



CRUSTACEAN TOXICITY TEST FOR FRESHWATER

BENCH PROTOCOL

Principle:

The **Daphtoxkit F magna** contains all the materials, including the test species *Daphnia magna* in the form of "dormant eggs (ephippia)", to perform 6 complete acute toxicity tests according to internationally accepted Standard Methods (e.g. OECD and ISO). The tests make use of the "neonates" which are hatched in about 3 days from the eggs.

1. Preparation of Standard Freshwater (ISO formula according to ISO 6341) as hatching and dilution medium:

Fill a 2 liter volumetric flask with approximately one liter deionized (or distilled) water and add the contents of one of the two sets of 4 vials of concentrated salt solutions, in the sequence 1 to 4 (as indicated on the labels). Add deionized (or distilled) water up to the 2000 ml mark and shake to homogenize the medium. Two liter Standard Freshwater largely suffices to perform 3 complete bioassays.

N.B. The Standard Freshwater medium can also be prepared in "double strength" in a one liter flask and subsequently diluted by half at the time of use.

2. Storage of the medium:

If the 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. Hatching of the ephippia:

Hatching of the ephippia should be initiated 3 days prior to the start of the toxicity test.

Pour the contents of one vial of ephippia into the microsieve and rinse thoroughly with tap water to eliminate all traces of the storage medium. Transfer the ephippia into the hatching petri dish in 15 ml Standard Fresh-water (one can also use a 10 cm diameter dish with 50 ml Standard Freshwater), pre-aerated by air bubbling. Cover the petri dish and incubate for 3 days, at 20-22°C, under continuous illumination of min. 6000 lux.

The largest percentage of hatching will occur between 72h and 80h of incubation. Since standard testing procedures indicate that the neonates should not be older than 24h at the start of the toxicity test, the young Daphnids must be collected at the latest 90h after the start of the incubation.

4. Preparation of the toxicant dilution series:

Dilution series of the test compound or effluent should be prepared according to standard procedures. A minimum volume of 50 ml is needed for each toxicant dilution.

5. Pre-feeding of the neonates prior to the test

"Starvation to death" of the weakest individuals of the test population may occur when the exposure period is prolonged from 24h to 48h. In order to avoid this problem (which can make the 48h assay invalid due to too high control mortality) a 2h pre-feeding with dry algae can be applied.

Fill one of the tubes containing Spirulina powder with Standard Freshwater and shake thoroughly to homogenize the contents. Pour the contents of the tube into the hatching petri dish 2 hours prior to collecting the neonates for the toxicity test. Swirl the petri dish gently to distribute the algal food evenly.

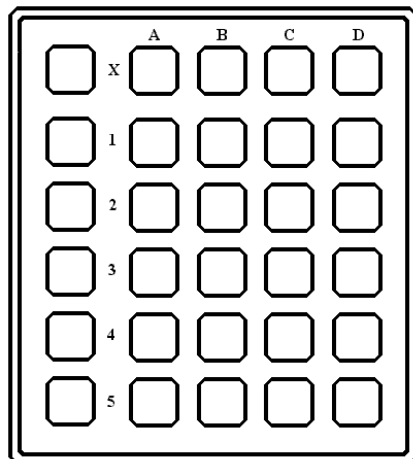
6. Filling of the test plate:

The bioassays are conducted in disposable multiwell test plates with 30 test wells (see Figure). Each plate is provided with 4 wells for the controls and 4 wells (A,B,C,D) for each toxicant concentration. Additionally, the plates are provided on the left side with a column of "rinsing wells" to prevent dilution of the toxicant during the transfer of the neonates from the hatching petri dish to the test wells. The wells are labelled vertically as rows X (for the controls) and 1 to 5 for the toxicant dilutions.

Each well of the test plates has to be filled with **10 ml** toxicant solution (or Standard Freshwater in the control column).

7. Transfer of the neonates to the test wells:

Put the hatching petri dish on the stage of a dissection microscope or on a light table evt. provided with a black strip to enhance the contrast (see figure). Transfer 20 (actively swimming) neonates with a micropipette into each rinsing cup in the sequence : row X (control), row 1 to row 5 (increasing concentrations of toxicant).



Rinsing wells

Test wells

Put the multiwell plate on the stage of the dissection microscope or on a light table and transfer exactly 5 neonates from the rinsing wells into each of the 4 wells of each column. This transfer shall also be performed in the order of increasing test concentrations.

LIGHT TABLE

For a better contrast between the test organisms and the white back-ground of the light table, one can use a strip of black paper placed in the middle of the viewing surface.



Important remark:

SURFACE FLOATING

Daphnids are quite susceptible to being trapped at the surface of the liquid medium in the test wells, by "surface tension" phenomena. "Floating" test organisms may die and hence jeopardize the outcome of the bioassay. In order to avoid "surface floating", it is important, during the transfer of the neonates, to put the tip of the micropipette into the medium and not to drop them onto the surface of the medium in the wells.

8. Incubation of the test plate and scoring of the results:

Put the Parafilm strip and the cover on the multiwell plate and incubate in darkness at 20°C.

After 24h and 48h incubation, put the test plate under the dissection microscope or on the transparent stage of the light table with dark light strip, and determine the number of dead and immobilized* test organisms.

* *The neonates which are not able to swim after gentle agitation of the liquid for 15 seconds shall be considered to be immobilized, even if they can still move their antennae.*

Score the data on the Results Sheet and calculate the % effect and the 50% effect threshold, using any standard data processing method.

9. Validity of the test:

Besides all other specific validity conditions prescribed in standard Daphnia bioassay protocols, the number of dead + immobile organisms in the controls should not exceed 10%.

10. Reference test:

In order to check the correct execution of the test procedure and the sensitivity of the test animals, it is advised to perform a reference test from time to time.

Quality control tests can e.g. be performed with the reference toxicant potassium dichromate ($K_2Cr_2O_7$), using the following dilution series : 3.2 - 1.8 - 1 - 0.56 - 0.32 mg/l.

The 24h EC_{50} in the quality control should be situated within the limits stipulated in the specification sheet of each Daphtoxkit.