 5: **Filling of the Test Plate**.

B. C1 - C5 spans two orders of magnitude.

***Remark* : only one test tube of the C1 concentration has to be prepared.**

1. Add the volumes of dilution water as indicated in Table 6 to the respective test tubes.

2. Add the volumes of toxicant concentration C1 as indicated in Table 6.

3. Cap and shake the test tubes.

Table 6 : Dilution series C1 - C5

Test tube	dilution water (ml)	C1 (ml)
C1	**0**	**10**
C2	**6.8**	**3.2**
C3	**9.0**	**1.0**
C4	**9.7**	**0.3**
C5	**9.9**	**0.1**

4. Calculate the actual concentrations of C1, C2, C3, C4, and C5 (these figures are needed for the LC50 estimation).

C1 = ...mg/l

C2 = 0.32 × C1 = ...mg/l

C3 = 0.10 × C1 = ...mg/l

C4 = 0.03 × C1 = ...mg/l

C5 = 0.01 × C1 = ...mg/l

5. Proceed to section 5: **Filling of the Test Plate**.

5. FILLING OF THE TEST PLATE

The bioassay is conducted in a specially developed, disposable, PVC "multiwell" test plate. Each plate has one hatching well, 6 rinsing wells and 36 test wells (see figure). The rinsing wells and the test wells are labelled as columns A to F across, and rows X and 1 to 5 down. The distribution of the test solutions should always be carried out starting from the control (X, top row) towards the highest concentration (5, bottom row).

Procedure (see Figure) :

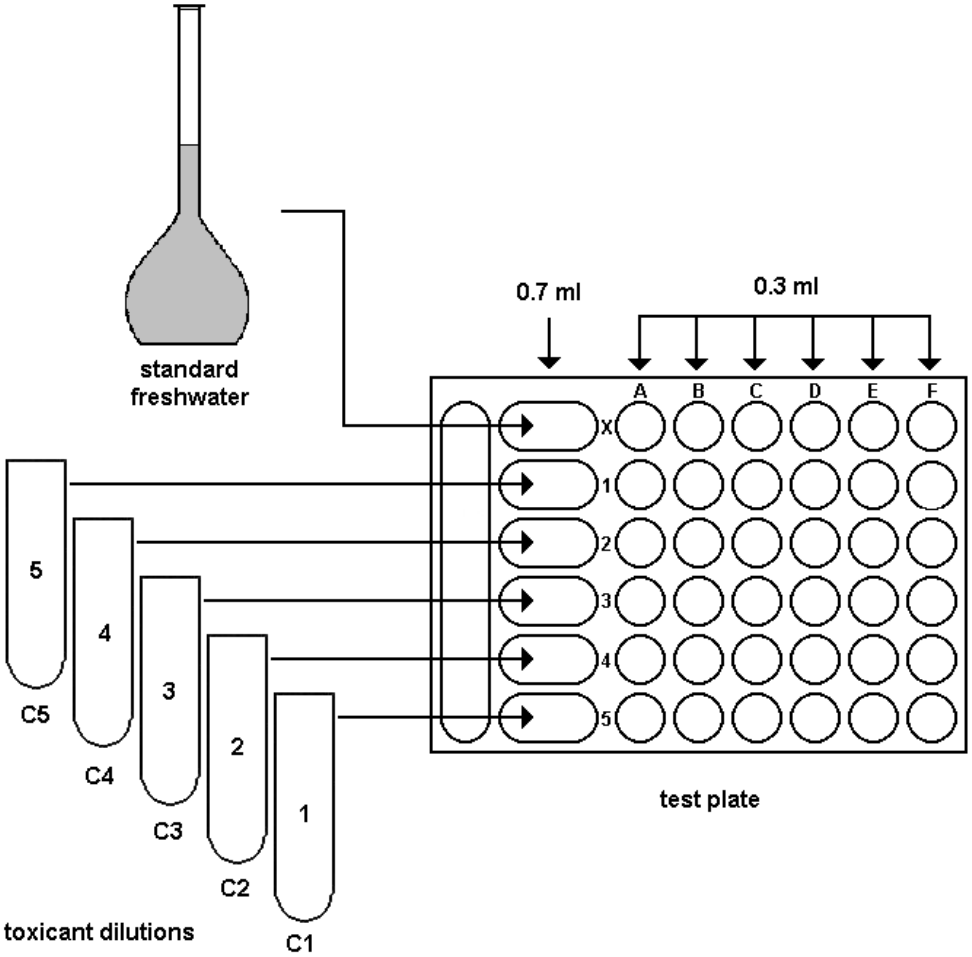
Controls

1. Add 0.7 ml dilution water (Standard Seawater or "brackish" water) to the rinsing wells of the top row (X)
2. Add 0.3 ml dilution water to each of the six test wells of the top row (X).

