REPORT ON THE THIRD INTERNATIONAL INTERLABORATORY COMPARISON OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

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EXECUTIVE SUMMARY

Detailed information on the development of the *Spirodela polyrhiza* microbiotest and its sensitivity to toxicants in comparison to duckweed toxicity tests with *Lemna* spp. is given in the "Report on the International Interlaboratory Comparison of the *Spirodela* duckweed microbiotest", which is accessible in the section "Publications – Extensive Reports" on the website www.microbiotests.be .

The former report also highlights the findings and the conclusions of the first and the second ringtests which were organized in 2013 and during the first half of 2014, and which have allowed to refine the test methodology and the selection of appropriate validity criteria.

Besides confirming the simplicity and the practicality of the test procedure, both International Interlaboratory Comparisons also clearly showed the high degree of reliability and robustness of the "stock culture independent" microbiotest with the duckweed *Spirodela polyrhiza*.

It was therefore concluded that a proposal could be submitted to ISO, for consideration of this toxicity test as an International Standard. Yet, this idea had to be postponed ! According to ISO 5725-1 (1994) requirements, supporting data for the accuracy of a new method must originate from laboratories "which have a previous experience with the application of the proposed test procedure".

This was, however, and unfortunately, not the case, because most of the participants in the first as well as in the second (extensive) ringtest were "first time users". It was therefore decided to organize a third International Interlaboratory Comparison with a (limited) number of laboratories which had participated in the second ringtest.

A call for participation was hence issued in May 2014 by Dr. Baudo of the I.S.E. - who kindly agreed to (again) coordinate the ringtest - to 10 laboratories from 10 different countries that had participated in the second ringtest. A detailed Operational Procedure was worked out for this third ringtest, based again on performance of one test with the reference chemical KCl.

The test kits were manufactured and sent by the company MicroBioTests Inc in the beginning of June 2014. As per the request of the organizer, the participants were asked to perform the test within one month of reception of the test kit.

This condition has been respected by all the 10 participating laboratories which all turned in their results to the organizer by mid July 2014.

Similarly to the second International Interlaboratory Comparison, Dr. Baudo made an extensive statistical analysis of the data, first by the linear regression approach, but also by the Hill model (with two different softwares).

Comparison of the data of the third ringtest with those of the second ringtest, revealed that, even with a limited previous experience with the *Spirodela* microbiotest, the 10 labs found consistent results, much better than the "first time users". In fact and contrary to the second ringtest, no outliers were detected in this third ringtest.

The summary of the data elaboration of the third ringtest shows that the EC50 estimates calculated according to the 3 applied statistical methods, provide different values, albeit that the differences are not statistically significant. The log regression calculation procedure has, as could be expected, the largest variability.

The outcome of this third ringtest again confirmed that the test procedure of the *Spirodela polyrhiza* microbiotest is simple, practical, reliable and robust, and that this "stock culture independent" assay can hence now be proposed to ISO for consideration as an International Standard for evaluation of the hazard of toxicants to floating higher water plants.

INTRODUCTION

An extensive Introduction on the development of the *Spirodela polyrhiza* microbiotest is given in the "Report on the International Interlaboratory Comparison on the *Spirodela* duckweed microbiotest" which can be found in the section "Publications – Extensive Reports" on the website www.microbiotests.be .

The simple and practical test procedure which was eventually worked out for this "stock culture independent" microbiotest is based on the following steps:

1. Three days germination of the turions in a small Petri dish, in Steinberg medium, at 25°C and with continuous illumination (6.000 lux).
2. Transfer of the germinated turions to a 6 x 8 cup multiwell, of which the cups are filled with the toxicant solutions prepared with Steinberg medium, and in which one germinated turion is placed per cup.
3. Shooting of a photo of the multiwell with a digital camera, at the start of the toxicity test (= t0h).
4. Incubation of the multiwell for 3 days at 25°C with continuous illumination (6.000 lux).
5. Shooting of a photo of the multiwell with a digital camera, at the end of the incubation period (= t72h).
6. Transfer of the photos to a computer file.
7. Measurement in each cup of the area of the first frond of the germinated turions, with an Image Analysis programme (such as e.g. Image J).
8. Calculation of the growth of the first frond for the 8 replicates in each test row after 72h incubation of the germinated turions (= t72h – t0h area values).
9. Calculation of the % inhibition of the growth of the first frond in each test concentration versus the growth in the control.
10. Calculation of the 72h EC50 with an appropriate statistical programme.

