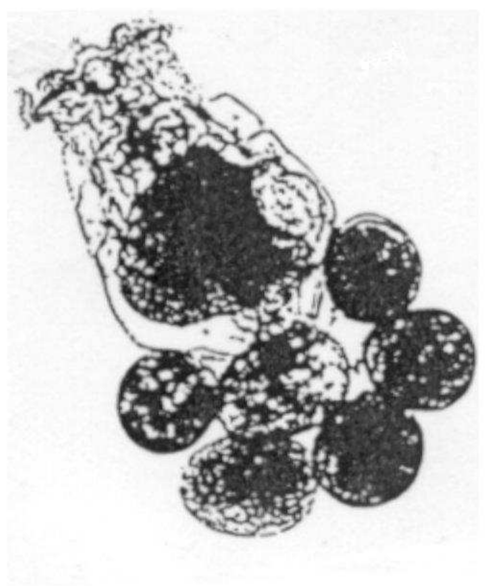
Chronic Toxicity Test

for Freshwater



STANDARD OPERATING

PROCEDURE

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INTRODUCTION TO THE ROTOXKIT F CHRONIC

A Chronic Rotifer Toxicity Test for Freshwater

Origin : The 2-day chronic rotifer test originates from the research team of Prof. Dr. T. Snell at the University of Tampa, Florida, USA and has been adapted to a Toxkit microbiotest by the research team of Prof. Dr. G. Persoone at the Laboratory for Biological Research in Aquatic Pollution at the University of Ghent in Belgium.

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

Advantages of TOXKIT tests : The major advantage of Toxkit microbiotests, in comparison to “conventional” bioassays, is that the test organisms are incorporated in the kits in a “dormant” 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 practical and user friendly microbiotests which can be performed with conventional lab materials and equipment, on little bench space.

Principle : A 48 h EC_{50} bioassay is performed in a multiwell test-plate using rotifer juveniles hatched from cysts.

Features : Each ROTOXKIT F CHRONIC contains all the (disposable) materials to perform 3 complete 48h toxicity tests with the freshwater rotifer *Brachionus calyciflorus*. The equipment needed comprises an incubator (25°C), a laboratory centrifuge, a dissection microscope (magnification 10-12x) and conventional laboratory glassware.

Sensitivity : The chronic rotifer microbiotest has been developed specifically as a “second tier” test for the acute Rotoxkit F microbiotest. The latter assay is based on mortality of the test organisms after 24h exposure to toxicants. The chronic Rotoxkit F measures the reproduction of the rotifers during 48h exposure, and uses “growth inhibition” as a (more sensitive) criterion for the evaluation of toxic effects.

Precision : Since the chronic Rotoxkit F contains standard test (bio)materials and test media, 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 several months as indicated on the expiry date label on each kit.

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

CONTENTS OF THE CHRONIC ROTOXKIT F

Vials with rotifer cysts
Three small plastic vials containing reference cysts of the rotifer *Brachionus calyciflorus*, to be stored in a refrigerator (4°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
Three specially designed polycarbonate test plates, composed of one hatching trough, 6 rinsing troughs and 48 test wells.

Concentrated salt solutions
Five small glass bottles, each containing a concentrated solution of one particular salt, to make up 1 litre Standard Freshwater (medium hard EPA reconstituted water) with deionized water, for both the cyst hatching and the preparation of the toxicant dilutions.
Composition :
Vial 1 : $NaHCO_3$ (96 mg - dissolved in 1 l. = 96 mg/l)
Vial 2 : $CaSO_4.2H_2O$ (60 mg - dissolved in 1 l. = 60 mg/l)
Vial 3 : $CaSO_4.2H_2O$ (60 mg - dissolved in 1 l. = 60 mg/l)
Vial 4 : $MgSO_4.7H_2O$ (123 mg - dissolved in 1 l. = 123 mg/l)
Vial 5 : KCl (4 mg - dissolved in 1 l. = 4 mg/l)

Vials with algal beads
Three tubes containing small beads of green algae (*Selenastrum capricornutum* (first renamed as *Raphidocelis subcapitata* and presently as *Pseudokirchneriella subcapitata*)) immobilized in an inert matrix. The tubes with the algal beads in their storage medium must be kept in the refrigerator (at 4°C) in darkness until use. The green algae will be used as food for the rotifers.

Matrix dissolving medium
Two glass bottles containing a special medium for dissolving the matrix in which the microalgae are immobilized. This medium must be stored in the refrigerator (at 4°C) in darkness.

Vials with Roti-Rich powder
Three tubes with Roti-Rich powder for "pre-feeding" the test organisms prior to the toxicity test.

Lugol solution

One small glass bottle with Lugol fixative solution, to fix and stain the rotifers for easy counting at the end of the exposure period.

Micropipettes

Three polyethylene micropipettes for transfer of the rotifers and one for the pre-feeding of the rotifers.

Parafilm strips

Three strips of Parafilm for sealing the multiwell plate to minimize evaporation during the hatching and incubation periods

Standard Operational Procedure manual

A detailed brochure with all instructions for the performance of range finding and/or definitive tests, on pure chemicals or effluents/wastes.