Toxicant dilutions

3. Shake each (capped) test tube thoroughly before the transfer of the toxicant
4. Transfer 0.7 ml of test tube 5 to the rinsing well of row 1.
5. Transfer 0.3 ml of test tube 5 to each of the 6 test wells in row 1.
6. Repeat this procedure (steps 4 and 5) with test tubes 4, 3, 2, and 1 to fill the rinsing wells and the test wells of rows 2, 3, 4, and 5 respectively.

FILLING OF THE TEST PLATE



6. TRANSFER OF THE ROTIFERS INTO THE TEST WELLS

Rotifers are small animals; at 0.25 mm in length they are about 1/4 the size of e.g. a new-born *Daphnia*. **Do not panic** when you first see them under the microscope at 10 X magnification. Their small size has some advantages for manipulation, and since they swim slowly, rotifers are easy to catch with a micropipette.

Newly hatched rotifers are white, so they are most visible **against a dark background**. If your microscope has a dark field setting, this is the best type of illumination. A dark background can also be obtained with most microscopes by adjusting the angle of illumination and its intensity.

Since they are moderately phototactic, rotifers tend to **congregate around the edges of the hatching well**.

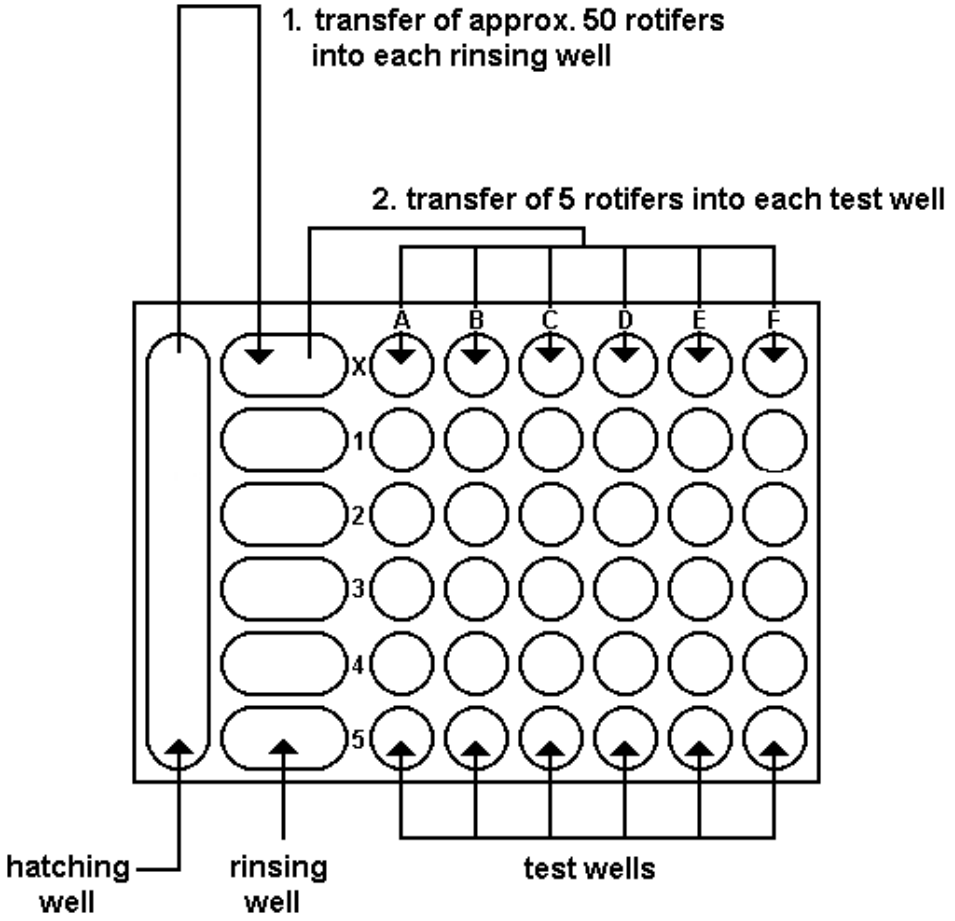
There are several ways to make rotifer manipulation easy and more efficient.

The micropipette should be held like a pencil with the index finger and thumb providing pressure on the bulb. This position usually provides the best control and produces the least fatigue, but any position may be used if it feels more comfortable. The bulb should be **squeezed gently** to provide adequate suction. It will take a little practice to develop a feel for just the right pressure.

Micro-pipetting of rotifers is an easily acquired skill. After about 15 minutes of practice, most people become sufficiently skilled at collecting, counting, and transferring rotifers rapidly to successfully complete the bioassay. Once accustomed to it, micro-pipetting becomes comfortable for most people.

