DUCKWEED TOXKIT F

Growth inhibition microbiotest
with *Spirodela polyrhiza*

STANDARD OPERATING

PROCEDURE

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INTRODUCTION TO THE *SPIRODELA* DUCKWEED MICROBIOTEST

ORIGIN

The conventional duckweed tests prescribed by national and international organisations are mostly based on the test species *Lemna minor* and *Lemna gibba*. These assays require a large number of test containers, they are time consuming and space demanding, and they are all dependent on the (continuous) culturing and maintenance of live stocks of the test species.
A technology for an alternative duckweed toxicity test has been developed by the company MicroBioTests Inc. in collaboration with the Laboratory of Ecology and Environmental Sciences of the Agricultural University of Athens in Greece. The innovation and major asset of this alternative duckweed test is that similarly to the other Toxkit microbiotests the assay departs from “dormant stages” of the test species (in casu vegetative buds called “turions”) which can be stored for long periods of time and “activated” (germinated) at the time of performance of the bioassay. This hence also makes the *Spirodela* duckweed microbiotest totally independent of the culturing/maintenance of stocks. *Spirodela polyrhiza* has been selected as test species since it is one of the very few duckweed species which produces vegetative buds. The technique for the *Spirodela* duckweed assay has been streamlined to a “microbiotest” since the assay is performed in a small multiwell test plate. Determination of the growth of the aquatic plants is made by image analysis and does not require any manipulation of the organisms in the test cups for the measurements.

SCOPE

The *Spirodela* duckweed microbiotest measures the decrease (or the absence) of growth of the germinated “turions”, after 3 days of exposure to toxicants or to contaminated water samples, in comparison to the controls in a not polluted medium.

PRINCIPLE

Vegetative buds (turions) of the duckweed species *Spirodela polyrhiza* stored in test tubes are "germinated" in a petri dish containing growth medium. The petri dish is incubated for 3 days at 25 °C under continuous illumination of 6 000 lux.
The germinated turions are transferred into the cups of a 48 cups multiwell test plate containing the toxicant dilutions, and a "digital picture" of the test plate is then taken and saved on a computer. The test plate is subsequently incubated for 3 days at 25 °C with continuous 6 000 lux illumination, after which a "digital picture" is then again taken.
The size (= the area) of the small "first frond" of the duckweeds is measured in the test cups at the start of the toxicity test and after 3 days incubation, with the aid of an Image Analysis program. The growth of the duckweeds is calculated by subtracting the mean "initial" size of the first frond from the mean "final" size, in the control and in the different toxicant concentrations. The percentage growth inhibition of the duckweeds in the respective toxicant concentrations can then be calculated, followed by the evaluation of the 72h EC50.

ASSETS OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

The *Spirodela* duckweed microbiotest has multiple advantages over conventional *Lemna* duckweed tests :

- 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 the conventional *Lemna* tests)
- The selected 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 on a computer 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

FEATURES

The *Spirodela* duckweed microbiotest is based on germination of the dormant vegetative buds (turions) of *Spirodela polyrhiza*, followed by 3 days growth of the plants in a multiwell test plate containing a dilution series of the toxicant (or the polluted water sample).
The effect parameter selected for the *Spirodela* duckweed microbiotest is the assessment of the inhibition of the growth of the duckweeds in increasing test concentrations by measurement of the area of the first frond of the germinated turions at the start of the test and after 3 days incubation. The growth effect parameter was found to give sensitivity results which are similar to the counting of the total number of fronds or the measurement of the total area of the fronds, which are prescribed as effect criteria in conventional *Lemna* tests
The area measurements are made by Image Analysis of the photos of the test plates. The Image J program (free of charge) is a simple and practical program for the area measurements of the first fronds.
The use of a multiwell plate with 48 cups (6 rows and 8 columns) allows to perform the assay with 1 control and 5 test concentrations.
The equipment needed for the *Spirodela* duckweed microbiotest is an incubator or a temperature controlled room at 25 °C, provided with an illumination system which gives at least 6 000 lux at the surface of the turions and the growing fronds.
A picture of the test plate at the start and at the end of the 3 days exposure has to be taken with a digital camera.
Each Toxkit allows to perform 2 complete toxicity tests with 5 test concentrations, with either chemical compounds or effluent dilutions.

SHELF LIFE

If stored properly (i.e. in the refrigerator and in darkness) the majority of the turions will still germinate after several months of storage.

SENSITIVITY

The sensitivity of the *Spirodela* duckweed microbiotest has been determined on a substantial number of inorganic and organic chemicals, with calculation of the 72h EC50.
The 72h EC50 values of the *Spirodela* duckweed microbiotest have been compared with the 7 days EC50's of conventional *Lemna* tests. A regression of the data pairs (which ranged over 4 orders of magnitude) revealed an R^2 of 0,97 and shows that the *Spirodela* duckweed microbiotest has basically the same sensitivity as the conventional *Lemna* tests.

CONTENTS OF THE SPIRODELA DUCKWEED TOXKIT

Multiwell test plates
Two polystyrene plates (9 x 13 cm) with 48 cups (1 ml) which will serve as test containers.

Petri dishes
Two polystyrene petri dishes (9 cm diameter) with cover, to be used for the germination of the turions.

Tubes with turions
Two polystyrene test tubes (10 ml) each containing *Spirodela polyrhiza* turions in a storage medium.

Microsieve
A small sieve with 100 µm mesh for eliminating the storage medium.

Spatulas
2 small plastic spatulas for easy transfer of the turions

Concentrated salt solutions
Three 25 ml glass bottles labeled A, B and C, and two 12 ml glass bottles labeled D and E, for preparation of the Steinberg growth and test dilution medium.

