SPIRODELA DUCKWEED TOXKIT

A SIMPLE AND PRACTICAL GROWTH INHIBITION MICROBIOTEST WITH *SPIRODELA POLYRHIZA*

BENCH PROTOCOL

PRINCIPLE AND FEATURES

The *Spirodela polyrhiza* microbiotest measures the growth inhibition of duckweed fronds germinated from "dormant vegetative buds" (called turions), after 3 days of exposure to toxicants or to contaminated water samples. *Spirodela polyrhiza* is one of the very few species of duckweeds known to produce turions, and these "dormant" stages can be stored for long periods of time, and germinated at the time of performance of the bioassay. Similarly to all other Toxkit tests, the *Spirodela polyrhiza* microbiotest is hence also "stock culture free", and can be started anytime and anywhere from the stored turions.

Turions stored in test tubes are "germinated" for 3 days at 25 °C and with continuous illumination, in a petri dish containing (Steinberg) growth medium. The germinated turions are then transferred into the cups of a 48 cups multiwell test plate containing the toxicant dilutions. A "digital" picture of the test plate is then taken (at t0h) and transferred to a computer file. The test plate is subsequently incubated for 3 days at 25 °C and under continuous illumination, after which a digital picture of the test plate (with the grown duckweeds) is again taken (at t72h).

The effect parameter for the growth of the duckweeds is the measurement of the "size" (= the area) of the first fronds developing from the germinated turions, at the start of the toxicity test versus their size at the end of the 3 days exposure. The area measurements are made directly on the 2 digital pictures of the test plate taken at t0h and at t72h, with the aid of an Image Analysis program.

The mean growth of the first fronds in the test concentrations is calculated by subtracting the "initial" area size of the first fronds (at the start of the toxicity test) from the "final" area size (at the end of the 3 days exposure). The inhibition percentage of the duckweed growth is then calculated for each test concentration, with subsequent calculation of the 72h EC50.

ASSETS OF THE *SPIRODELA POLYRHIZA* MICROBIOTEST

- The assay is totally independent of the culturing/maintenance of live stocks of the test species
- The germination of the turions and their transfer to the test plate are very simple operations
- The test plates for the toxicity test are small, require little bench space and incubation space, and allow to set up multiple tests concurrently
- The test duration (after the germination of the turions) is only 3 days (instead of 7 days for *Lemna* tests)
- The effect parameter (= the duckweed growth inhibition) is the measurement of the area of the first fronds of the germinated turions at the start and at the end of the test, which is simple and rapid with the aid of image analysis
- The photos of the test plates with the grown duckweeds are stored as computer files which allows to postpone the area measurements
- The test procedure is highly standardized and its precision has been evaluated in an extensive "International Interlaboratory Comparison"
- Validity criteria have been selected for the assay and a methodology has been worked out for a reference test (quality control test) with potassium chloride (KCl)

The sensitivity of the *Spirodela* duckweed microbiotest has been determined on a substantial number of inorganic and organic compounds and was found to be very similar to that of conventional *Lemna* tests

TEST PROCEDURE

1. Preparation of duckweed growth and test dilution medium

The "Steinberg" growth medium prescribed by ISO for *Lemna* toxicity tests (ISO 20079) has been selected for the *Spirodela* microbiotest. This medium is prepared by transferring 10 ml of concentrated solutions from the vials A,B and C and 0.5 ml from vials D and E in 300 ml pure water (e.g. distilled or deionized water) in a 500 ml liter volumetric flask. The flask is then filled to the mark with pure water.

N.B. The prepared Steinberg medium has a relatively short shelf life and should be used within a few weeks after preparation. A similar (500 ml) volume of Steinberg medium shall be prepared at the time of performance of the second toxicity test.

2. Germination of the turions

The contents of a tube with turions are poured in the microsieve and rinsed with pure water to remove the storage medium. The microsieve is turned upside down above a Petri dish containing 10 ml Steinberg medium, and 10 ml Steinberg medium are then poured over the sieve to transfer the turions into the Petri dish. The Petri dish is then filled by adding 10 ml Steinberg medium. The covered Petri dish is incubated for 3 days at 25 °C with continuous illumination (at least 6 000 lux at the surface of the dish).