***Important remark* : The transfer of the rotifers into the test wells should be made within 2 hours after cyst hatching. It is indeed important to have test animals of the same age (i.e. 0 to 2 hours old) to start the bioassay. For this reason the hatching well has to be checked hourly after 28 hours incubation, to find out whether enough cysts have hatched to start the transfer.**

TRANSFER OF THE ROTIFERS INTO THE TEST WELLS



Procedure (see Figure) :

1. Take the test plate out of the incubator and put it on the stage of the dissection microscope.

The next steps are executed under a dissection microscope at magnification 10-12x.

2. Transfer approximately 50 rotifers with the micropipette from the hatching well into the rinsing well of control row X (top row).
3. Repeat this operation for rows 1, 2, 3, 4 and 5. Make sure not to touch the toxicant solution in the rinsing wells with the tip of the micropipette to avoid contamination in the next steps of the procedure. Eventually rinse the micropipette with deionized water.
4. Wait for approximately one hour to allow the rotifers to adapt to the eventual salinity change (e.g. from 20 ppt in the hatching medium to the salinity in the test medium). The rotifers are fully adapted when they resume active swimming.
5. Transfer 5 rotifers from the rinsing well to each of the six wells in row X. Take care during this operation to carry over as little as possible medium along with the rotifers. It is advised to count the rotifers as they exit the micropipette, to make sure that exactly five organisms are put in each test well.
6. Repeat this operation (step 5) for rows 1, 2, 3, 4 and 5, in this sequence (i.e. in increasing order of concentration of the toxicant).
7. After completion of the last transfer, empty the hatching well to avoid spilling of the hatching medium into the test wells during subsequent transportation of the test plate.

***Remark** : The intermediate transfer of the rotifers from the hatching well to the wells via a rinsing well "washes" the neonates in the appropriate test solution before they enter the actual test well, thus minimizing dilution of the test solution during rotifer transfer.*

7. INCUBATION OF THE TEST PLATE

Procedure

1. Put the strip of Parafilm back on the test plate, cover the multiwell and incubate at 25°C in darkness, **for 24 and 48 hours**.

8. SCORING OF THE RESULTS

Procedure

1. Take the test plate out of the incubator and put it under the dissection microscope.
2. Check each test well of columns A, B, C, D, E and F, and record the number of dead* and living rotifers.

* *The organisms are considered dead if they do not exhibit any movement in 5 seconds of observation.*

3. Score the mortality figures on the RESULTS SHEET.

4. Total the number of dead rotifers for each concentration and calculate the % mortality*.

* *If the mortality in the controls exceeds 10 %, the bioassay is considered invalid and the test must be repeated !*

ROTOXKIT M - RESULTS SHEET

Name of operator : .....

Date of performance of test : .....

Toxicant tested : .....

Type of test : O range finding
O definitive

Salinity of the test medium : ..... ppt

Dilution series tested : concentration 1 = ...
concentration 2 = ...
concentration 3 = ...
concentration 4 = ...
concentration 5 = ...

		A	B	C	D	E	F	Total	% mortal.
Control	24h							/ 30	
	48h							/ 30	
Conc. 5	24h							/ 30	
	48h							/ 30	
Conc. 4	24h							/ 30	
	48h							/ 30	
Conc. 3	24h							/ 30	
	48h							/ 30	
Conc. 2	24h							/ 30	
	48h							/ 30	
Conc. 1	24h							/ 30	
	48h							/ 30	

Mortality scores

9. ESTIMATION OF THE LC50

There are many procedures for calculating 50% effect thresholds. A data treatment program to calculate the 24h and the 48h EC_{50} for the Rotoxkit M microbiotest is available on demand from MicroBioTests Inc.

10. REFERENCE TEST

In order to check the correct execution of the test procedure and the good physiological condition of the test animals, it is advisable to regularly perform a reference test for quality control.

Such a quality control test can e.g. be performed with the reference toxicant potassium dichromate ($K_2Cr_2O_7$)

Procedure

1. Weigh 100 mg potassium dichromate on an analytical balance, transfer it to a 100 ml volumetric flask and fill to the mark with Standard Seawater.
2. Add Standard Seawater up to the mark, cap the flask and shake to dissolve and homogenize the 1 g/l stock solution of potassium dichromate.
3. Make a dilution series of the reference toxicant (e.g. in 10 ml plastic tubes), according to the procedure indicated in section 4 : **Chemical compounds - Definitive test**.

The dilution series to be made and used for the test is the following :

C1 : 1000 mg/l
C2 : 560 mg/l
C3 : 320 mg/l
C4 : 180 mg/l
C5 : 100 mg/l

4. Proceed to section 5. **Filling the Test Plate**.

From the data obtained in the quality control test, a 24/48 hr LC50 has to be calculated, the value of which should be situated within the limits (range) stipulated in the specification sheet.

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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