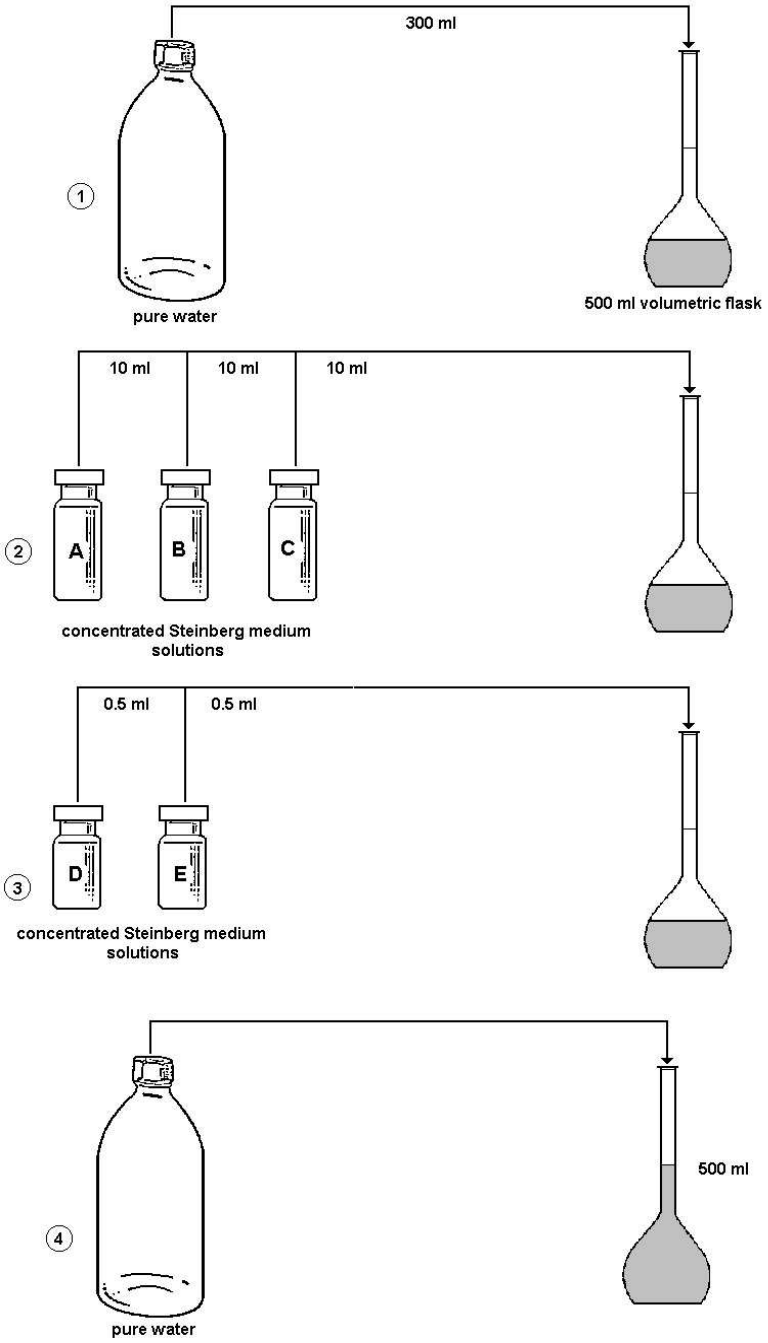
Standard Operational Procedure manual
A detailed brochure with all instructions for performance of “range finding” and/or “definitive” assays on pure chemicals or polluted water samples.

Bench protocol
An abbreviated version of the Standard Operational Procedure manual.

Specification sheet

A sheet indicating the batch number of the turions, the batch number of the concentrated salt solutions, the expiry date of the Toxkit and the 72h EC50 for the test with the reference chemical potassium chloride KCl.

FIGURE 1 : PREPARATION OF DUCKWEED GROWTH AND TEST DILUTION MEDIUM



TEST PROCEDURE

General remark **:** *The tubes with the turions and the vials with the solutions must be stored in the refrigerator prior to use.*

1. PREPARATION OF DUCKWEED GROWTH AND TEST DILUTION MEDIUM

*The growth medium will be used for the germination of the Spirodela turions and as growth medium for the duckweeds and at the same time as dilution medium for the toxicants in the toxicity test. The composition of the growth medium is that of the "**Steinberg medium"** prescribed by ISO for Lemna toxicity tests (ISO 20079).*

Procedure (see Figure 1)

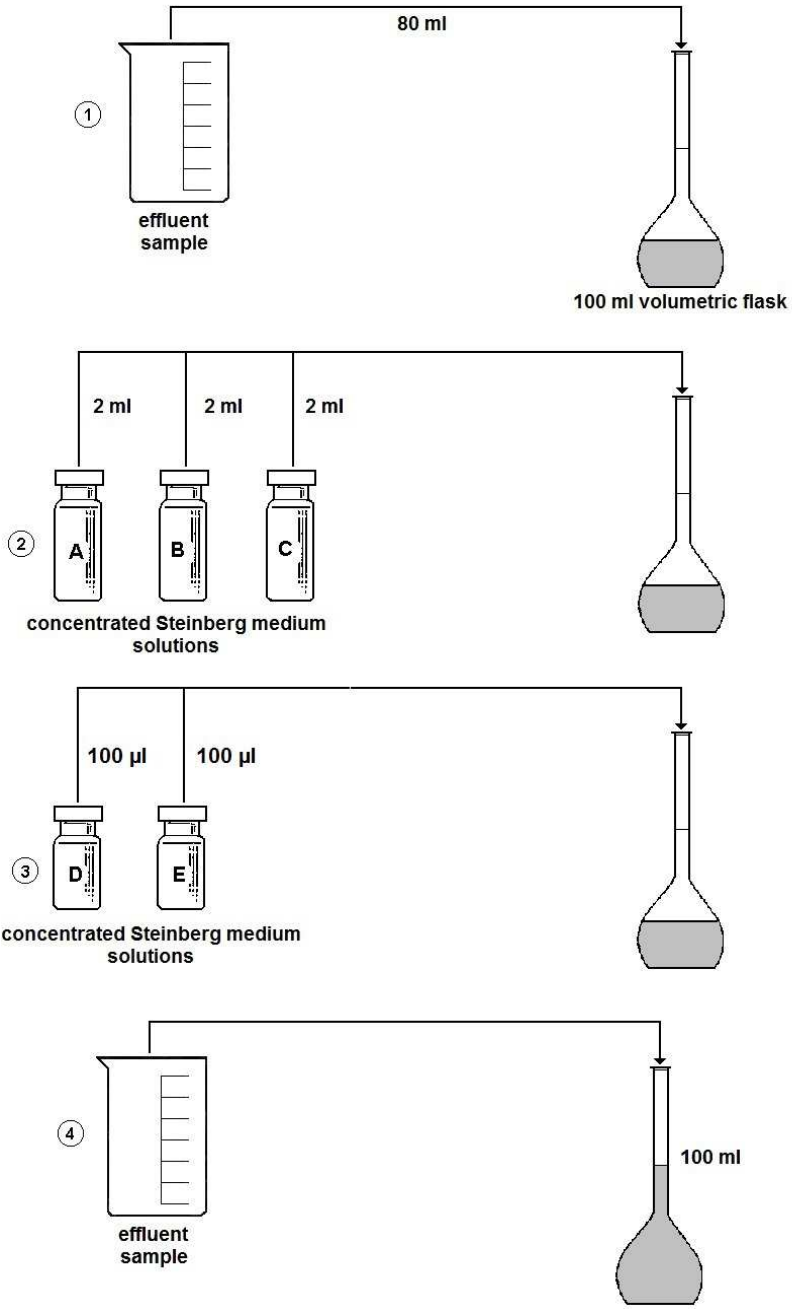
1. Transfer about 300 ml pure water (deionized or distilled) in a 500 ml volumetric flask.
2. Uncap one each of vials A, B and C and transfer 10 ml from each bottle in the volumetric flask.
3. Uncap vials D and E and transfer 0,5 ml from each bottle in the volumetric flask.
4. Fill the flask up to the 500 ml mark with pure water, stopper the flask and shake thoroughly to homogenize the medium.
5. Store the prepared Steinberg medium in the refrigerator in darkness until use.

