ROTOXKIT F CHRONIC

SHORT-CHRONIC TEST WITH A FRESHWATER ROTIFER

BENCH PROTOCOL

Principle :
The Rotoxkit F chronic contains all the materials to perform standardized, simple and low cost bioassays for toxicity screening of chemicals, effluents, sediments or wastes. Using juveniles of the rotifer Brachionus calyciflorus hatched from cysts, a **short-chronic reproduction inhibition test** is executed in 48 hours. Each chronic Rotoxkit provides for 3 complete tests (range finding or definitive 48hr EC_{50}). The Standard Operational Procedure brochure included in each kit describes this microbiotest in all details, including a quality control test with a reference toxicant.

1. Preparation of the Standard Freshwater
Fill a 1 litre volumetric flask with approximately 800 ml deionized water and add the contents of the five vials with concentrated salt solutions, in the sequence 1 to 4 as indicated on the flask labels*. Add deionized water up to the 1000 ml mark and shake to homogenize the medium. Store the Standard Freshwater in the refrigerator at 5 °C (+/- 2 °C) until use. Take care to bring the cooled medium back to room temperature and aerate it for 15 minutes prior to use.

** Note that there are 2 vials with $CaSO_4.2H_2O$, both of which must be used !*

2. Hatching of the rotifer cysts :
Rotifer cyst hatching should be initiated 16-18 hours before the start of the toxicity test.
Add 1.5 ml Standard Freshwater to the hatching trough. Empty the contents of one vial of rotifer cysts into the hatching trough of the test plate (see figure); to secure complete carryover of all the cysts, the cyst vial should be rinsed with 0.5 ml Standard Freshwater.

Put a strip of Parafilm on the test plate, cover with the lid and incubate at 25°C **for 16-18 hours, under continuous illumination (light source of min. 3000-4000 lux)**.

If hatching is delayed (which in most cases is due to lower temperatures than prescribed), check the hatching hourly after 18 h incubation in order to collect the test animals within 2 hours of their hatching.

3. 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.

Fill one of the tubes containing Roti-Rich food with 1 ml Standard Freshwater and shake thoroughly to homogenize the contents. Suck up a small volume of the Roti-Rich food suspension with a micropipette and - holding it vertically - add "one" drop to the hatching trough with the freshly hatched rotifers. Allow the rotifers to feed for exactly two hours before the start of the toxicity test.

4. Preparation of concentrated algal suspension
Take a tube with algal beads, pour out the storage medium and add 7 ml matrix dissolving medium. Cap and shake the tube at regular intervals till the matrix has fully dissolved (which takes about 15 minutes.). Centrifuge the tube at 3000 rpm for 10 minutes, pour out the supernatant and add 10 ml deionized water. Resuspend the algae and centrifuge again at 3000 rpm for 10 minutes. Pour out the rinsing water “to the last drop” and add 1.8 ml Standard Freshwater. Shake thoroughly to resuspend the algae

5. Preparation of the toxicant dilution series :
Prepare the dilution series of the test compound or effluent according to the detailed instructions given in the Standard Operational Procedure brochure.
For tests on effluents, add 100 µl algal mix to each of the test tubes containing 10 ml of toxicant dilutions and control medium. For range finding tests on chemical compounds, add 90 µl algal mix to the tubes containing 9 ml test medium, for definitive tests add 100 µl algal mix to 10 ml test medium.

6. Filling of the test plate :
The bioassays are conducted in specially designed, disposable "multiwell" plates.

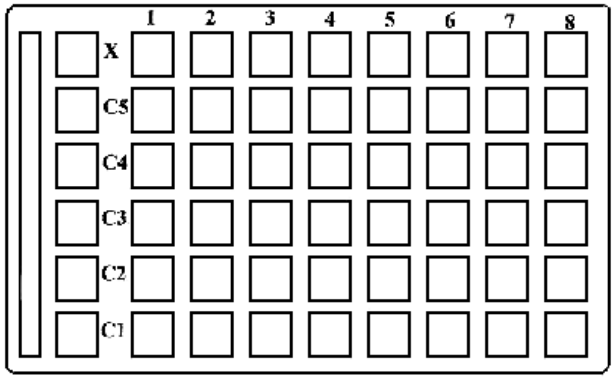

FIGURE : Multiwell test plate composed of (from left to right) : 1 hatching trough, 6 rinsing wells and 48 test wells.

The test design of the multiwell plate is based on one control and five toxicant concentrations, each with 8 replicates of 1 test organism.
Each plate has one hatching well, 6 rinsing wells, and 48 test wells (see figure). The test wells are labelled as columns 1 to 8 across, and rows X and C5 to C1 down.
The wells in each row of the test plates have to be filled with 1 ml of the respective toxicant concentrations (+ algal food), and those in the top row with 1 ml Standard Freshwater (+ algal food).

7. Transfer of the rotifers to the test wells :
Using a dissection microscope at magnification 10-12x, transfer 10 - 15 rotifers with a micropipette from the hatching trough into each rinsing well in the sequence : row X (controls) and row C5 down to C1. Take care to carry over as little hatching medium as possible during the transfer.
After thorough rinsing of the micropipette, subsequently transfer exactly "**one**" rotifer from each rinsing well into the 8 test cups of the corresponding row, starting with the control row X and subsequently downwards from C5 to C1.

8. Incubation of the test plate and scoring of the results :
Put the strip of Parafilm back on the test plate, cover the multiwell with the lid and incubate at 25°C in darkness.
After 48 hours, count the number of live rotifers in each test well and score the data on the results sheet.
To facilitate the counting the rotifers can first be fixed (and stained) with Lugol solution by adding "one drop" of this fixative (equivalent to approximately 40-50 µl) to the test wells. The fixed organisms sink to the bottom within a few minutes and can then be counted very easily.

N.B. In case of fixation with Lugol, one has, however, always to first determine the number of "dead" organisms in each test well and subtract this number from the total count after fixation !!

9. Determination of the "population growth rate (r)" in the controls
For a test to be valid, the population growth rate r (calculated with the formula given hereunder) must be at least 0.55.

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

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. Since this value, corresponds very closely with a mean of 3 "live" rotifers in the control test wells after 48h incubation, one can, in practice, bypass the former calculation and use the mean value 3 in the controls as one of the validity thresholds of the bioassay

10. 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.

N.B. In case the percentage inhibition is above 50% in the lowest toxicant concentration, no (reliable) 48h EC_{50} can be calculated and the bioassay should be repeated with a new series of (lower) test concentrations.

11. Validity of the test
Three conditions have to be fulfilled for the short-chronic rotifer test to be valid : reproduction of rotifers must have occurred in at least 7 of the 8 control test wells the mean growth rate r in the controls must be at least 0.55 (or the mean number of live rotifers at least 3) the percentage effect in the lowest toxicant concentration must be below 50%

12. 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.

13. Reference test
It is recommended to regularly perform a quality control test to check proper adherence to the test protocol, as well as test sensitivity.

A reference test can be carried out with potassium dichromate ($K_2Cr_2O_7$). The 48hEC_{50} of the quality control test should be within the 95% confidence limits stipulated on the specification sheet.

The dilution series to be prepared for the reference test with potassium dichromate is 1.8 - 3.2 – 5.6 - 10 - 18 mg/l.