O S T R A C O D T O X K I T F

" DIRECT CONTACT " TOXICITY TEST
FOR FRESHWATER SEDIMENTS

BENCH PROTOCOL

Principle :

The Ostracodtoxkit is the very first **" direct sediment contact " microbiotest** with a crustacean test species.
Each Ostracodtoxkit contains all the materials to perform standardized, simple and low cost bioassays for toxicity screening of freshwater sediments (and by extension also contaminated soils).
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 (or soils). The Ostracod-toxkit tests are performed on non-diluted sediments and allow to determine the % mortality and the % growth inhibition of the test organisms in comparison to a (non-contaminated) reference sediment.
Since growth is measured as a second effect criterion, this assay is in fact a "sub lethal" toxicity test.
The tests are performed in multiwell test plates in 6 replicates. Depending of the concurrent or separate performance of the assays, one Ostracodtoxkit allows to perform assays on 3 to 5 test sediments.

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. Put 8 ml Standard Freshwater in the hatching Petri dish, fill a vial with cysts with 1 ml. Standard Freshwater and empty the contents of the vial into the Petri dish. To secure the complete transfer of the cysts, rinse the vial twice with 1 ml Standard Freshwater.
Cover the Petri dish and incubate it **at 25 °C (+/- 1 °C) for 52 h, under continuous illumination (3000-4000 lux)**.

3. Pre-feeding of the freshly hatched ostracods

Take one tube 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 and 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. The newborn ostracods have a size of 150-250 µm.

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, add 10 ml Standard Freshwater and shake. Transfer the algal suspension into a 25 ml volumetric flask and add Standard Freshwater to the mark. Shake the flask thoroughly to resuspend the algae and homogenize the algal suspension.

6. Addition of Standard Freshwater, sediment, algal food and ostracods to the test plate

Transfer 2 ml Standard Freshwater into the 6 wells of a multiwell test plate. Take the pot filled with reference sediment and fill the spoon on the lid with this sediment. Strike off the excess sediment with the spatula and transfer the remaining 500 µl sediment into the first well of the multiwell plate. Repeat this operation in order to have 1000 µl sediment into the cup. Do the same operation for the other 5 cups of the test plate and subsequently add 2 ml algal food suspension to each well.
Proceed the same way to put 1000 µl test sediment and 2 ml algal food in the 6 wells of a second test plate.

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. Then transfer **exactly 10 ostracods into each test cup** of the two test plates.
Cover the 2 multiwells with a piece of Parafilm and their lid and put them in the incubator at 25 °C (+/- 1 °C), in darkness, for 6 days.

7. Recovery of the ostracods from the test plate with reference sediment

At the end of the 6 days exposure period the living ostracods can be recovered directly from the 6 wells with a glass micropipette, and transferred into the cups of the "length measurement multiwell" for subsequent length measurement, after addition of 1 drop of Lugol solution.

8. Recovery of the ostracods from the test plate with test sediment

In case the test sediment contains fine sediment particles, a sieving process has to be applied to recover the ostracods from the test wells.

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 with tap water (in a wash bottle), 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 its contents back into the Petri dish with Standard Freshwater. Make sure the full contents of the microsieve are transferred !
Subsequently transfer the "living" ostracods from the Petri dish into a cup of the "length measurement multiwell", for length measurements after fixation with 1 drop of Lugol solution.
Repeat the sieving and transfer operations for the 5 other wells of the test plate.

9. Determination of the percentage mortality

Count the number of ostracods in each cup of the length measurement multiwell and score the figures on the Results Sheet A - Mortality. Calculate the total (= A) for the 6 wells of the 2 test plates and subtract this number from 60, to obtain the total number of dead ostracods (B = 60 - A). Calculate the percentage ostracod mortality in the reference sediment and in the test sediment with the formula : % mortality = B/60*100.

10. Determination of the percentage growth inhibition

The "sub lethal" effect criterion growth inhibition of the ostracods in the test sediment is determined by comparing the length of the surviving organisms in the test sediment in comparison with their length in the reference sediment at the end of the test.

The "sub lethal" growth inhibition effect shall, however, only be measured for test sediments for which the percentage mortality of the ostracods (= the lethal criterion) is less than 30%.

N.B. Length measurements must, however, in any case be performed on the ostracods from the wells with reference sediment to calculate their "growth increment", which is a validity criterion for the Ostracodtoxkit microbiotest.

1. Measure the length of each ostracod and score the data on the Results Sheet B - Growth inhibition.
2. Calculate the mean length of the 10 (freshly hatched) ostracods measured at the start of the experiment.
3. Calculate the mean length of the (live) ostracods found back in each test well, and the mean length for all the replicates with reference sediment and test sediment respectively.
4. Calculate the mean length increment of the ostracods in the reference sediment and the test sediment with the formula :
   $L_{increment} = L_{end} - L_{start}$.
5. Calculate the % growth inhibition of the ostracods in the test sediment with the formula :
   growth inhibition = 100 - [(Growth in toxicant/Growth in control) x 100]

11. Validity of the test

a) the mean % mortality of the ostracods in the control test with the reference sediment must not exceed 20%.
b) the mean length of the ostracods in the reference sediment should have increased by a factor 1.5 to the mean length of the organisms at the start of the test.

12. Reference test

A quality control test can be performed with the reference toxicant **copper sulphate ($CuSO_4.5H_2O$)**.
The 6d LC50 for this assay should be in the range 2.21 - 9.37 mg/l as determined by an International Interlaboratory Comparison on the *Heterocypris* incongruens microbiotest.

13. Standardization

Subsequent to the International Interlaboratory Comparison which had revealed its high degree of standardization, the ostracod microbiotest has been taken into consideration by the ISO (International Organization for Standardization) as a new toxicity test for freshwater sediments under the name "ISO 14371 - Water quality - Determination of freshwater sediment toxicity to *Heterocypris incongruens* (Crustacea, Ostracoda).