N.B. This medium has a relatively short shelf life and should be used within 2 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 *SPIRODELA POLYRHIZA* TURIONS

1. Take a tube with *Spirodela polyrhiza* turions and shake it slightly to resuspend the turions.

FIGURE 2 : ADDITION OF CONCENTRATED STEINBERG MEDIUM TO THE EFFLUENT



2. Pour the contents of the tube in the microsieve and rinse with pure water to remove the storage medium.
   Make sure that all the turions are transferred to the microsieve.
3. Put 10 ml Steinberg medium in one of the 9 cm Petri dishes.
4. Turn the microsieve upside down and flush all the turions in the Petri dish, by pouring 10 ml Steinberg medium over the surface of the microsieve.
5. Fill the petri dish further by adding 10 ml Steinberg medium.
6. Cover the petri dish with the transparent lid and place it in the incubator.
7. Incubate the petri dish for 3 days (72h ± 1h) at 25 °C with continuous "top"illumination (at least 6 000 lux at the surface of the petri dish).

N.B. Both germination of the turions and the growth of the first fronds are "very substantially" dependent on temperature and illumination conditions.
It is therefore most important that the prescribed values (25 °C and 6 000 lux) be respected "as closely as possible.

3. PREPARATION OF THE TOXICANT DILUTIONS

A. TESTS ON EFFLUENTS

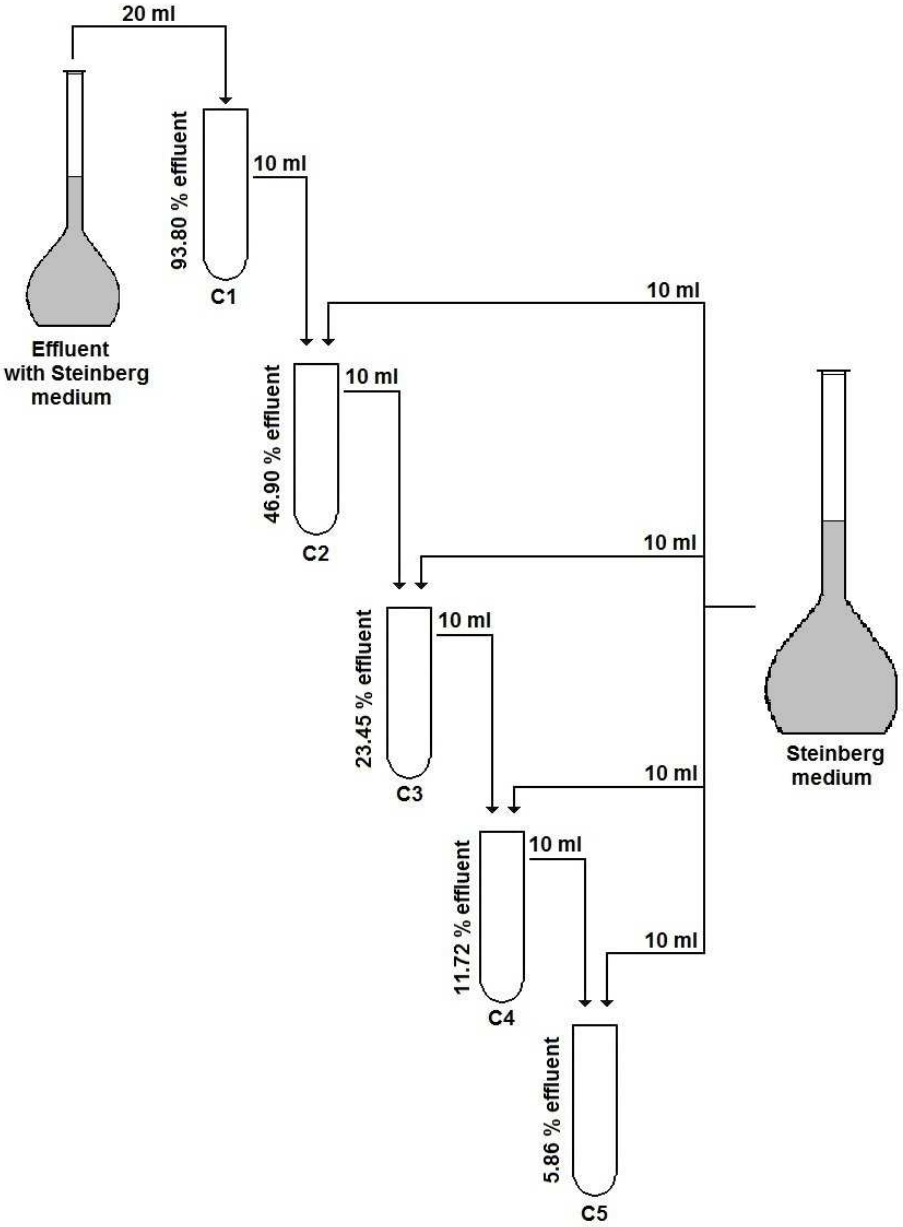
For a test on an effluent, a small volume of concentrated Steinberg growth medium must first be added to the effluent prior to making the dilution series.

Addition of concentrated Steinberg growth medium solutions to the effluent

Procedure (see Figure 2)

1. Transfer about 80 ml effluent in a 100 ml calibrated flask.
2. Uncap one each of vials A, B and C and transfer 2 ml from each vial in the calibrated flask.
3. Uncap vials D and E and transfer 100 µl from each vial in the calibrated flask.

FIGURE 3 : PREPARATION OF THE TOXICANT DILUTIONS



4. Fill the flask up to the 100 ml mark with effluent, stopper the flask and shake thoroughly to homogenize the contents.

Preparation of the effluent dilutions

Procedure (see Figure 3 and Table 1)

N.B. The addition of 6,2 ml growth medium to 93,8 ml effluent dilutes the effluent sample by about 6 %, which means that the highest effluent concentration which will be tested is about 94 % of the original effluent.
A 1:1 dilution series is prepared from this 94 % effluent.