ORGANIZATION OF A THIRD INTERNATIONAL INTERLABORATORY COMPARISON ON THE *SPIRODELA* DUCKWEED MICROBIOTEST

During the summer of 2013, a preliminary ringtest has been organized of which the effect parameter (at that time) was "the final size" of the first fronds at the end of the 3 days exposure.

Six laboratories from 6 different countries participated in this first ringtest with the reference chemical KCl.

The 72h EC50 of this first comparative exercise (as calculated by linear regression) was 6 593 mg/l KCl, with a variation coefficient of 6,2%.

The test procedure of the *Spirodela* microbiotest was subsequently adapted and "growth" was selected as the effect criterion.

A second (extensive) International Interlaboratory Comparison has then been launched in early 2014, with the same reference chemical, and 56 laboratories from 22 countries participated in this ringtest..

The outcome of the second International Interlaboratory Comparison confirmed the practicality and the robustness of the new microbiotest, with a mean 72h EC50 of 5 879 mg/l KCl (as calculated by linear regression) and a variation coefficient of 22%.

Taking into account the numerous assets of the stock culture independent *Spirodela polyrhiza* microbiotest in comparison to the duckweed toxicity tests with *Lemna* spp., it was eventually decided to propose this new microbiotest to ISO, as an interesting additional toxicity test with duckweeds, for the evaluation of the hazard of toxicants in water to higher aquatic plants.

However, according to ISO 5725-1(1994)[11] requirements, supporting data for the accuracy of the proposed method must originate from laboratories "which have a previous experience with the application of the proposed test procedure".

Since this was not the case with most of the participants in the second ringtest (which were indeed "first time users"), it was decided to organize a third International Interlaboratory Comparison, with a (limited) number of laboratories which had participated in the second ringtest.

A call for participation was hence issued in May 2014 to 10 laboratories from 10 different countries which participated in the 2nd International Interlaboratory Comparison.

It was stipulated in the invitation that no subscription charge would be asked, that the participants would receive free of charge a kit containing the materials to perform one assay on the reference chemical KCl, but that they had to send their results within 1 month of reception of the kit.

Dr. Baudo of the Istituto per lo Studio degli Ecosistemi (I.S.E), Verbana, Pallanza in Italy kindly agreed to also coordinate the third *Spirodela* ringtest and to perform the statistical analysis of the results.

An "Operational Procedure" was again worked out describing in detail the experimental procedure, including the preparation of the toxicant dilution series of the reference chemical.

A request was made by I.S.E. to the company MicroBioTests to work out a model kit for the *Spirodela* International Interlaboratory Comparison, containing all the materials for performance of two complete tests (the second test only to be performed in case of problems with the first assay).

The company MicroBioTests was then asked to prepare the test kits for all the participants and to send them at the timing indicated by the I.S.E.

PARTICIPATION IN THE THIRD INTERNATIONAL INTERLABORATORY COMPARISON OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

The ten participating laboratories, institutes, organisations and companies are given hereunder, per country.

Belgium	• Institut Scientifique de Service Public ISSEP – Liège • MicroBioTests Inc. – Mariakerke (Ghent)
Estonia	• National Institute of Chemical Physics and Biophysics – Tallinn
France	• Groupe CARSO, Laboratoire Ecotoxicologie– Lyon Cedex 07
Greece	• Agricultural University of Athens, Laboratory of Ecology and Environmental Sciences – Athens
Italy	• Ecobioqual – Torino
Lithuania	• Institute of Botany at Nature Research Centre – Vilnius
Poland	• Medical University of Warsaw, Dept. of Environmental Health Sciences, Faculty of Pharmacy – Warsaw
Romania	• Ovidius University of Constanta, Dept. of Natural Sciences, Faculty of Natural and Agricultural Sciences – Constanta
Spain	• Xenobiotics, S.L. University of Valencia Science Park (PCUV) – Paterna, Valencia

[1] ISO 5725-1:1994/TECHNICAL CORRIGENDUM 1:1998. Accuracy (trueness and precision) of measurement methods and results -- Part 1: General principles and definitions

TEST PROTOCOL OF THE THIRD INTERNATIONAL INTERLABORATORY COMPARISON OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

The detailed test protocol which was sent to all those participating in the *Spirodela* ringtest is added in Annex 1 of this Report.