3. Preparation of the toxicant dilutions

Prepare a dilution series of the test compound or effluent according to standard methods.

4. Filling of the test plate with the toxicant dilutions

1 ml Steinberg growth medium is put into the 8 cups of the control row, and 1 ml toxicant solution into each cup of the corresponding rows for the 5 toxicant concentrations, starting in sequence from the row under the control row (row B at the top of the test plate) towards the row with the highest test concentration (row F, at the bottom of the test plate).

5. Transfer of the germinated turions in the test cups
With the aid of a spatula, transfer 1 germinated turion into each cup of the test plate.

N.B. Germinated turions can easily be distinguished from those which have not germinated by the presence of a (small) initial frond with small roots.

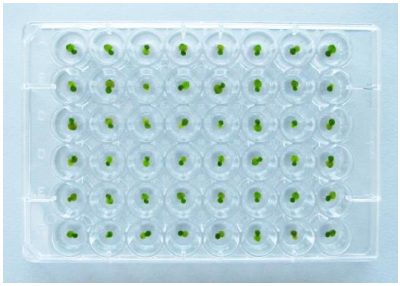
A photo of the multiwell plate is taken with a digital camera (= at t0h) and transferred to a computer file.

6. Incubation of the test plate
The covered test plate is incubated for 3 days at 25 °C and with (continuous) 6 000 lux illumination, after which a digital picture of the multiwell is taken again (at t72h) after removal of the lid.

7. Area measurement of the fronds
The size (= the area) of the first fronds is measured directly on the 2 photos of the multiwells, with the aid of an "Image Analysis" program.

N.B. "Image J" is a very convenient Image Analysis program and can be down-loaded free of charge from the Internet.

The area measurements of the first fronds have to be made two times :
1. Measurement of the area of the initial (small) frond of the germinated turions (from the photo of the test plate taken at the start of the toxicity test (t0h).
2. Measurement of the area of the (grown) first fronds of the germinated turions (from the photo of the test plate taken after 3 days of incubation (t72h).



Left : Photo of the germinated turions with the (small) first frond, in the cups of the multiwell test plate, at the start of the toxicity test (t0h)
Right : Photo of the grown duckweeds in the cups of the multiwell test plate, in increasing concentrations of a toxicant, after 3 days incubation (t72h)

N.B. The measurements must be restricted to the area of the fronds, (i.e. "without" the area of the turion to which the frond is attached). At the end of the test, a second frond (or even more) can also already have developed in some cups, but the area measurement shall only be made on the "largest" of the fronds.

The data of the area measurements must be transferred to an Excel file for subsequent data treatment.

8. Data treatment
The mean area of the "Initial" fronds is calculated in each row (= I), as well as the mean area of the "Final" fronds (= F). These mean data are scored on the Data Treatment Sheet. Subtracting I from F gives "the growth" of the duckweeds in each row of the test plate.
The percentage growth inhibition is calculated in each test concentration for subsequent
Calculation of the 72h EC50 with an appropriate computer program for toxicity tests.
NOTE : A data treatment Excel sheet has been worked out by MicroBioTests Inc, and can be obtained free of charge from MicroBioTests Inc. This program calculates the growth of the first fronds, the percentage inhibition in each test concentration, and the 72h EC50 with 95% confidence limits.

9. Validity criteria
For the assay to be valid the mean area of the first fronds in the control row after 3 days incubation at 25 °C and under 6 000 lux illumination, should be at least 10 mm^2.

10. Reference test
A quality control test can be performed with the reference toxicants indicated in the ISO 20079 standard for *Lemna* tests, i.e. 3,5 dichlorophenol or KCl.
For a reference test with KCl the following test concentrations are recommended : 18 000 - 10 000 - 5 600 - 3 200 - 1 800 mg/l. The 72h EC50 of the quality control test should be in the range stipulated in the Specification Sheet of the *Spirodela* duckweed microbiotest.