Bench protocol

An abbreviated version of the extended Standard Operational Procedure manual.

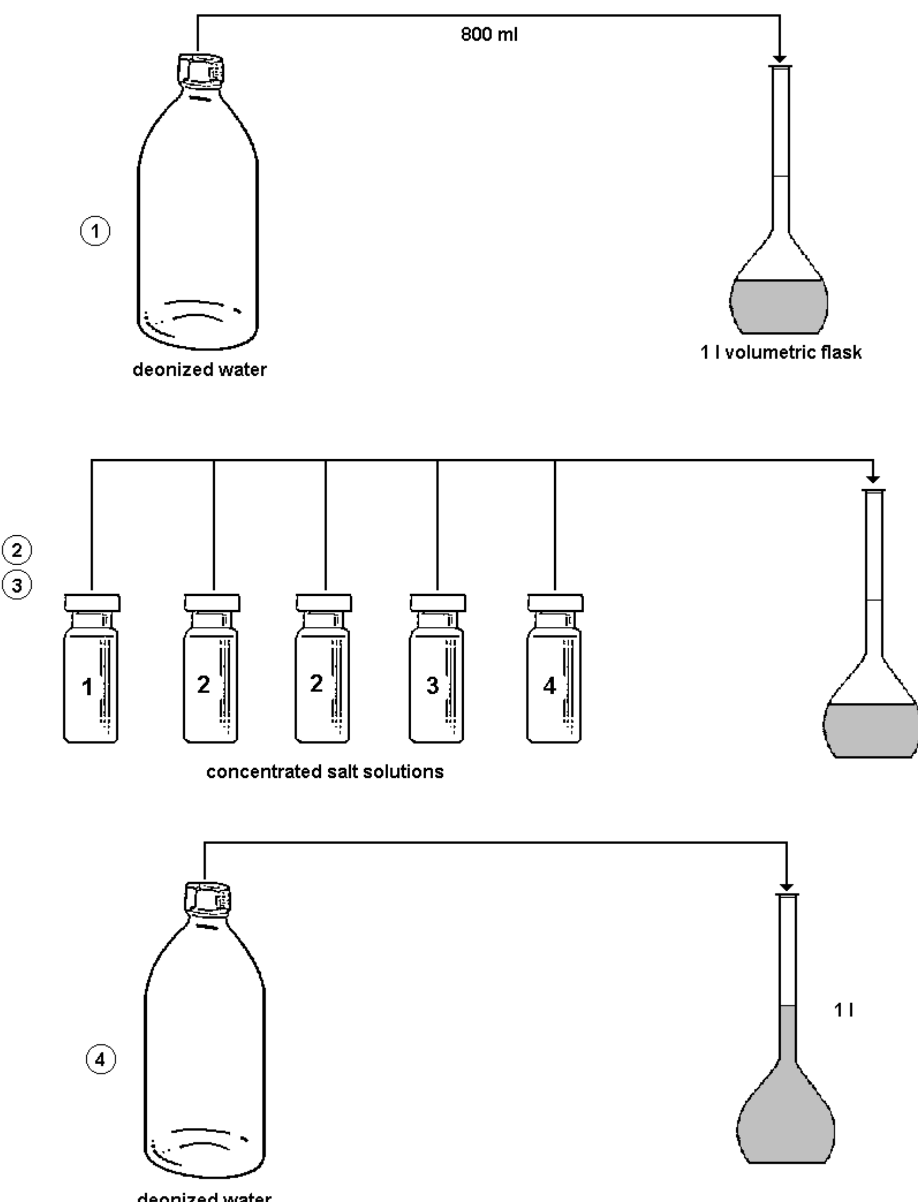
Results sheets

Sheets for scoring of the results and calculation of the growth rate of the rotifers and the toxic effect percentages in the test concentrations used.

Specification sheet

A sheet indicating the batch number and shelf life of the cysts and the algal beads, the batch number of the concentrated salt solutions, the expiry date of the Rotoxkit F chronic and the $48hEC_{50}$ value for tests with the reference chemical potassium dichromate.

PREPARATION OF STANDARD FRESHWATER



1. PREPARATION OF STANDARD FRESHWATER

General remark : The solutions described hereunder are prepared with deionized water or distilled water. To avoid repetition, only the wording "deionized water" will be used further on.

The vials with concentrated salt solutions provided in the kit are used to prepare 1 litre Standard Freshwater. The reconstituted freshwater selected for the Rotoxkit tests is a "moderately hard water" (US EPA formula). The Standard Freshwater is used as hatching medium for the cysts and as the medium for preparation of the toxicant dilution series.