The test kit also contained a USB stick with the "Image J"[2] analysis programme for measurement of the areas of the first frond, and an Excel programme and Results sheets for the data storage and treatment and calculation of the EC50.

SHIPMENT OF THE TEST KITS FOR THE INTERNATIONAL INTERLABORATORY COMPARISON OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

All the test kits were prepared in MicroBioTests and sent to the 10 participants the first week of June 2014 by courier service.

According to the conditions for participation, this meant that the results of the test had to be sent to the organizer by mid July 2014 "at the latest".

RESULTS OF THE THIRD INTERNATIONAL INTERLABORATORY COMPARISON OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

All 10 participants performed the prescribed *Spirodela* test and sent their results. Four labs repeated a second time the test, so that eventually a total of 14 data sets have been received.

In order "to put the same weight" on the results of each participating laboratory for the EC50 estimate, it was decided to make the statistical analysis (and the discussions) on "only one" result from each participant, i.e. 10 data sets.

For this International Interlaboratory Comparison the considered endpoint has been the EC50 at 72 h.

MEASUREMENTS

As indicated in the test protocol (see Annex I), the participants were requested to measure the area of the germinated turions in each test plate (with the aid of the Image J program) at the start of the toxicity test (= time t0h) and after 72 h exposure (t72h). The results have to be filled out on specific Excel Result Sheets, and sent by e-mail to the organiser.

DATA TREATMENT

The Excel sheet automatically calculates the EC50 by linear regression of the turions growth (area at time 72 h minus the corresponding area at time 0 h) versus the log concentrations. This calculation is easily done by using the built-in formulas of the Excel sheet.

Statistical analysis

It was stipulated by the organiser that the statistical analysis of all the data would be made by I.S.E.

The data sheet used for this International Interlaboratory Comparison allows to compute the regression of the mean % inhibition versus the log concentrations, by using the Excel built-in data regression. However, this calculation does not give "confidence limits".

[2] NB : Image J is a public domain Java based image processing program developed in 1997 at the National Institutes of Health in the USA. Image J can be easily downloaded from the Internet.

It was therefore decided to make a recalculation of the 72h EC50 values, by using the original % inhibition data, instead of the mean % inhibition, versus the log transformation of the concentration data. This procedure, which uses more than one value of Y for each X (log concentration)[3], provides practically identical results for the EC50 values as those of the Excel regression, but in addition gives the upper and lower 95 % confidence limits.

Therefore, these results for the log regression EC50 estimates have been used in the following data elaboration, instead of the Excel log regression.

However, a statistical approach using linear regression may be not the best estimate of the EC50 and its 95 % confidence limits of toxicity tests based on "growth reduction".

It was therefore decided to make additional calculation by using the Hill model, according to the suggestion of the ISO/TS 20281[4]. For this calculation, the data of the "growth" area have been used, instead of the % of inhibition (statistically this transformation leads to non-independent data, since each value takes into accounts both the area at 72 h and the mean value of the negative control).

This type of calculation requires a specific software, such as the Benchmark Dose Software (BMDS) provided by USEPA, freely downloadable from http://www.epa.gov/ncea/bmds/, or the Macro Regtox, freely downloadable from http://www.normalesup.org/~vindimian/en_download.html.
The statistical analysis furthermore produced the repeatability and reproducibility of this International Interlaboratory Comparison, according to the ISO 5725-2[5] procedure. The data treatment is reported in Annex 2.

VALIDITY CRITERION FOR THE REFERENCE TEST WITH KCl

For the reference test to be valid, the mean area of the first fronds in the cups of the control row after 3 days incubation (t72h) at 25°C and under 6 000 lux illumination must be ≥ 10 mm^2.

The 10 results all respect this validity criterion.

REPEATABILITY AND REPRODUCIBILITY

The statistical analysis for the repeatability and reproducibility of this International Interlaboratory Comparison follows the ISO 5725-2 (2002) procedure. The data treatment is explained in Annex 3.

This statistical analysis has been made both for the estimates of the EC50 obtained by linear regression of the % inhibition of the turions growth (area at time 72 h minus the corresponding area at time 0 h) over the log transformed concentrations data (in the following called log regression), and for those computed from the same data, but by using the Hill model (both with the BMDS software and the Regtox macro).

