



FRESHWATER TOXICITY TEST WITH MICROALGAE

BENCH PROTOCOL

Principle :

The **Algaltoxkit F** contains all the materials, including the test species *Selenastrum capricornutum* (first renamed as *Raphidocelis subcapitata* and presently as *Pseudokirchneriella subcapitata*) immobilized in algal beads, to perform 2 complete algal growth inhibition assays, according to internationally accepted Standard Methods. The tests can be started within 30 minutes after de-immobilization of the microalgae from the beads.

Optical density (at 670 nm) is used as the parameter for algal growth inhibition. The tests are performed in disposable spectrophotometric cells of 10 cm path-length (= "long cells") as test vials, which allows for direct and rapid scoring of the OD in any spectrophotometer equipped with a holder for 10 cm cells.

1. Preparation of the algal culturing medium :

Fill a 1 liter volumetric flask with approximately 800 ml deionized water and add 10 ml of "Nutrient Stock" vial A, and 1 ml of Nutrient Stock vials B, C and D. Add deionized water up to the 1000 ml mark and shake to homogenize the contents.

2. De-immobilization of the algae :

Take one tube containing algal beads, pour out the liquid and add 5 ml "Matrix dissolving Medium". Cap the tube and handshake vigorously

every 2 minutes to dissolve the matrix. A Vortex shaker may be used to speed up the process.

As soon as the matrix is totally dissolved (which takes from 20 to 40 minutes), centrifuge the tube for 10 minutes at 3000 rpm. Pour out the supernatant and add 10 ml deionized water; cap and shake to re-suspend the algae. Centrifuge again for 10 minutes at 3000 rpm and decant the supernatant. Add 10 ml algal culturing medium, cap and shake to re-suspend the algae.

3. Preparation of the concentrated algal inoculum :

Pour the contents of the tube into a 25 ml calibrated flask and add algal culturing medium to the mark. Stopper and shake to homogenize the algal suspension. Take the two long cells marked "Calibration cell" and "Algal Stock cell". Fill the calibration cell 1 with 25 ml algal culturing medium, close with the lid and measure the OD at 670 nm. Transfer the algal suspension into the Algal Stock cell, close the cell tightly and shake to distribute the algal suspension evenly*.

* SHAKING PROCEDURE OF LONG CELLS WITH ALGAL SUSPENSIONS

- Place the thumb in the middle on the bottom of the cell and all other fingers on the lid at equal distance from each other. Press firmly to ensure a tight closing of the cell. Turn the cell upside down and shake gently for approx. 10 seconds and turn the cell upwards again. Score the OD in the spectrophotometer after 10 seconds.

Put the algal stock cell in the spectrophotometer and read the optical density (OD1). Take the OD/N sheet and look up the number of algae (N1) corresponding with OD1. With N2 equal to 1.10^6 algae/ml, calculate from the N1/N2 ratio the dilution factor needed to reach an optical density equal to OD2, corresponding to an algal density of 1.10^6 cells/ml. Transfer the algal suspension from the Algal Stock cell into a 100 ml flask and add the volume of algal culturing medium needed to make up a 1.10^6 cells/ml suspension. Stopper and shake the container to distribute the algae evenly.

Rinse the Algal Stock cell, transfer 25 ml algal suspension from the flask into this cell, close and shake, and read the OD. Check on the measured OD/N graph whether the OD corresponds with the desired OD₂ value (1.10⁶ algae/ml).

4. Preparation of the toxicant dilution series

Prepare the dilution series of the effluent or chemical according to the detailed instructions given in the Standard Operation Procedure Manual. Inoculate each flask with microalgae from the concentrated suspension to obtain 1.10⁴ cells/ml as the test start concentration.

5. Transfer of the algae-toxicant dilutions into the test vials :

Take one of the two holding trays with long cells, remove the rubber bands and the two plastic strips, and mark all the cells in sets of 3 (a, b, c), corresponding to the concentration range C0-C5. Open all the cells and after thorough shaking, transfer 25 ml of the algae-toxicant dilutions from each flask into the corresponding long cells.

6. Incubation of the test vials :

Close all the cells and put them back in the holding tray in a random way (*i.e. not in the sequence C0-C5, and not all 3 parallels next to each other*). Slightly lift up the lid of each cell on the same side and slide the plastic strip over the open part, in order to leave some opening near the middle of the long cells for gas exchange. Incubate the tray with the cells for 72 h in an incubator or in a temperature controlled room with constant uniform illumination (10000 lux for sideway illumination or min. 3000-4000 lux for bottom illumination), at 21-25°C.

7. Scoring of the results :

Determine the OD in each cell after (after applying the shaking procedure outlined above) after 24h, 48h and 72h incubation and score the data on the Results Sheets.

8. Data treatment :

Calculate the mean daily OD values for the 3 replicate cells and determine the algal growth inhibition in each test concentration. Calculate the 72h ErC₅₀ according to internationally accepted procedures (e.g. ISO 8692 or OECD Guideline 201). A computer programme for automatic data treatment can be obtained from the distributors of Toxkit microbiotests.

Validity of the test :

Besides other prerequisites specific for individual guidelines, the number of algae in the control test vials must have increased by at least a factor 16 (OECD Guideline) or 67 (ISO norm) during the 72h exposure period for the toxicity test to be acceptable.

9. 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 the test sensitivity.

A reference test can be carried out with potassium dichromate (K₂Cr₂O₇). On the corresponding specification sheet one can find the values of the reference test performed by MicroBioTests Inc.

The dilution series to be prepared for the reference test with potassium dichromate is : 0.18 - 0.32 - 0.56 - 1 - 1.8 mg/l.