Procedure (see figure)

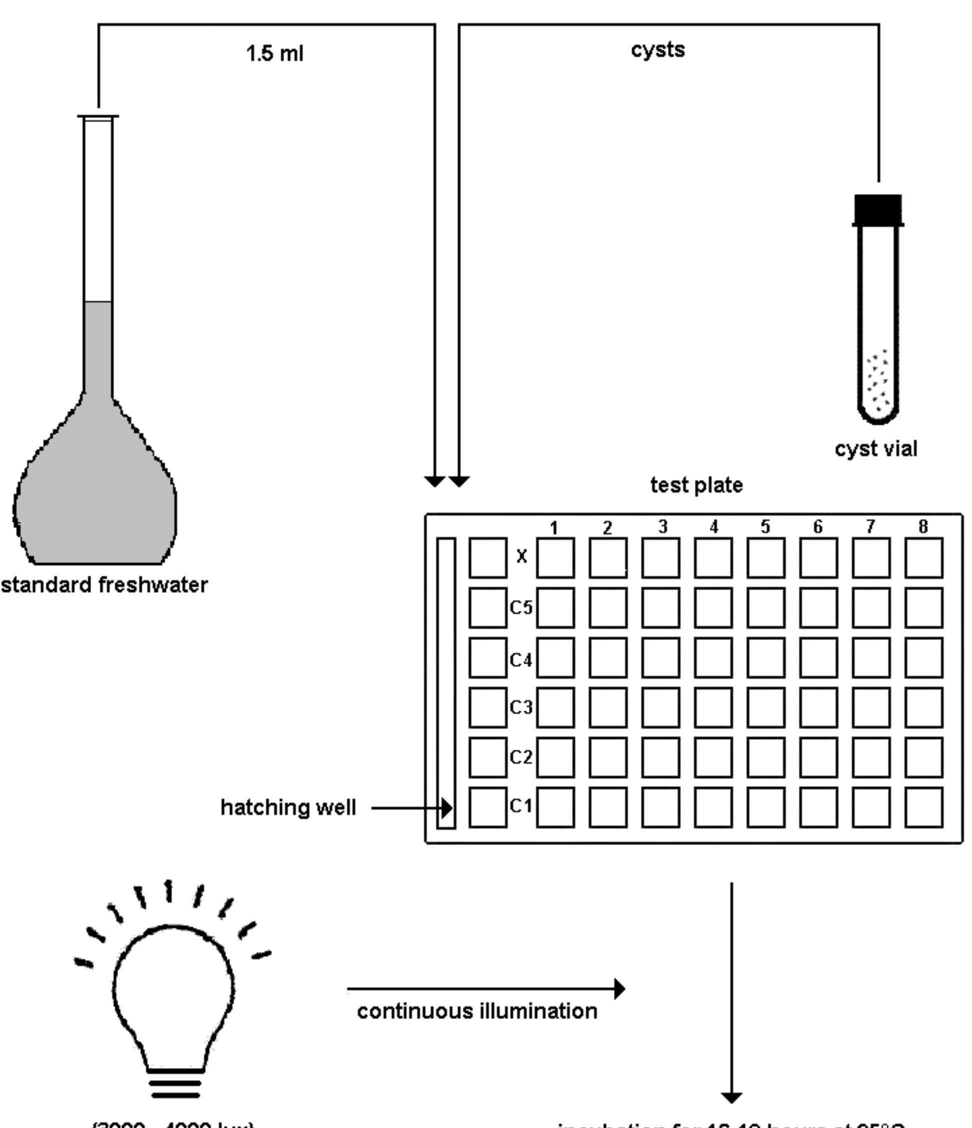
1. Fill a 1 litre volumetric flask with approximately 800 ml deionized water.
2. Uncap the vial with concentrated salt solution labelled number 1 ($NaHCO_3$), and pour the contents in the flask.
3. Repeat step 2 for the other vials with concentrated salt solutions, i.e. two vials number 2 ($CaSO_4.2H_2O$), one vial number 3 ($MgSO_4.7H_2O$) and one vial number 4 (KCl), respecting this sequence.
4. Add deionized water up to the 1 litre mark and shake to homogenize the medium.

2. STORAGE OF THE STANDARD FRESHWATER

The 1 litre Standard Freshwater suffices for the 3 bioassays which can be performed with one chronic Rotoxkit F kit. If all 3 tests are not carried out within a few days after preparation of the medium, store the Standard Freshwater in the refrigerator in darkness. Take care to bring the cooled medium (gradually) back to room temperature prior to use.

3. PRE-AERATION OF THE STANDARD FRESHWATER

It is strongly advised to aerate the Standard Freshwater for at least 15 minutes prior to use it for the hatching of the cysts and for the preparation of the toxicant dilutions.
Pre-aeration can be performed very easily by air bubbling through a tube connected to an aquarium pump.