1. Take five 20 ml test tubes and label them C1, C2, C3,C4 and C5.
2. Add 20 ml effluent (containing growth medium) to test tube C1.
3. Add 10 ml Steinberg medium (as dilution medium) to the tubes C2, C3, C4 and C5.
4. Transfer 10 ml effluent from tube C1 to tube C2. Cap and shake the test tube.
5. Transfer 10 ml test dilution from tube C2 to tube C3. Cap and shake the test tube.
6. Repeat this procedure for the next dilutions, i.e. 10 ml from tube C3 to tube C4, and 10 ml from tube C4 to tube C5.
7. Proceed to section 4. “Filling of the Test Plate with the toxicant dilutions”.

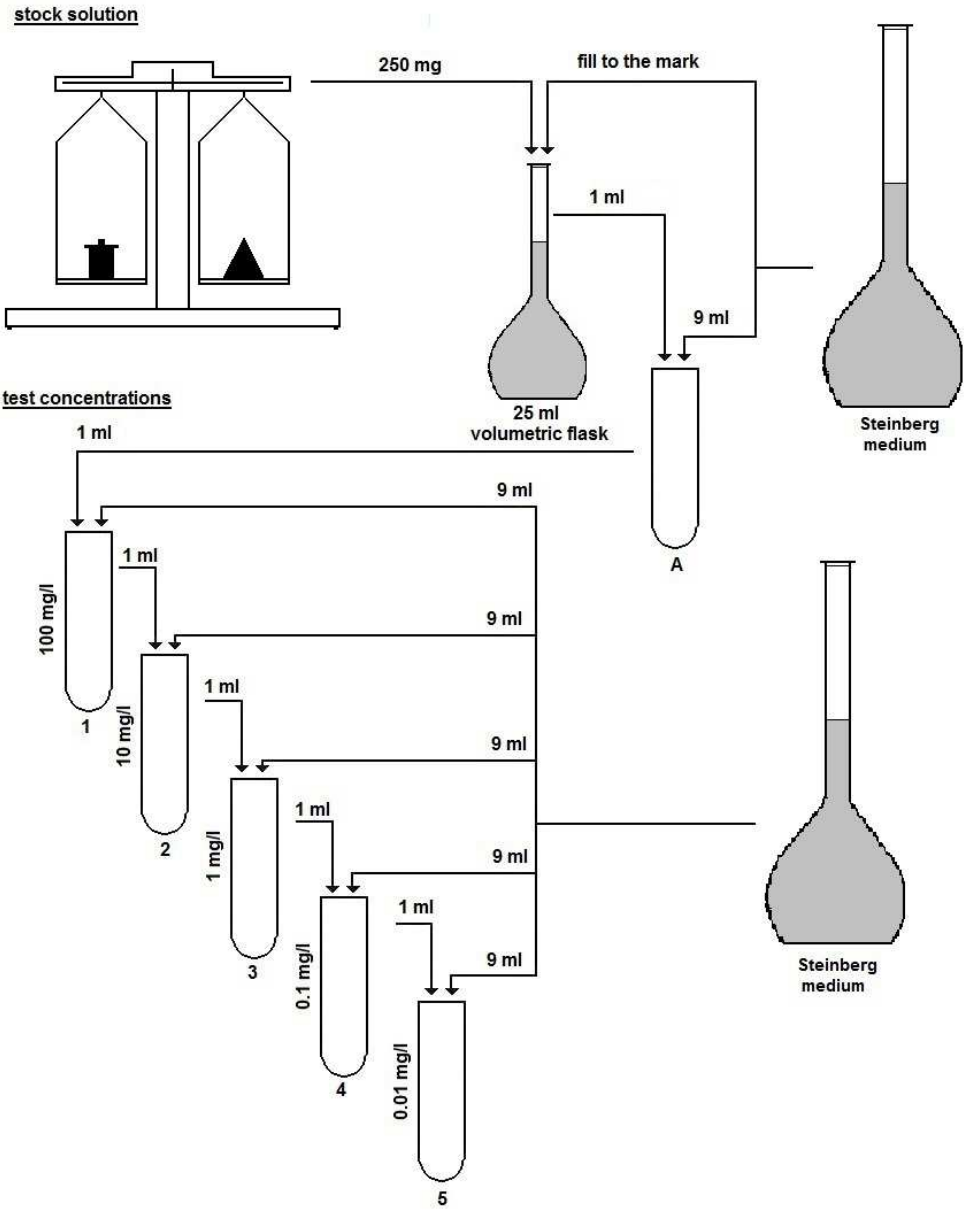
Table 1 : Dilution series of the effluent

Test tube	Effluent concentration (%)
1	93,80
2	46,90
3	23,45
4	11,72
5	5,86

FIGURE 4 : PREPARATION OF TOXICANT DILUTIONS

(in Steinberg growth medium as dilution medium)

RANGE FINDING TEST



B. TESTS ON CHEMICAL COMPOUNDS

RANGE FINDING TEST

If the "approximate toxicity" (= order of magnitude) of the chemical compound under investigation is not known, a "Range Finding Test" has to be performed first to determine the 0-100 % tolerance range of the duckweeds to the toxicant.
A dilution series 1:10 is prepared in test tubes of 10 and 20 ml, in Steinberg growth medium (as dilution medium).
An example is given below for a concentration range from 100 mg/l down to 0,01 mg/l.

Preparation of the toxicant dilutions

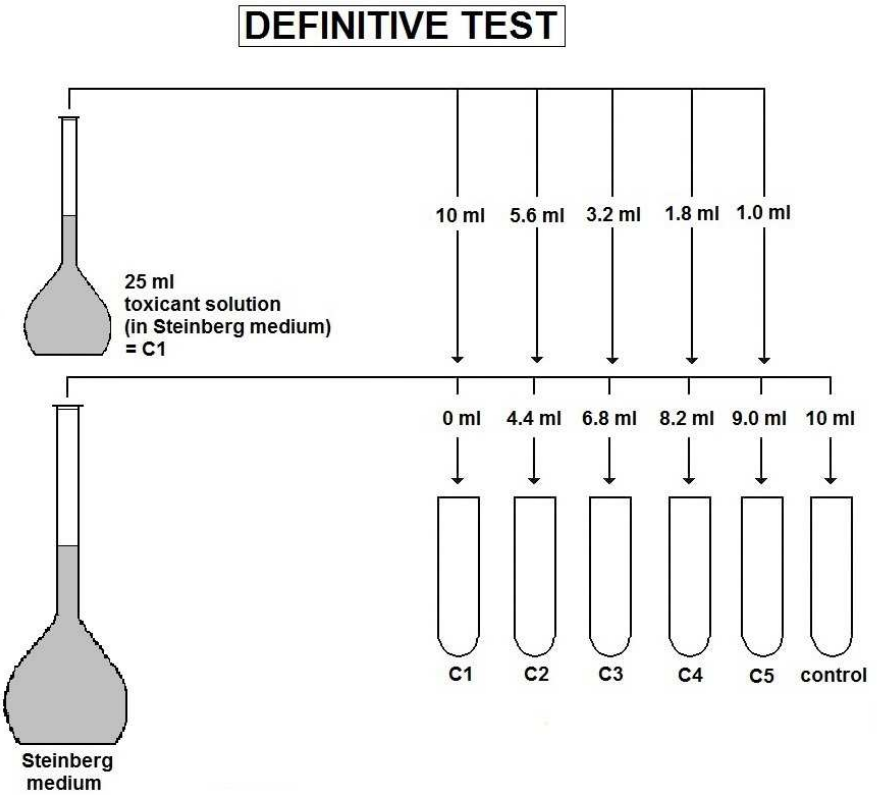
Procedure (see Figure 4)