Statistical treatment of ecotoxicity data can be made with a variety of data treatment programmes and different users will in the future apply different models, that very likely will produce EC50 estimates which can be quite different !

However, if the users, in addition to their own model, will apply either the log-regression, or the Hill model, they will be still able to compare their results (for the reference toxicant KCl) with the repeatability and reproducibility figures of this International Interlaboratory Comparison given in this Report.

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[3] R.R. Sokal and F.J. Rohlf. 2009. Introduction to biostatistics, 2nd Edition. Dover ed.
[4] ISO/TS 20281: 2006, Water quality – Guidance document on the statistical analysis of ecotoxicity data
[5] ISO 5725-2:1994/Cor 1:2002. Accuracy (trueness and precision) of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

Log-regression EC50

The EC50 data calculated with this model for the 10 labs have been summarized in figure 2, as histograms ordered from the lowest to the highest average of each laboratory, and the data on repeatability and reproducibility are reported in table 1. Since there are no outliers, nor stragglers, "all data" values only are reported.

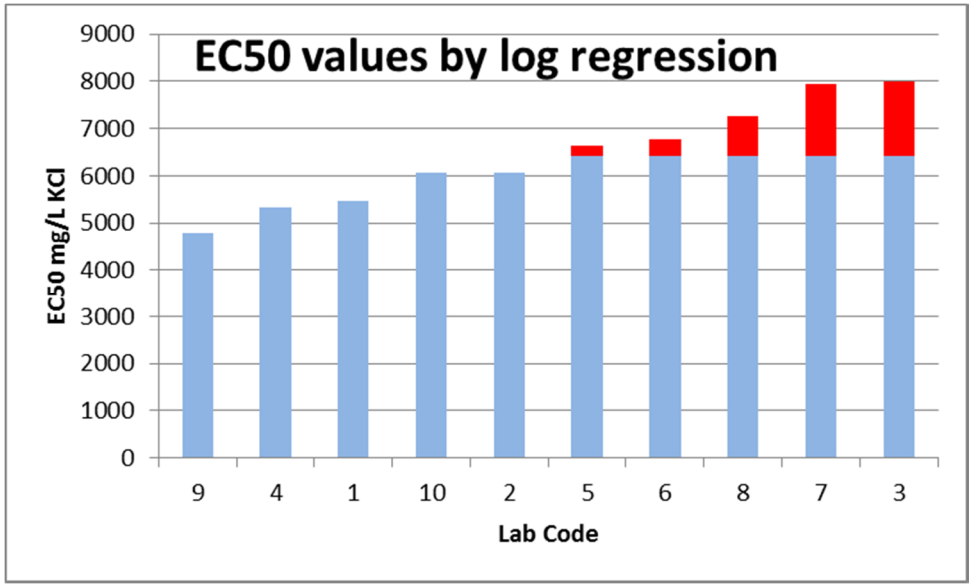


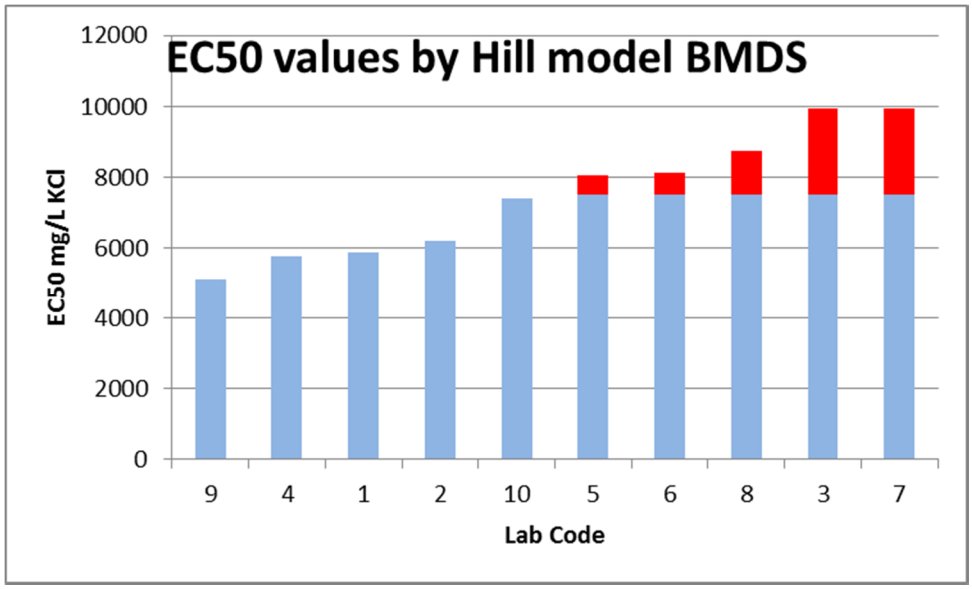
Fig. 2 – EC50 (log regression) from 10 labs, in increasing order (results above the overall mean in different colour)