HATCHING OF THE ROTIFER CYSTS
1.5 ml
cysts
cyst vial
test plate
standard freshwater
1
2
3
4
5
6
7
8
X
C5
C4
C3
C2
C1
hatching well
continuous illumination
(3000 - 4000 lux)
incubation for 16-18 hours at 25°C

4. HATCHING OF THE ROTIFER CYSTS

*Rotifer cyst hatching should be initiated **16-18 hours** prior to the start of the toxicity test.*

Procedure (see Figure)

1. Add 1.5 ml Standard Freshwater to the hatching trough.
   One can also use a 5 cm petri dish (with 10 ml Standard Freshwater) to perform the hatching.
2. Empty the contents of one vial with cysts into the hatching trough of a multiwell test plate.
   Make sure most of the cysts are carried over during the transfer ! To secure complete transfer of the cysts, the vial should be rinsed with 0.5 ml Standard Freshwater.
3. Put a strip of Parafilm on the test plate and cover with the lid.
   Put the multiwell in an incubator **for 16-18 hours (*) at 25°C, 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.

5. PRE-FEEDING OF THE ROTIFER CYSTS

"Pre-feeding" of the freshly hatched rotifers for 2 hours with a specific inert rotifer food (Roti-Rich) prior to the start of the short-chronic toxicity test, substantially increases the reproduction rate of the test organisms during the test, in comparison to "non-pre-fed" rotifers.

General remarks :

1. ***The Roti-Rich food suspension should be prepared just before the pre-feeding of the rotifer is carried out.***
2. ***The pre-feeding must be carried out as soon as the rotifer cysts have hatched, i.e. after 16-18h incubation.***

Procedure

1. Take one of the 3 tubes with Roti-Rich food and add 1 ml Standard Freshwater.

2. Close the tube and mix the contents thoroughly to obtain a homogenous Roti-Rich food suspension.
3. Take the micropipette and suck up a small volume of Roti-Rich food suspension.
4. Keeping the micropipette vertically, add "one" drop of Roti-Rich food suspension to the hatching well with the freshly hatched rotifers (add 5 drops if a 5 cm petri dish is used).
5. Allow the rotifers to feed for exactly "two hours" before the start of the toxicity test.

N.B. The Roti-Rich food suspension deteriorates rapidly and cannot be stored for any subsequent use !

6. PREPARATION OF CONCENTRATED ALGAL FOOD SUSPENSION

1. Take one tube with algal beads, pour out the storage medium and add 7 ml matrix dissolving medium.
2. Cap the tube and shake the contents intermittently (mixing on a Vortex mixer is advised) until the matrix has fully dissolved and the microalgae are totally set free. This will take approximately 10-15 min.
3. Centrifuge the tube at 3000 rpm for 10 minutes.
4. Pour out the supernatant, add 10 ml deionized water and re-suspend the algae by shaking.
5. Centrifuge the tube again at 3000 rpm for 10 minutes.
6. Pour out the rinsing water.
   Make sure that all the water is removed "to the last drop" !
7. Add 1.8 ml Standard Freshwater, cap the tube and shake thoroughly to re-suspend the algae.

The concentrated food stock contains 2.10^8 algal cells per ml. A very small amount of this food (2.10^6 algal cells per ml) will be provided to the rotifers during the test, to allow for their growth during the 48h exposure period.

7. PREPARATION OF THE TOXICANT DILUTIONS

7.1. TESTS ON EFFLUENTS

***General remark** : The procedure described below for the testing of effluents (or liquid wastes) can also be applied to any other type of contaminated liquids (e.g. surface waters, ground-waters, sediment pore waters, soil or waste leachates, etc.).*

1. Sample preparation

In order to avoid interference by particulate matter and/or biota which may be present in particular effluent samples, it is recommended to clean the samples prior to testing.
Preference is given to centrifugation with use of the supernatant for testing.
Fifty ml of the sample is centrifuged at 10.000 g for 15 minutes. The upper half (at least 25 ml) of the supernatant shall be collected immediately after centrifugation for performance of the test. Alternatively 25 ml effluent can be centrifuged at 15.000 g for 15 minutes and all the supernatant collected by simply pouring it out in another vessel (centrifuging at 15.000 g results in a stable pellet on the bottom of the tube).
In case centrifugation is not possible, one can filter approximately 25 ml on a low porosity glass fiber filter (nominal pore size of about or less than 1 µm).

2. Preparation of toxicant dilutions

A dilution series (100% - 50% - 25% - 12.5% and 6.25%) of the centrifuged (or filtered) effluent sample is prepared by serial 1:1 dilution with Standard Freshwater.

1. Take six 25 ml test tubes (preferably in glass) and label them from C1 to C5, and the last tube as “control”. Tube C1 will contain the undiluted effluent, tube C5 the highest dilution (see Table 1).

Table 1 : Dilution series of the effluent

Test tube	Effluent concentration (%)
C1	100
C2	50
C3	25
C4	12.5
C5	6.25
Control	0

2. Put 20 ml of the undiluted (treated) effluent in tube C1.
3. Put 10 ml Standard Freshwater in tubes C2 to C5 and the control tube.
4. Transfer 10 ml effluent from C1 to C2 and mix.
5. Repeat this operation for C2 to C3, C3 to C4 and C4 to C5.
6. Remove and discard 10 ml of the diluted effluent from tube C5.

3. Addition of algal food to the toxicant dilutions and the control

Shake the tube with the concentrated algal food thoroughly and add 100 µl to all the test tubes.