1. Take a 25 ml volumetric flask.
2. Take 6 test tubes of 10 ml contents and label them A, 1,2,3,4 and 5.
3. Weigh 250 mg of the chemical on an analytical balance and transfer it to the 25 ml volumetric flask.
4. Add Steinberg medium (as dilution medium) to the mark and shake vigorously to dissolve the chemical.
5. Add 9 ml Steinberg medium to test tube A and test tubes 1,2,3,4 and 5.
6. Transfer 1 ml of the volumetric flask to test tube A; cap and shake this test tube.
7. Transfer 1 ml of test tube A to test tube 1; cap and shake this test tube.
8. Transfer 1 ml of test tube 1 to test tube 2; cap and shake this test tube.
9. Repeat this operation for the dilutions in test tubes 3, 4 and 5.
10. Proceed to section 4. "Filling of the test plate with the toxicant dilutions".

DEFINITIVE TEST

The dilution series to be prepared spans the range of the lowest concentration producing 100 % effect to the highest one producing less than 10 % effect in the range finding test.

FIGURE 5 : PREPARATION OF TOXICANT DILUTIONS
(in Steinberg growth medium as dilution medium)



Preparation of the toxicant dilutions

Procedure (see Figure 5 and Table 2)

1. Take a 25 ml volumetric flask and prepare in Steinberg medium (as dilution medium) 25 ml toxicant solution at the concentration which was the lowest one giving 100 % effect in the Range Finding Test.
   This will be the C1 test concentration.
2. Take 6 test tubes (10 ml contents) and label them C1,C2, C3, C4 and C5 and the last tube as “control”.
3. Add the volumes of toxicant concentration C1 indicated in Figure 5 and Table 2 to the test tubes C1 to C5.
4. Add the volumes of Steinberg medium (= dilution medium) indicated in Figure 5 and Table 2 to the test tubes C2, C3, C4, C5 and “control”.
5. Cap and shake all the test tubes to obtain a homogenous distribution of the toxicant in the tubes.

Table 2 : Dilution series C1 - C5

Test tube	C1 (ml)	Steinberg medium (ml)
C1	10	0
C2	5,6	4,4
C3	3,2	6,8
C4	1,8	8,2
C5	1,0	9,0
Control	0	10

6. Calculate the actual concentrations of the toxicant in the tubes (which will be needed for the EC50 calculation)
   C1 = …..mg/l
   C2 = 0,56 x C1 = ….mg/l
   C3 = 0,32 x C1 = ….mg/l
   C4 = 0,18 x C1 = ….mg/l
   C5 = 0,10 x C1 = ….mg/l

7. Proceed to section 4. “Filling of the test plate with the toxicant dilutions”

4. FILLING OF THE TEST PLATE WITH THE TOXICANT DILUTIONS

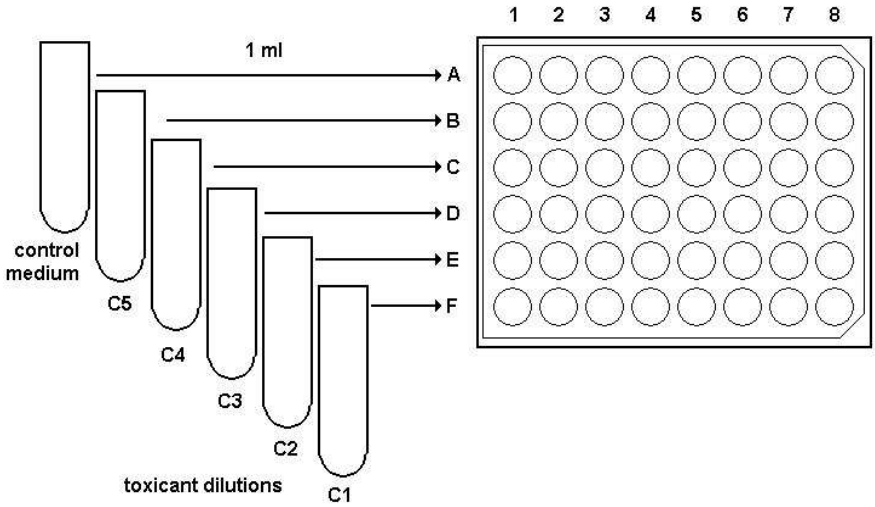
Each toxicant dilution has to be transferred into all the cups of one row in the multiwell plate. The rows are labeled from A to F and the cups from 1 to 8.

N.B. The distribution of the test solutions must always be carried out starting with the control row (row A) on top of the multiwell plate with Steinberg growth medium (= dilution medium), followed in sequence by the rows containing increasing toxicant concentrations, up to the highest test concentration in the bottom row (= row F).

Procedure (see Figure 6)

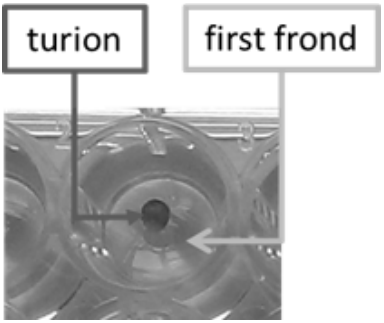
1. Put 1 ml Steinberg medium from the control tube in the 8 cups of row A (= the control row).
2. Put 1 ml of the tube containing the C5 toxicant concentration in the 8 cups of Row B.
3. Repeat this procedure with the tubes C4, C3, C2 and C1 for the 8 cups in the rows C,D,E and F respectively.

FIGURE 6 : FILLING OF THE TEST PLATE WITH THE TOXICANT DILUTIONS



5. TRANSFER OF THE GERMINATED TURIONS IN THE TEST CUPS

Take the petri dish with the germinated turions out of the incubator and check if the turions have germinated.



N.B. Germinated turions can easily be distinguished from those which have not germinated, by the presence of a (small) first frond on one side of the turion (see photo) and small roots.

Procedure

1. With the aid of the spatula, transfer 1 germinated turion into each cup of the Control row (row A)
2. Repeat this operation with the other rows "from the top to the bottom of the multiwell plate", i.e. starting with the row containing the lowest test concentration (C5 in row B) down to the row with the hightest test concentration (C1 in row F).

