DAPHTOXKIT F™ *PULEX*

CRUSTACEAN TOXICITY TEST FOR FRESHWATER

BENCH PROTOCOL

Principle:

The **Daphtoxkit F *pulex*** contains all the materials, including the test species ***Daphnia pulex*** 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 3-4 days from the eggs.

1. Preparation of Standard Freshwater ("moderately hard" water, US EPA formula) as hatching and dilution medium:
Fill a 1 liter volumetric flask with approximately one liter deionized (or distilled) water and add the contents of one of the two sets of 5 vials of concentrated salt solutions, in the sequence 1 to 4 (as indicated on the labels). Add deionized (or distilled) water up to the 1000 ml mark and shake to homogenize the medium. One liter Standard Freshwater suffices to perform 3 complete bioassays.

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 4 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 Freshwater. Cover the petri dish and incubate for 4 days, at 20◦C, under continuous illumination of 10 000 lux.

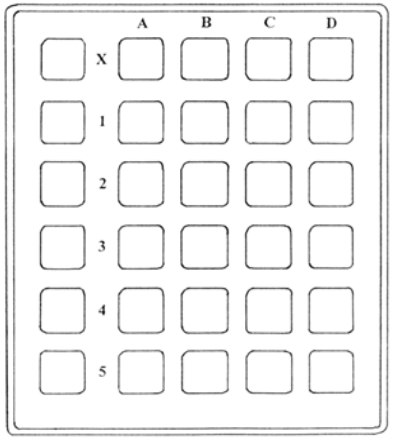
"Time to hatching" is not synchronous and extends over a period of several days. The largest percentage of hatching, however, occurs between 72h and 96h from the start of the incubation of the ephippia. In order to perform the assays with test organisms less than 24h old, the neonates should be collected 96h after the start of the incubation of the ephippia.

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 organisms in 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. Additionnally, 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.

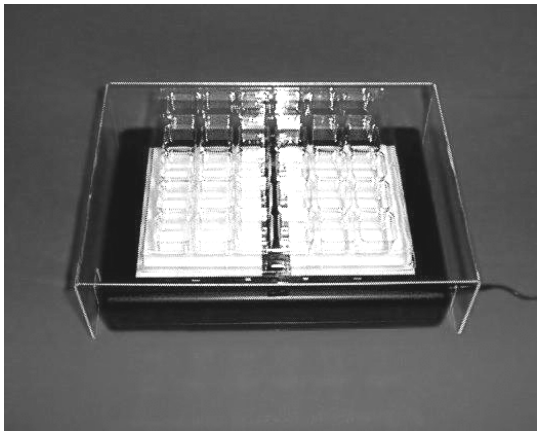


Each well of the test plates has to be filled with **5 ml** toxicant solution (or Standard Freshwater in the control column).

7. Transfer of the neonates to the test wells:
Put the hatching petri dish on the stage of a dissection microscope or on the transparent stage of a light table provided with a black strip to enhance the contrast (see figure).

LIGHT TABLE WITH TRANSPARENT STAGE

The transparent stage, which can easily be made with any kind of transparent material, should be at a distance of approximately 3 cm from the light table. The width of the black strip shall be around 3 cm.



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).

Put the multiwell plate on the stage of the dissection microscope or on the transparent stage of the 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.

Important remarks:

A. PRE-CONCENTRATION OF THE TEST-ORGANISMS
The neonates can be concentrated into a smaller volume of hatching medium by pouring the contents of the hatching petri dish into the microsieve, placed on the bottom of one of the small petri dishes. This intermediate step can facilitate the collection of the test organisms substantially.

B. 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 liquid. If, despite all precautions, surface floating occurs, put a small pellet of cetylalcohol (a non-toxic surface-active agent with very low solubility) on the surface of the medium in each test well. The pellets may be removed after a while and be used again after thorough rinsing and drying.

8. Incubation of the test plate and scoring of the resuls:
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 are considered immobilized if they lay on the bottom and do not resume swimming within 15 seconds of observation.*

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 : 1.8 - 1 - 0.56 - 0.32 - 0.18 mg/l.

The 24h-48h EC50 in the quality control should be situated within the limits stipulated in the specification sheet of each Daphtoxkit.