7.2. TESTS ON CHEMICAL COMPOUNDS

*If the acute toxicity of the chemical compound to rotifers has been determined, a **definitive** test can be performed immediately. If no such data are available two consecutive assays must be performed :*

*a) a **range finding test** to determine the tolerance range of Brachionus calyciflorus to the toxicant,*

*b) a **definitive test** to determine the 50% effect concentration and the NOEC.*

A. RANGE FINDING TEST

An example is given below for a concentration series ranging from 100 mg/l down to 0.01 mg/l.

1. Preparation of chemical stock solution

Prepare a 1 g/l stock solution by weighing 100 mg of the substance to be tested on an analytical balance, and dissolve it in deionized water in a 100 ml calibrated flask.
Poorly soluble compounds should first be dissolved in an organic solvent (e.g. DMSO, acetone, ethanol or methanol).

Important remark : In order to avoid (toxic) interference by solvents, the amount of solvent in the highest test concentration may not have any toxic effect on the test organisms. The maximum solvent concentration that can be used must be determined in a separate bioassay on the solvent selected for dissolving the chemical compound.

2. Preparation of toxicant dilutions

A 1:10 dilution series with Standard Freshwater will be prepared, starting from 100 mg/l as the highest test concentration.

1. Take six 15 ml test tubes (preferably in glass) and label them from C1 to C5, and the last tube as "control".
   C1 is the highest and C5 the lowest concentration of the compound to be tested (see Table 2).

Table 2 : Dilution series of the chemical compound

Test tube	Chemical concentration (mg/l)
C1	100
C2	10
C3	1
C4	0.1
C5	0.01
Control	0

2. Put 9 ml Standard Freshwater into all the test tubes.
3. Transfer 1 ml stock solution (1000 mg/l) into tube C1 and mix.
4. Transfer 1 ml from tube C1 into C2 and mix.

5. Repeat the 1 ml transfer from tube C2 to C3, and subsequently from C3 to C4 and from C4 to C5.
6. Remove 1 ml from tube C5 and discard.

3. Addition of algal food to the toxicant dilutions and the control

Shake the tube with the algal food thoroughly and add 90 µl to all the test tubes.

B. DEFINITIVE TEST

Case I. The acute toxicity of the chemical to *Brachionus calyciflorus* is known.

If the acute toxicity of the toxicant to Brachionus calyciflorus (i.e. the 24hLC$_{50}$) is known, a definitive chronic test can be performed without a prior range finding chronic test.

1. Preparation of toxicant dilution series

A dilution series will be prepared of which the highest concentration, is the lowest concentration in the acute toxicity test which produced a significant effect (i.e. more than 20% mortality).
The dilution series of the chronic test shall cover one order of magnitude, with the concentrations (preferably) at "log-equidistant" intervals, as conventional in ecotoxicology (e.g. 10 - 5.6 - 3.2 - 1.8 -1).

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

1. Prepare, in a calibrated flask and with Standard Freshwater, 25 ml of the highest test concentration (C1) to be used, and transfer it into a test tube or a beaker.
2. Take six 15 ml tubes (preferably in glass) and label them from C1 to C5 and the last one as “control”.
3. Add the volumes of Standard Freshwater indicated in Table 3 to the respective test tubes.

Table 3 : 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
Control	10	0

4. Add the volumes of toxicant concentration from the tube (or beaker) C1, to the tubes C1 up to C5 and the control tube, as indicated in Table 3.
5. Cap and shake the test tubes.

Since the exact actual concentrations of C1 up to C5 are needed for the EC_{50} estimation they shall be calculated as follows :

C1 = ...mg/l
C2 = O.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

2. Addition of algal food to the toxicant dilutions

Shake the tube with the algal food thoroughly and add 100 µl to all the test tubes.

N.B. The addition of 0.1 ml algal food suspension to the tubes containing 10 ml of the toxicant dilutions leads to a 1% error in the toxicant concentrations.
This small error has been deliberately admitted for reasons of convenience in the preparation of the toxicant dilution series.

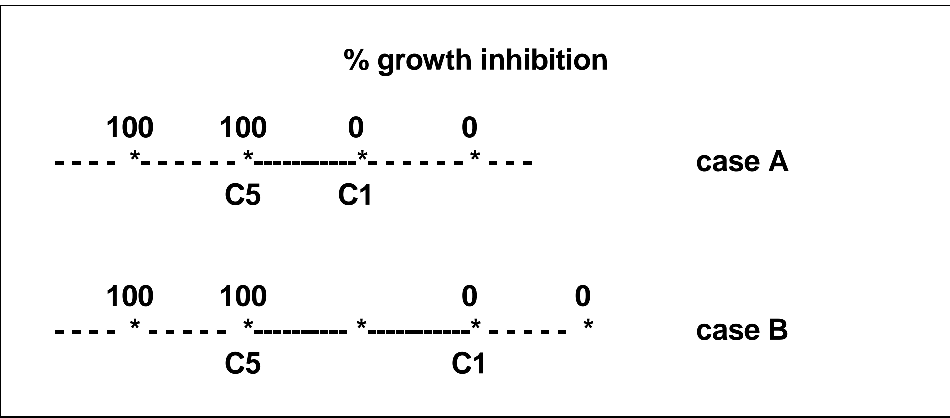
Case II. The acute toxicity of the chemical to *Brachionus calyciflorus* is not known.