Important remark : The transfer of the germinated turions to the cups of the multiwell must be carried out "randomly", i.e. one has to avoid picking up and transferring first the germinated turions which have "the largest fronds".

6. TAKING OF A PHOTO OF THE MULTIWELL AT THE START OF THE TOXICITY TEST

A digital photo of the multiwell plate containing the germinated turions (with their small first fronds) has to be taken at the start of the 3 days toxicity test).

Procedure

1. Place the test plate on a horizontal surface.
2. Take a photo of the multiwell plate with a digital camera (see photo).

N.B.1 : If possible, put the multiwell plate on a light table to take the photo. This will increase the contrast between the germinated turions and the first small fronds, and allow for a better distinction between the turion and the first frond.
Alternatively the multiwell plate can be placed on a white background, but by no means the plate should be placed on a "dark" background to take the photo.

N.B.2 : To take the photo, the digital camera should not be held too close to the multiwell plate, since this will lead to a "distortion" of the view of the cups in the columns on the left and right side of the multiwell plate. It is important that the edges of all the lateral wells also have a round (and not an oval) look !

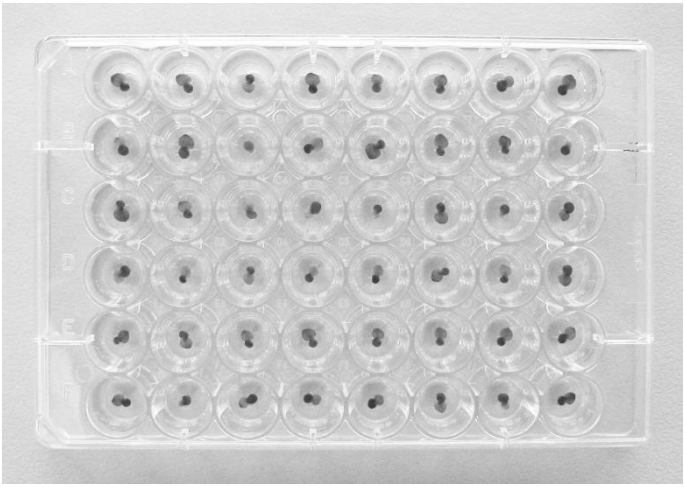


Photo of the multiwell plate with the germinated turions and their small first fronds (at t0h)

3. Transfer the photo of the multiwell plate to a computer file.

7. INCUBATION OF THE TEST PLATE

1. Put the cover on the multiwell plate and put the plate in the incubator.
2. Incubate the test plate at 25 °C for 3 days (72h ± 1h), with a continuous illumination of 6 000 lux (at the top of the multiwell).

N.B. Same remark as for the germination conditions : the prescribed 25 °C and 6 000 lux illumination must be respected "as closely as possible" !

8. TAKING OF A PHOTO OF THE MULTIWELL AT THE END OF THE TOXICITY TEST

A digital photo of the multiwell plate containing the grown fronds has to be taken again at the end of the 3 days toxicity test. It is also advised to again put the plate on a "light table" (or on a white background) to take the photo, for a better distinction between the turions and the first fronds.

Procedure

1. Take the multiwell plate from the incubator and remove the lid.

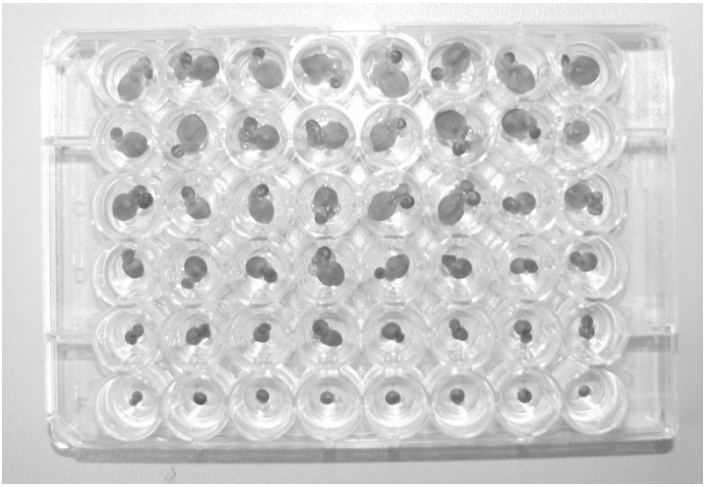


Photo of the multiwell plate with the grown first fronds, taken after 3 days incubation (t72h)

2. Take a quick look at the fronds in each cup. If some fronds are not laying totally "horizontally" (and hence don't show their total surface) they have to be put in a horizontal position with the aid of the spatula.
3. Take (again) a photo of the multiwell plate (see photo) and transfer the photo to a computer file.

9. MEASUREMENT OF THE AREA OF THE FIRST FRONDS

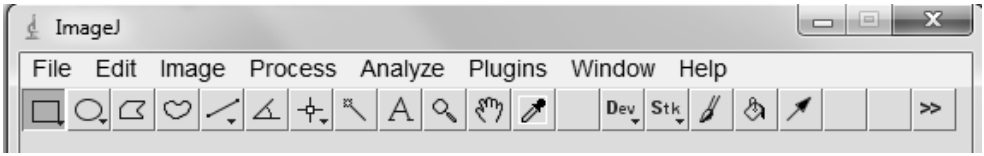
The measurement of the areas of the first fronds can be made immediately after taking the photo of the multiwell, or can be postponed to any appropriate time.
The area measurements are made with the aid of an appropriate "Image Analysis" program (such as e.g. "Image J" which is accessible free of charge on the Internet).
The procedure to be followed for the area measurements and the subsequent data saving is detailed hereunder for specific use of ***"Image J".***

PROCEDURE FOR MEASUREMENT OF THE AREA OF THE FRONDS WITH IMAGE J

As indicated above, the area measurements have to be made "a first time" on the photo with the germinated turions and their small first fronds, at the start of the toxicity test (t0h) and a second time on the photo with the "grown" first fronds at the end of the 3 days toxicity test (t72h).

1. Access to the photo of the multiwell plate

1. Open the ImageJ program - A horizontal bar appears with a number of icons and words.



2. Go to Analyze and click Set Measurements. Make sure that only the

checkbox Area is ticked (all the other checkboxes should be unticked). Then click OK (to save the new settings)
3. Go to File and click Open image
4. Select the Directory and the file with the saved picture of the multiwell plate.

2. Calibration of the measurements

1. Click the 5th icon (/) from the left (and select "straight")
2. Draw a straight line from the top border down to the lower border of the test plate.
3. Click Analyze and Set scale
4. Fill out the box Known distance with the figure **80** (which is the length of the calibration line), and type "**mm**" in the box Unit of length.
5. Click OK (*this will subsequently automatically make the calculation of the areas in mm^2*).