Tab. 1 – Mean EC50 values estimated by log regression and their 95 % upper (UCL) and lower confidence limits (LCL). Since there are no outliers, nor stragglers, "all data" values only are reported.

	All data
n lab	10
Mean	6421
s_r	4758
CV%	74.1
s_R	4758
CV%	74
h straggler	0
h outlier	0
k straggler	0
k outlier	0
95 % UCL	15746
95 % LCL	0

Hill model EC50 BMDS

The EC50 data calculated with this model by the BMDS software for the 10 labs have been summarized in figure 3, as histograms ordered from the lowest to the highest average of each laboratory, and the data on repeatability and reproducibility are reported in table 2.



Fig, 3– EC50 (Hill model BMDS) from 10 labs, in increasing order (results above the overall mean in a different colour).

Tab. 2 – Mean EC50 values estimated by the Hill model (BMDS) and their 95 % upper (UCL) and lower confidence limits (LCL).

	All data	Without h and k stragglers
n lab	10	9
Mean	7502	7433
s_r	1233	1153
CV%	16.4	15.5
s_R	2085	2122
CV%	28	28.6
h straggler	0	0
h outlier	0	0
k straggler	1	0
k outlier	0	0
95 % UCL	11588	11593
95 % LCL	3415	3273

Also using this model the labs seem to perform quite well, with results very close to each other; in fact, no outliers have been identified, and only one straggler (Lab. 6).

Hill model EC50 Macro Regtox

The EC50 data calculated with the Macro Regtox for the 10 labs have been summarized in figure 4, as histograms ordered from the lowest to the highest average of each laboratory, and the data on repeatability and reproducibility are reported in table 3.

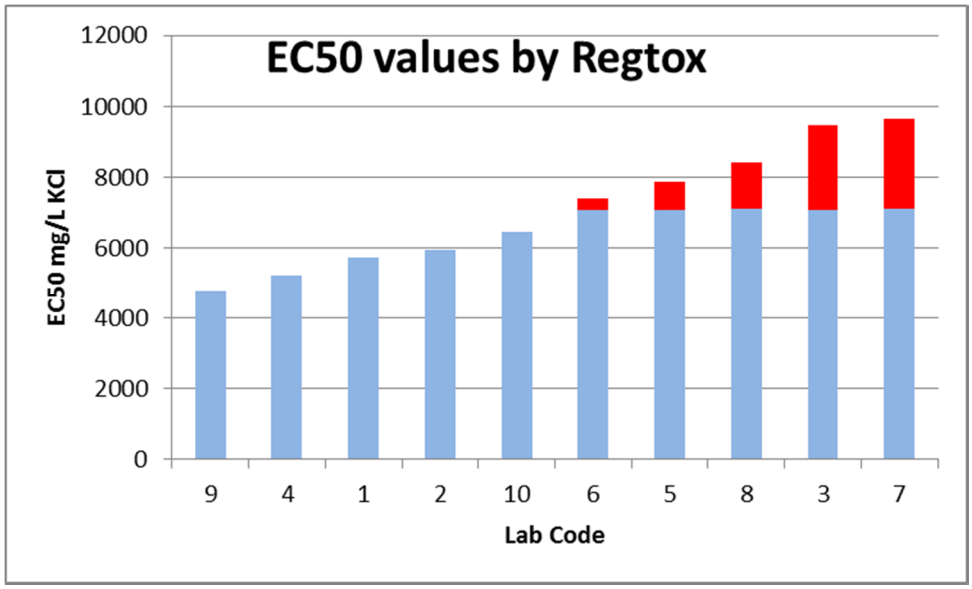


Fig. 4 - EC50 (Hill model Macro Regtox) from 10 labs, in increasing order (results above the overall mean in a different colour)

Tab. 3 – Mean EC50 values estimated by the Hill model Macro Regtox) and their 95 % upper (UCL) and lower confidence limits (LCL).