In this case a range finding chronic test must always be performed first, following the procedure described in the section "Range Finding Test", to determine the (chronic) tolerance range (0-100% effect range). For the definitive chronic test a dilution series will subsequently be prepared which must span the range of the lowest concentration producing 80-100% effect and the highest concentration producing less than 20% effect in the range-finding test.

As shown in Table 4, this range can span one order of magnitude (case A) or two orders of magnitude (case B).

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

Table 4 : Schematic presentation of the 0-100% effect range determined in the range finding test



Preparation of toxicant dilutions

A. - C1-C5 spans one order of magnitude

Follow exactly the procedure described above for the "definitive test", starting as the highest concentration (C1) with the lowest concentration which, in the range finding test produced 80-100% growth inhibition.

B. - C1-C5 spans two orders of magnitude

Proceed exactly as in A, except for the following :

Add the volumes of Standard Freshwater and toxicant concentration C1 indicated in Table 5 to each tube.

Table 5 : Dilution series C1 - C5

Test tube	EPA 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
Control	10	0

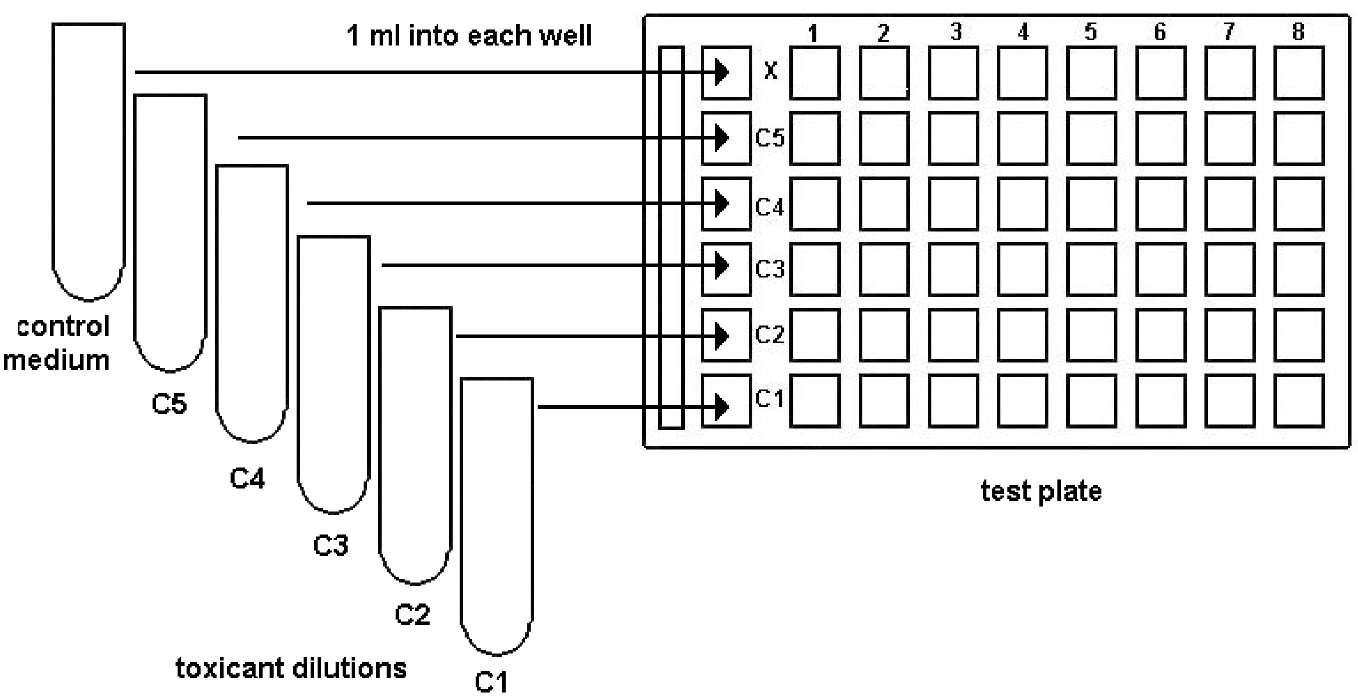
8. FILLING OF THE TEST PLATE

General remarks :
For a statistical acceptable evaluation of the effects, each test concentration as well as the control is assayed in 8 replicates. Each multiwell plate is therefore provided with 8 test wells for the controls as well as with 8 wells for each toxicant concentration (see Figure Configuration of the multiwell plate).