3. Enlargement of the picture of the individual wells

1. Enlarge the picture of the multiwell plate by clicking the + key several times until one cup almost totally fills up the computer screen.
2. Move the picture on the screen horizontally or vertically by pressing the space bar (*and keeping it pressed*) and moving "the mouse" of the computer in the desired direction (*when keeping the space bar pressed, the + sign changes to a small hand (✋).*
3. Move the picture to bring the view of the A1 cup (the first cup of the top row = the "control row) on the computer screen and then release the space bar (*the ✋ changes again to the + sign).*

4. Measurement of the "contour" of the first frond in the cups

1. Click the 4th icon from the left (♡) (*which indicates the choice "free hand selection").*
2. Move the + cursor on the screen with the mouse so that it is placed exactly on the edge of the first frond in the cup; then draw a line around the whole contour of the first frond till the total area of the first frond is surrounded by a yellow line.

N.B. This operation has to be made "in one continuous movement". In case of a problem during the measurement, stop drawing the line and click the + sign. This will eliminate the yellow line.
Then start drawing again the line around the contour of this frond.

The area measurement must be restricted to the area of the first frond, i.e. "without" the area of the turion (to which the first frond is attached).

N.B. For the area measurements "at the end of the test" one will see that in some cups a second (or even more) frond has also already developed from the germinated turion.
Only the largest of these fronds must be measured.

3. Click simultaneously Ctrl and the letter M on the keyboard. This will open the Result box and show the first result.

N.B. as long as "Ctrl and M" are not clicked, measurement values will not be saved in the Result box.

4. Move the picture to the second cup (= A2) in the top row and proceed similarly to make the area measurement of this frond.
5. Save the result by clicking Ctrl and M.
6. Proceed further with the measurements of the first fronds in the other 6 cups of the control row.

IMPORTANT REMARK

It will only be possible to treat the results of the area measurements in Excel if the data (copied from the Image J program) are transferred with a "decimal point" i.e. "a dot" (.) and not with a "comma" (,).

Depending on your computer, the configuration for the decimals has been set either to "dot" or to "comma".

In case the decimals setting of your computer is "comma" you first have to change this to "dot" through the control panel of your computer.

5. Data Treament

NOTE : A Data Treament Excel sheet has been worked out by MicroBioTests Inc.
This program allows to transfer the data of the "initial first frond areas" and the "final first frond areas" into this Excel sheet which then calculates the growth of the first fronds, the percentage growth inhibition in each test concentration, and the 72h EC50 with the 95 % confidence limits.
This Excel sheet can be obtained free of charge from MicroBioTests Inc. .

A. Area measurements of the (small) first fronds at the start of the toxicity test

1. Go to the Results box (which contains the 8 area values of row A (= control), and select all the values (which will highlight on a black background).
2. Right-click the mouse and select Copy.
3. Open the Data Treatment Excel file and go to "Table 1 - Area measurements first frond t0h".
   Replace C5-C1 with your test concentrations.
4. Select "Paste here" in the column "Control" and paste the set of 8 data to this column.
5. Go back to the Results box in the Image J programme (showing the data on the black background).
6. Click Edit and Clear to eliminate all the data and to obtain a "blank" Result box for the area measurements of the second row (row B) of the multiwell.
7. Perform the area measurements of the first fronds in all the cups of row B and transfer the data into the column with your lowest concentration (= previously C5).
8. Proceed further similarly with the area measurements of the first fronds in the cups of rows C to F and transfer each time the data into the corresponding column.
9. Save the Excel file with the name of your choice.

B. Area measurements of the (grown) first fronds at the end of the toxicity test

The procedure for the area measurements at the end of the toxicity test is the same as that of the area measurements at the start of the toxicity test.
Open the (saved) Excel file, go to "Table 2 - Area measurements first frond t72h" and paste the area data for each row in the corresponding columns.
The Excel file then to be saved again (under the same name).

In Table 3 of the Data Treatment Excel file "the growth" of the first fronds in the 6 rows of the multiwell plate will be shown for each test cup. This growth is calculated by subtracting" the size of the (small) first fronds at the start of the toxicity test (t0h) from the size of the first fronds after 3 days exposure to the toxicant (t72h).
Table 3 also displays "the mean growth" (in mm^2) of the first fronds for the 8 replicates in the control row and in the rows with the 5 toxicant concentrations and the calculated % inhibition of the growth of the first fronds in the 5 test concentrations versus the control.

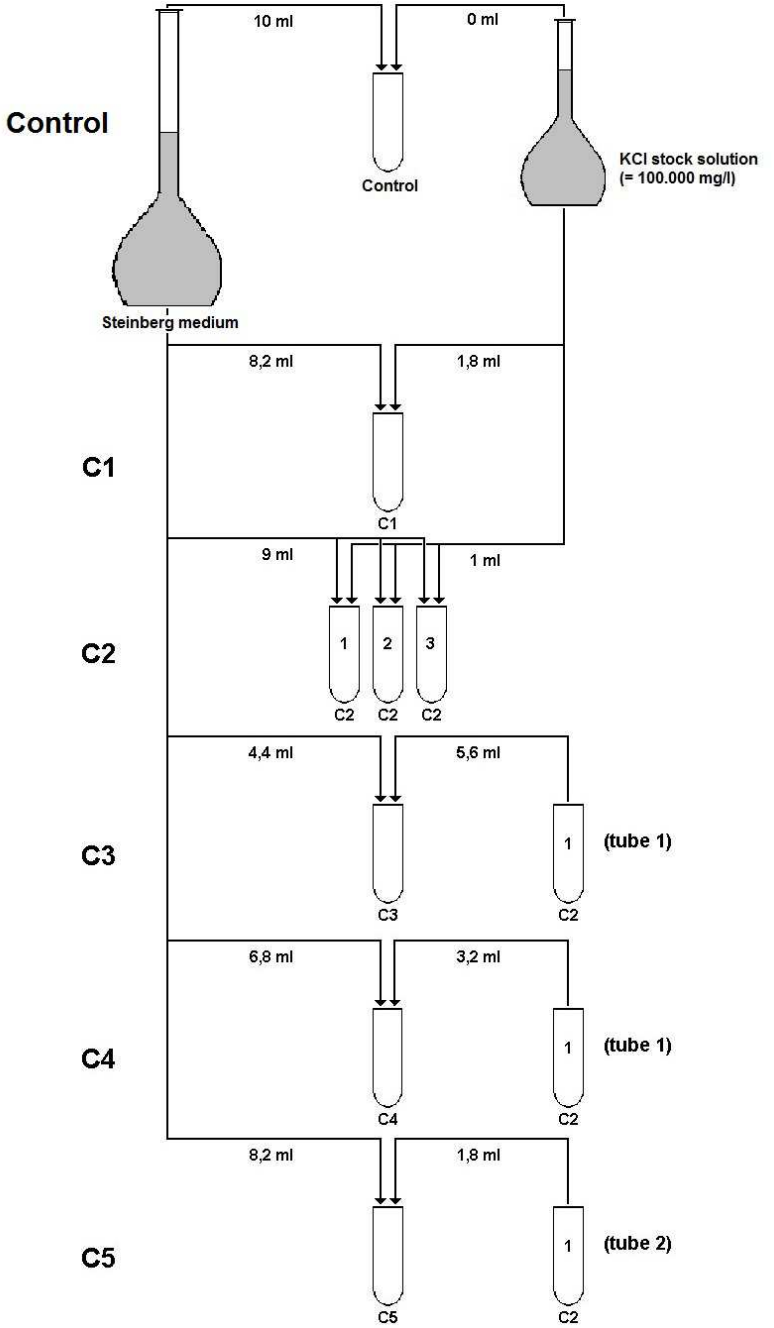
Table 4 displays the logaritmic concentrations versus % the inhibition of the growth of the first fronds.

