OSTRACODTOXKIT F™

CHRONIC « DIRECT CONTACT » TOXICITY TEST
FOR FRESHWATER SEDIMENTS

BENCH PROTOCOL

Principle :

The Ostracodtoxkit is the very first **chronic « direct sediment contact » microbiotest.**
It contains all the materials to perform standardized, simple and low cost bioassays for toxicity screening of freshwater sediments.
The tests are based on mortality and growth inhibition of neonates of the ostracod crustacean *Heterocypris incongruens* hatched from cysts, which are exposed for 6 days to contaminated sediments. The Ostracodtoxkit tests are performed on non-diluted sediments and give the % mortality and the % growth inhibition of the test organisms in comparison to a (non-contaminated) reference sediment.
The tests are performed in 3 x 2 multiwells, in several parallels. Depending of the concurrent or separate performance of the assays, one Ostracodtoxkit allows to perform tests on 3 to 5 different sediment samples.

1. Preparation of Standard Freshwater
Fill a 1 liter volumetric flask with approximately 800 ml deionized (or distilled) 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 for 15 minutes prior to use.
** There are 2 vials with $CaSO_4$ both of which must be used !*

2. Hatching of the ostracod cysts
Hatching of the cysts should be initiated 52h before the start of the toxicity test. Add 8 ml Standard Freshwater in the hathcing petri dish and empty the contents of one of the vials with cysts into the petri dish. To secure the complete transfer of the cysts, rince the vial twice with 1 ml Standard Freshwater. Cover the petri dish and incubate it **at 25°C for 52 h, under continuous illumination (+ 4.000 lux)**. Start the test as soon as possible when enough neonates are available.

3. Pre-feeding of the freshly hatched ostracods
Take one of the tubes with Spirulina powder and fill it with Standard Freshwater. Mix the contents (preferably on a Vortex) to homogenize the suspension and pour it into the hatching petri dish 48 h after the start of the incubation of the cysts. Put the petri dish back in the incubator and continue to incubate for 4 hours.

4. Length measurement of freshly hatched ostracods
Pick up 10 ostracods from the hatching petri dish with a glass micropipette and transfer them into one cup of the “length measurement” multiwell. Add one drop of Lugol solution and wait for a few minutes till the organisms are immobile. Put the special « coverslip with micrometer » exactly in the middle of the bottom stage of the dissection microscope, and fix it to the glass plate with transparent tape.

N.B. The two perpendicular axes of the micrometer are exactly 1 cm in length. They are subdivided in 10 graduations of 1 mm which are subdivided further in 100 µm. The 100 µm are subdivided further in 2, so that the distance between the smallest graduations is exactly 50 µm.

Rotate the multiwell on the bottom stage of the dissection microscope in order to position the ostracods one after the other with their length axis exactly on top of one of the two micrometer lines and measure the length of the organisms.

5. Preparation of algal food suspension
Take one tube with algal beads, pour out the storage medium, add 7 ml matrix dissolving medium and shake intermittently by hand (for 5 to 15 minutes), or mix on a Vortex mixer till the matrix surrounding the algae is fully dissolved and the algae set totally free. Centrifuge the tube for 10 min. at 3000 rpm and pour out the supernatant. Add 10 ml distilled water, resuspend the algae and centrifuge again for 10 min. at 3000 rpm. Pour out the rinsing water and transfer the algal pellet to a 25 ml volumetric flask. Add Standard Freshwater to the 25 ml mark and shake the flask thoroughly to resuspend the algae and homogenize the algal suspension.

6. Addition of sediment, algal food suspension and ostracods to the test plate
Transfer 2 ml Standard Freshwater into each well of a 3 x 2 multiwell test plate. Take one pot filled with sediment and add 2 x 500 µl sediment (1000 µm in total) to each well (use the spatula to strike off any eventual excessive sediment). Then add 2 ml algal food suspension to each cup.

Put the hatching petri dish on the bottom stage of the dissection microscope (magnification 10-12 x) and transfer, with a glass micropipette, part of the ostracod neonates into the lid of the hatching petri dish (*this intermediate step facilitates the transfer of the ostracods into the wells of the multiwell test plate*). Then transfer 10 ostracods into each test cup.

When all the test cups are filled with 1000 µl sediment, the multiwell shall be covered with a piece of Parafilm and the lid.
Put the multiwell plate in the incubator at 25°C, in darkness, for 6 days.

7. Transfer of the ostracods to a petri dish

Take a "large mouth" micropipette and very gently mix the sediment in the first test cup with the water layer. Suck up part of the sediment suspension and transfer it into the microsieve. Gently rinse the contents of the microsieve under the tap, to eliminate all the fine sediment particles. Repeat this operation till most of the sediment has been transferred. Add a few ml Standard Freshwater to the cup, mix it with the remaining sediment and transfer it to the microsieve for rinsing. Repeat this operation several times if necessary to make sure that all the sediment and the ostracods have been transferred. Turn the microsieve upside down above a small petri dish and rinse the contents back into the petri dish with Standard Freshwater. Make sure the full contents of the microsieve are transferred !

N.B. The former "transfer" operation has to be performed for each individual test cup. However, the next step "8. Scoring of the Results - Part A. Mortality Scoring" (described hereafter) has to be completed first before one can proceed with the sediment transfer from the next cup into the same petri dish.

8. Scoring of the Results

A. Mortality scoring

Put the petri dish under the dissection microscope and observe the contents at magnification 10-12 x. Pick up all the live ostracods with a glass micropipette and transfer them into one cups of the multiwell plate for "length measurement". Score the number of live ostracods found, on the Results Sheet. Rinse the petri dish and proceed with the transfer of sediment and ostracods from the other test cups as indicated above.

B. Length measurement

Add one drop of Lugol to each cup of the "length measurement" multiwell containing the live ostracods and wait for a few minutes till the organisms are immobile. Measure the length of the ostracods following the procedure indicated above. Score the length results on the Results Sheet.

9. Data treatment

1. Calculate the number of dead ostracods in each cup with reference sediment and test sediment. This number is obtained by substracting the number of live ostracods found back in each cup, from the original number inoculated at the start of the experiment (i.e. 10).

N.B. In case a few ostracods are lost during the transfer operations, or escape attention during the microscopic observation, the calculation of the "real" mortality will be biased… Hence it is very important to perform all the operations with great care !

2. Calculate the mean mortality for all the replicate cups of the reference sediment and the test sediment respectively, and calculate the mean % mortality with standard deviation and variation coefficient.
3. Calculate the mean length of the 10 (freshly hatched) ostracods measured at the start of the experiment.
4. Calculate the mean length of the (live) ostracods found back in each test cup, and the mean length for all the replicate cups with reference sediment and test sediment respectively.
5. Calculate the mean length increment of the ostracods in the reference sediment and the test sediment with the formula :

$$L_{increment} = L_{end} - L_{start}$$

6. Calculate the % growth inhibition of the ostracods in the test sediment with the formula

$$\% \text{ Growth inhibition} = 100 - (L_{incr.test\ sediment}/L_{incr.control\ sediment}) \times 100$$

10. Validity of the test

For the toxicity test to be acceptable, the following 2 criteria must be fulfilled :

a) the mean % mortality of the ostracods in the control test with the reference sediment must not exceed 20%
b) the mean length increment of the ostracods in the control cups with the reference sediment must be at least 400 µm.