In addition the multiwell plates are provided on the left side with a "hatching well" and with a column of "rinsing wells". The rinsing wells serve to prevent dilution of the toxicant in the multiwell cups during the transfer of the test organisms from the hatching trough to the test wells. The 8 columns of test wells are labelled 1 to 8 and the 6 rows are labelled X for the controls and C5, C4, C3, C2 and C1 for the toxicant dilutions.

All the wells of each row will be filled with 1 ml of the respective toxicant concentrations, and the top row with 1 ml control medium.

FILLING OF THE TEST PLATE



Procedure (see Figure)

1. Shake the tube with the control medium (Standard Freshwater + algal food) and transfer 1 ml into the rinsing well and into each well of the control row.
2. Shake the tubes with the toxicant concentrations (and algal food) and transfer 1 ml into the rinsing well and the 8 test wells of each row, in the sequence of increasing toxicant concentration, i.e. from the top to the bottom of the multiwell, thus starting with C5.

N.B. For the range finding test, in which only 9 ml solution is available in each tube, it is advised) to fill the 8 test wells first, and the rinsing well as the last.

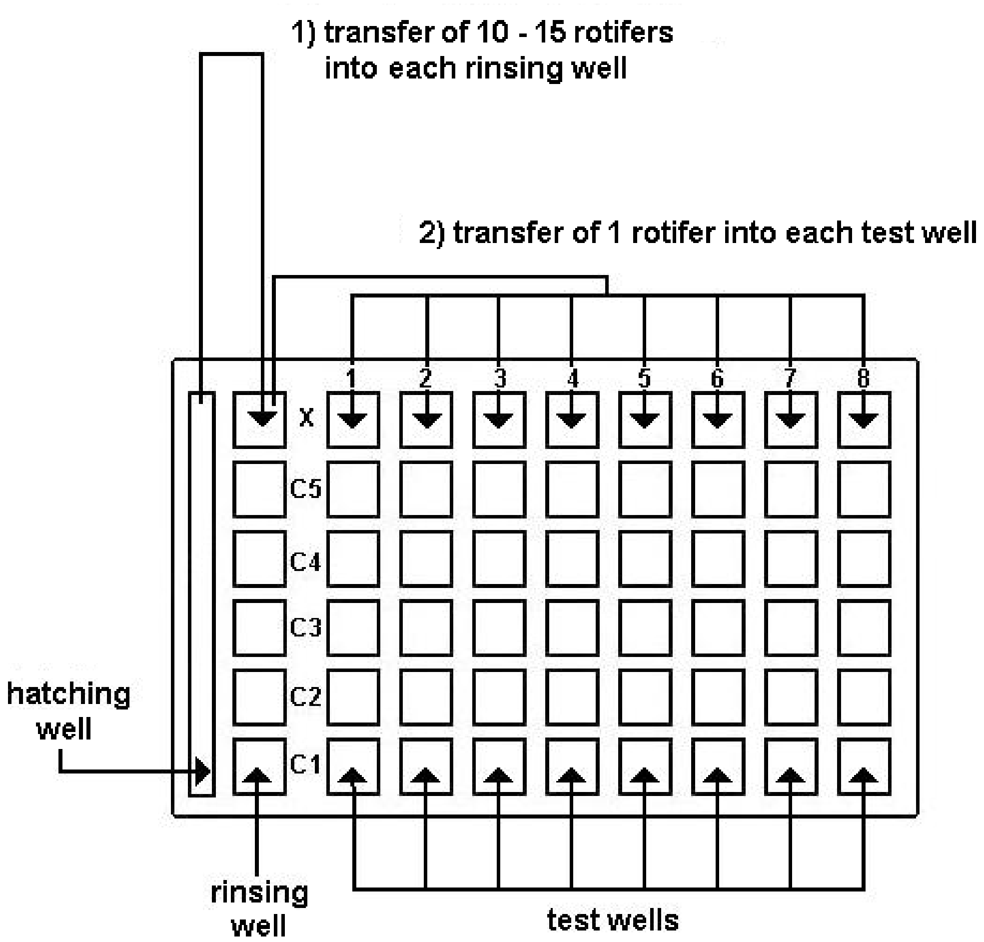
9. TRANSFER OF THE ROTIFERS TO THE TEST WELLS

General remarks :
The transfer of the rotifers into the test wells is performed at the aid of a micropipette. 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.

Because of their small size the transfer of the rotifers has to be carried out under a ***dissection microscope*** *at a magnification of 10-12x.*

Transfer of the rotifers to the test cups is accomplished in two steps :
1. transfer of a number of rotifers from the hatching trough into the rinsing wells.
2. transfer of one rotifer from the rinsing wells into each test well of the same row.

TRANSFER OF THE ROTIFERS INTO THE TEST WELLS



Procedure (see Figure)

1. Transfer 10 - 15 (actively swimming) rotifers from the hatching well into each rinsing well in the sequence : row X (controls), row C5, C4, C3, C2, C1, thus in order of increasing toxicant concentration.
   Try to carry over as little as possible liquid from the hatching trough to the rinsing wells during this transfer.
2. Transfer one rotifer from the rinsing trough into each test well of the same row.
   This transfer shall also be performed in the sequence of increasing test concentrations, i.e. from row C5 to C1.