In Table 5 the 72h EC50 with the 95% confidence limits is calculated.

VALIDITY CRITERIA FOR THE *SPIRODELA* DUCKWEED MICROBIOTEST

The "mean growth" of the first fronds in the cups of the control column after 3 days incubation at 25 °C and under 6 000 lux illumination (= the mean t72h - t0h area), must be at least **10 mm^2.**

FIGURE 7 : PREPARATION OF TOXICANT DILUTIONS FOR THE REFERENCE TEST



REFERENCE TEST

In order to check the correct execution of the test procedure and the good physiological condition of the growing duckweeds, it is advisable to perform a reference test from time to time.
Such a quality control test can e.g. be performed with one the two reference toxicants indicated in the ISO 20079 standard for *Lemna* tests : 3,5 dichlorophenol (DCP) or potassium chloride (KCl).

A description is given hereunder for a reference test with KCl.

PREPARATION OF THE TOXICANT SOLUTIONS

The reference test is carried out with the following KCl test concentrate-ions : 18 000 - 10 000 - 5 600 - 3 200 - 1 800 mg/l.

Procedure (see Figure 7)

1. Prepare a stock solution of 100 000 mg/l KCl by weighing 10 g KCl on an analytical balance.
2. Transfer the weighed amount of KCl into a calibrated 100 ml flask.
3. Fill the calibrated flask to the 100 ml mark with Steinberg medium (= KCl stock solution).
4. Stopper the flask and shake thoroughly till the salt is completely dissolved.
5. Take eight 10 ml test tubes and label them as C1, C2 (3 tubes), C3, C4, C5 and Control.
6. Put 10 ml Steinberg medium in the control tube.
7. Concentration C1 (= 18 000 mg/l) : Put 1,8 ml KCl stock solution (100 000 mg/l) in test tube C1 and add 8,2 ml growth medium.
8. Concentration C2 (= 10 000 mg/l) : Put 1 ml KCl stock solution in the 3 tubes labeled C2 and add 9 ml growth medium to each of them.
9. Use two of the three C2 tubes to prepare the C3, C4 and C5 test concentrations by adding the following volumes of C2 and of Steinberg medium to the tubes labeled C3, C4 and C5 :

Concentration C3 (= 5 600 mg/l) : 5,6 ml C2 + 4,4 ml growth medium
Concentration C4 (= 3 200 mg/l) : 3,2 ml C2 + 6,8 ml growth medium
Concentration C5 (= 1 800 mg/l) : 1,8 ml C2 + 8,2 ml growth medium

10. Proceed further as described in this Standard Operational Procedure manual for “Filling the test plate with the toxicant dilutions”, “Transfer of the germinated turions”, “Incubation of the test plate”, etc ...

Remark *: one will see that in the highest test concentration (18 000 mg KCl/l) the first fronds will not have grown during the 3 days exposure in this high concentration of the toxicant, and that they have lost their green color and are “whitish”.*
The area of the first fronds in the highest test concentration must nevertheless be measured and included in the data.

With the data obtained in the quality control test, calculate the 72h EC50. This EC50 value should be situated in the range stipulated in the Specification Sheet of the *Spirodela* duckweed Toxkit.

LIST OF TOXKIT MICROBIOTESTS

Tests for freshwater and soils

PROTOXKIT F : 24h reproduction inhibition test based on the ciliate protozoan *Tetrahymena thermophila*. This assay is under consideration as an OECD Guideline.

ROTOXKIT F : 24h mortality test, based on the rotifer *Brachionus calyciflorus*. This assay adheres to ASTM Standard Guide E1440-91.

ROTOXKIT F chronic : 48h reproduction inhibition test based on the rotifer *Brachionus calyciflorus*. This assay adheres to ISO norm 20666 and AFNOR norm T90-377.

THAMNOTOXKIT F : 24h mortality test, based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

CERIODAPHTOXKIT F : 24h mortality test, based on the cladoceran crustacean *Ceriodaphnia dubia*. This assay is in current practice in the USA as an EPA Method.

DAPHTOXKIT F: 24h-48h mobility inhibition test, based on the cladoceran crustacean *Daphnia magna*. This assay adheres to ISO norm 6341 and OECD Guideline 202.

OSTRACODTOXKIT F : 6 days chronic mortality and growth inhibition test with the ostracod crustacean *Heterocypris incongruens*. This assay adheres to ISO norm 14370.

RAPIDTOXKIT F Thamno: 30-60 min particle ingestion inhibition test based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

ALGALTOXKIT F : 72h growth inhibition test, based on the green alga *Selenastrum capricornutum* (presently named *Pseudokirchneriella subcapitata*). This assay adheres to ISO norm 8692 and OECD Guideline 201.

PHYTOTOXKIT solid samples: 3 days germination and root growth inhibition test with seeds of 3 higher plants.

PHYTOTOXKIT liquid samples: A short germination and root/shoot growth inhibition microbiotest for determination of the direct effect of chemicals on higher plants.

DUCKWEED TOXKIT F : 72h growth inhibition test with the duckweed species *Spirodela polyrhiza*.

Tests for estuarine/marine environments

ROTOXKIT M : 24h mortality test based on the rotifer *Brachionus plicatilis*. This assay adheres to ASTM Standard Guide E1440-91.

ARTOXKIT M : 24h mortality test based on the anostracan crustacean *Artemia salina* (renamed *Artemia franciscana*). This assay adheres to ASTM Standard Guide E1440-91.

ALGALTOXKIT M : 72h growth inhibition test based on the marine diatom *Phaeodactylum tricornutum*. This test adheres to ISO norm 10253.

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