	All data	Without h and k stragglers
n lab	10	7
Mean	7078	7017
s_r	1671	1333
CV%	23.6	19.0
s_R	2334	2423
CV%	33.0	34.5
h straggler	0	0
h outlier	0	0
k straggler	1	0
k outlier	0	0
95 % UCL	11652	11765
95 % LCL	2504	2268

The Macro Regtox produced comparable data, with no outliers and three stragglers (Labs. 5, 5, and 10).

Summarizing this data elaboration (Tab. 4), it is evident that the three EC50 estimates provide different values, albeit the differences are not statistically significant. However, as expected, the log regression has a larger variability. Conversely, the Hill model consistently produces less variable estimates, both using the BMDS software and the Macro Regtox. As a consequence, the estimates present a reduced variability in both the intra- and interlaboratory variability.

Tab. 4 – Synthesis of the EC50 data elaboration

	Total Labs	h and k outliers	EC50	95 % LCL	95 % UCL	s_r	CV_r%	s_R	CV_R%
log regression	10	0	6421	0	15746	4758	74.1	4758	74.1
Hill model BMDS	10	0	7502	3415	11588	1233	16.4	2085	27.8
Macro Regtox	10	0	7078	2504	11652	1671	23.6	2334	33.0

Conclusions

This interlaboratory comparison points out that, even with a limited previous experience of the *Spirodela* test, all labs found consistent results, much better than with "first time users". In fact, no outliers were detected in this third ringtest, whereas in the previous one some were identified.

Moreover, the statistical data treatment confirm that the EC50 estimates depend on the model used for the calculation.

A comparison of the three ringtest (Tab. 5) however indicates that, using the same model, the *Spirodela* test is very reliable.

Tab. 5 – Comparison between three ringtests.

	1st ringtest	2nd ringtest	3rd ringtest
N of labs	6	52	10
EC50 Excel	6593	5879	6340
CV %	6.2	22.3	15.6
EC50 log reg		5932	6421
CV %		22.4	17.0
EC50 BMDS		6320	7502
CV %		26.5	23.2
EC50 Regtox			7078
CV %			24.5

The conclusion of this third ringtest is (again) that the test procedure of the *Spirodela polyrhiza* microbiotest is reliable and robust.

This stock culture independent *Spirodela polyrhiza* microbiotest can therefore be proposed to ISO, as a sensitive, simple and practical additional toxicity test with a duckweed species.

Annex 1
PROCEDURE FOR THE PERFORMANCE OF A REFERENCE TEST WITH KCl

CONTENTS OF THE KIT FOR PERFORMING THE REFERENCE TEST

- 2 tubes with *Spirodela polyrhiza* turions
- 1 microsieve
- 2 Petri dishes
- 1 small spatula
- 2 multiwell plates (48 cups)
- 1 set of 5 vials with concentrated salt solutions to prepare the test and dilution medium (Steinberg medium)
- 1 vial with stock solution of the test chemical KCl (100 000 mg/l)
- 8 test tubes (10 ml)
- 1 USB stick with the test protocol, the Image J programme and the Excel file for the data treatment

N.B : The tubes with the turions and the vials with the solutions must be stored in the refrigerator prior to use.