   Count the rotifers as they exit the micropipette to be sure of the transfer of only one rotifer per test well.

10. INCUBATION OF THE TEST PLATE

Procedure

1. Put the Parafilm strip on top of the multiwell plate and put the cover on tightly.
2. Put the multiwell plate in the incubator at 25°C, in darkness, for 48h.

11. SCORING OF THE RESULTS

After 48h incubation, the number of live rotifers has to be counted in all the test wells.
Counting of live rotifers which are actively swimming takes some experience, especially if there are 7 - 8 organisms per well.

To facilitate the counting, one can fix (and at the same time stain) the organisms with a conventional zooplankton fixative (such as e.g. Lugol solution, a small bottle of which is included in each chronic Rotoxkit). The fixed organisms rapidly sink to the bottom of the test wells and can then be counted very easily.

Since a number of rotifers will have died during their exposure to the toxicant, ***one must, however, in case of application of the fixing and staining technique, always first determine the number of "dead" animals in each test well !***

1. Procedure for determining the number of live rotifers in the test wells well fixing and staining.

- Count the number of dead rotifers (N_{dead}) in each test well.
- Add 50 µl Lugol solution to each test well
- Wait for 5 minutes and count the total number of rotifers in each test well (N_{total}).
- Calculate the number of live rotifers in each test well (N_{total} - N_{dead}) and record the data on the results sheet.

2. Determination of the "population growth rate (r)" in the controls

The calculation of r is necessary to establish the validity of the test, i.e. to determine whether there has been sufficient reproduction of the rotifers. As indicated in the Section "Validity of the test", r should have a value of (at least) 0.55 for a test to be acceptable.

1. Calculate the mean number of live rotifers after 48h incubation in the control wells.
2. Calculate r by applying the formula :

$$\mathbf{r} = \frac{\ln N_{final} - \ln N_{start}}{T}$$

N_{final} = mean number of rotifers after 48h incubation
N_{start} = mean number or rotifers at the start (= 1)
T = time of exposure in days (= 2).

N.B. The threshold value 0.55 for the population growth rate, as one of the acceptability criteria for the validity of the test, corresponds to a mean value of 3 (live) rotifers in the control test wells after 48h incubation. One can thus, in practice, bypass the (scientifically correct but more cumbersome) calculation of r, by assessing whether the mean number of rotifers in the control wells is at least 3.

CHRONIC ROTOXKIT F - RESULTS SHEET

Name of operator : ....

Date of performance of test : ....

Toxicant tested : ....

Type of test : O range finding
O definitive

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

Number of live rotifers									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Mean**
Control									
Conc. 5									
Conc. 4									
Conc. 3									
Conc. 2									
Conc. 1									

3. Determination of the percentage growth inhibition

- Calculate the mean number of live rotifers in each test well of the rows containing toxicants.
- Calculate the percentage inhibition (I%) of the rotifer growth for each toxicant concentration by applying the formula :

$$I\ (\%) = \frac{N_{control} - N_{toxicant}}{N_{control}} \times 100$$

$N_{control}$ = mean number of live rotifers in the control test wells
$N_{toxicant}$ = mean number of live rotifers in the respective toxicant concentrations after 48h exposure.

Important remark ***:***
In case the percentage effect in the lowest toxicant concentration in any test is above 50%, the bioassay must be repeated with a new series of (lower) test concentrations (see Section " Validity of the test").

12. VALIDITY OF THE TEST

For the toxicity test to be acceptable, the following criteria must be fulfilled :

a) reproduction of rotifers must have occurred in at least 7 of the 8 control test wells.
b) the mean growth rate r in the controls must be at least 0.55
As said above, the threshold value of 0.55 corresponds to a mean number of 3 (live) rotifers in the control wells.
c) the percentage effect in the lowest toxicant concentration must be below 50%.

13. DATA TREATMENT

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

14. REFERENCE TEST

In order to check the correct performance of the test and the sensitivity of the test organisms, it is advised to regularly run a quality control test with the reference chemical potassium dichromate $K_2Cr_2O_7$

The toxicant dilution series to be prepared ranges from 18 mg/l down to 1.8 mg/l.

Procedure

1. Make a stock solution of 100 mg/l potassium dichromate by weighing 25 mg of the compound and dissolving it in deionized water in a 250 ml calibrated flask.
2. Transfer 10 ml of the stock solution into a 100 ml calibrated flask and fill to the 100 ml mark with Standard Freshwater.
3. Follow the procedure given for “Testing of chemicals - Definitive test - Case A : C1-C5 spans one order of magnitude”.

The 5 dilutions to be used for the reference test are the following :

C1 (18 mg/l); C2 (10 mg/l); C3 (5.6 mg/l); C4 (3.1 mg/l); C5 (1.8 mg/l)

From the data obtained in the quality control test, a $48hEC_{50}$ *has to be calculated, the value of which should be situated within the limits 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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