ROTOXKIT M

ESTUARINE / MARINE TOXICITY SCREENING TEST

BENCH PROTOCOL

Principle :
The **rotifer toxicity test-kit** contains all the materials to perform standardized, simple and cost-effective bioassays for screening toxicity in estuarine (brackish) water and seawater. Using juveniles of the rotifer *Brachionus plicatilis* hatched from cysts, an acute toxicity test is executed in 24 hours. The sensitivity of the ROTOXKIT M bioassay is comparable to that of acute tests with other marine invertebrates. Each ROTOXKIT provides for 6 complete tests (range finding or definitive 24hr LC50), or 5 bioassays and 1 quality control test with a reference toxicant.

1. Preparing Standard Seawater :
Fill a 1 liter volumetric flask with approximately 800 ml deionized water and add the contents of vial number 1 containing pure NaCl. When dissolved, add the contents of the other vials with concentrated salt solutions, in the sequence 2 to 7 as indicated on the flasks. Add deionized water up to the 1000 ml mark and shake to homogenize the seawater medium. The Standard Seawater has a salinity of 35 ppt.

2. Storing 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. In the latter case, the contents should preferably be distributed between several flasks, for separate use. Take care to bring the cooled medium (gradually) back to room temperature prior to use.

3. Hatching the rotifer cysts :
Rotifer cyst hatching should be initiated **28-30 hours** prior to the start of the toxicity test, and has to be carried out in seawater of reduced salinity (20 ppt).
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.
Empty the contents of one vial of rotifer cysts into the hatching trough of the test plate (see figure); one may rinse the cyst vial with 0.5 ml hatching medium to carry over all the cysts into the hatching trough.
Add 2.5 ml hatching medium to the hatching trough in the test plate (only 2 ml if the cyst vial has been rinsed as indicated above). Make sure that most of the cysts are submerged (e.g. by rinsing the sides of the hatching trough with a micropipet).
Put a strip of Parafilm on the test plate, cover the multiwell and incubate at 25◦C **for 28 hours, under continuous illumination (light source of 3000-4000 lux)**. If hatching is delayed (which in most cases is due to lower temperatures than prescribed), check the cysts hourly after 28 hours incubation to insure collecting the test animals within 2 hours of hatching. It is indeed important to have 0 to 2 hour old test animals to start the bioassay*

* *Because there is no feeding during the test the effects of starvation begin to cause mortality after about 32 hours at 25◦C, and if the rotifers are older than 32 hours at the end of the exposure period, excessive control mortality may result, invalidating the bioassay.*

4. Preparing the Toxicant Dilution Series :
As the rotifers are hatching, prepare a dilution series of the test compound or effluent according to standard methods (e.g. USEPA, 1985).
Tests can be carried out in normal (35 ppt) seawater to determine effects of the toxicant(s) in "marine" conditions, or at reduced salinity to determine effects in "estuarine" (brackish water) conditions. For the latter case, a medium of the desired salinity has first to be prepared, using deionised water and Standard Seawater in the appropriate ratio.

5. Filling the Test Plate :
The bioassay is conducted in a specially developed, disposable, PVC "multiwell" test plate. Each plate has one hatching trough, 6 rinsing troughs, and 36 test wells (see figure). The rinsing troughs 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). To fill control row X add 0.7 ml Standard Seawater (or brackish water) to the rinsing trough and 0.3 ml to each of the six test wells. Repeat this procedure for the other rows with the respective toxicant concentrations, progressing from low to high concentrations in rows 1 to 5.

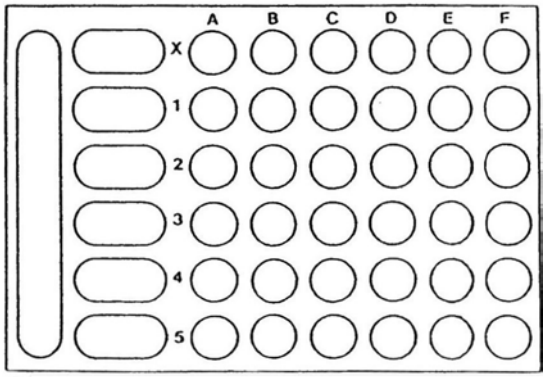

FIGURE : Multiwell test plate composed of (from left to right) : 1 hatching trough, 6 rinsing troughs and 36 test wells.

6. Adding the Rotifers :
Using a dissection microscope at magnification 10-12x, transfer approx. 50 rotifers with a micropipet from the hatching trough to the rinsing trough of control row X (top row). Repeat this operation for rows 1 trough 5. 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). Subsequently transfer 5 rotifers from the rinsing well to each of the six test wells of row X. Take care, during this operation, to minimize the transfer of medium along with the rotifers. Repeat this operation for rows 1 through 5*.

** The intermediate passage of the rotifers from the hatching trough to the wells via a rinsing trough "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.*

The test design of the multiwell plate is based on one control and five toxicant concentrations, each with 6 replicates of 5 animals. Each bioassay shall be performed in a new multiwell with a new micropipet.

7. Incubating the Test Plate and Scoring the results :
To avoid spilling of the hatching medium into the test wells during transportation of the multiwell plate, it is advised to empty the hatching trough after completing the transfer of the rotifers ; this operation can easily be performed with the micropipet.

Put the strip of Parafilm back on the test plate, cover the multiwell and incubate at 25°C in darkness. **After 24 hours**, count the dead* rotifers in each well and fill out the results sheet.

Calculate the % mortality** and, for definitive tests, the LC50 using any standard method (e.g. USEPA, 1985).

** Rotifers are considered dead if they do not exhibit any internal or external movement in 5 seconds of observation.*
*** For the ROTOXKIT test to be valid, control mortality (as is the rule in aquatic toxicity tests) must not exceed 10%.*

8. Reference test
It is recommended that every 5 to 10 assays, a quality control test be carried out to check proper adherence to the test protocol as well as test sensitivity.

Each ROTOXKIT M contains one vial with a reference chemical (potassium dichromate $K_2Cr_2O_7$) to perform an eventual quality control test. When performing this quality control test, the 24h LC50 should be within the 95% confidence limits stipulated on the specification sheet.

8.1. Preparation of stock solution and dilution series of the reference chemical.
Dissolve the contents of the vial with reference chemical into a 100 ml volumetric flask and add Standard Seawater to the 100 ml mark to make a 10 g/l stock solution. Make a dilution series 1000-560-320-180-100 mg/l for the quality control test.