PREPARATION OF DUCKWEED GROWTH AND TEST DILUTION MEDIUM

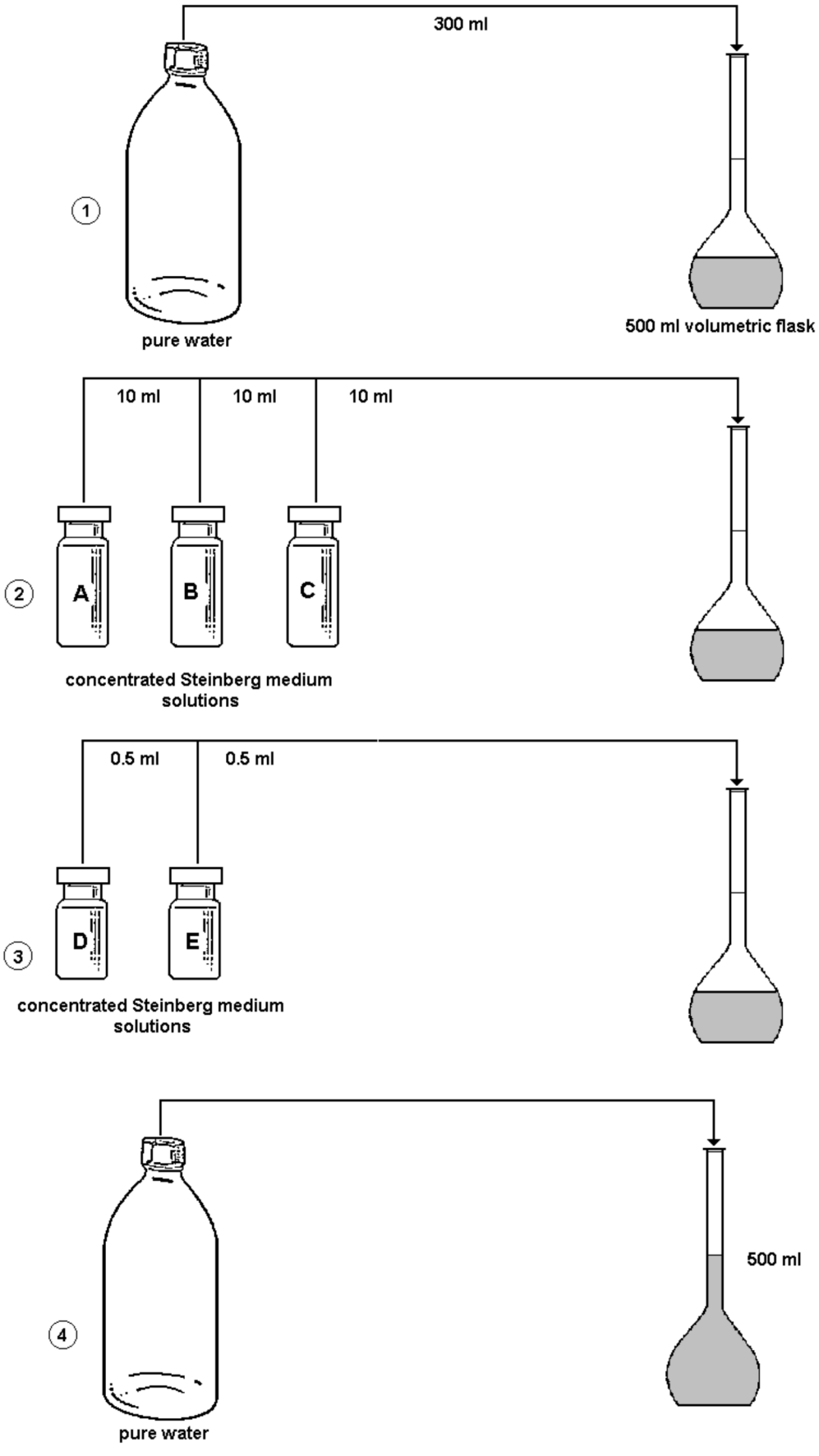
This medium will be used for the germination of the *Spirodela* turions and as growth medium for the duckweeds and dilution medium for the toxicants in the toxicity test. The composition of this medium is that of the "Steinberg medium" prescribed by ISO for *Lemna* toxicity tests (ISO 20079).

Procedure (see Figure 1)

1. Transfer 300 ml pure water (e.g. 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 therefore be prepared with the concentrated solutions from the 5 bottles, at the time of performance of the second toxicity test (if needed).

FIGURE 1 : PREPARATION OF DUCKWEED GROWTH AND TEST DILUTION MEDIUM



GERMINATION OF THE *SPIRODELA POLYRHIZA* TURIONS

1. Take a tube with *Spirodela polyrhiza* turions and shake it slightly to resuspend the turions.
2. Pour the contents of the tube in the microsieve to remove the storage medium. Make sure that all the turions are transferred to the microsieve.
3. Put 10 ml Steinberg medium in the 10 cm Petri dish.
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. Make sure that all the turions are transferred in the Petri dish.
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 the prescribed temperature and illumination. It is therefore most important that the prescribed values (25°C and 6 000 lux) be respected "as closely as possible" !

PREPARATION OF THE TOXICANT DILUTION SERIES

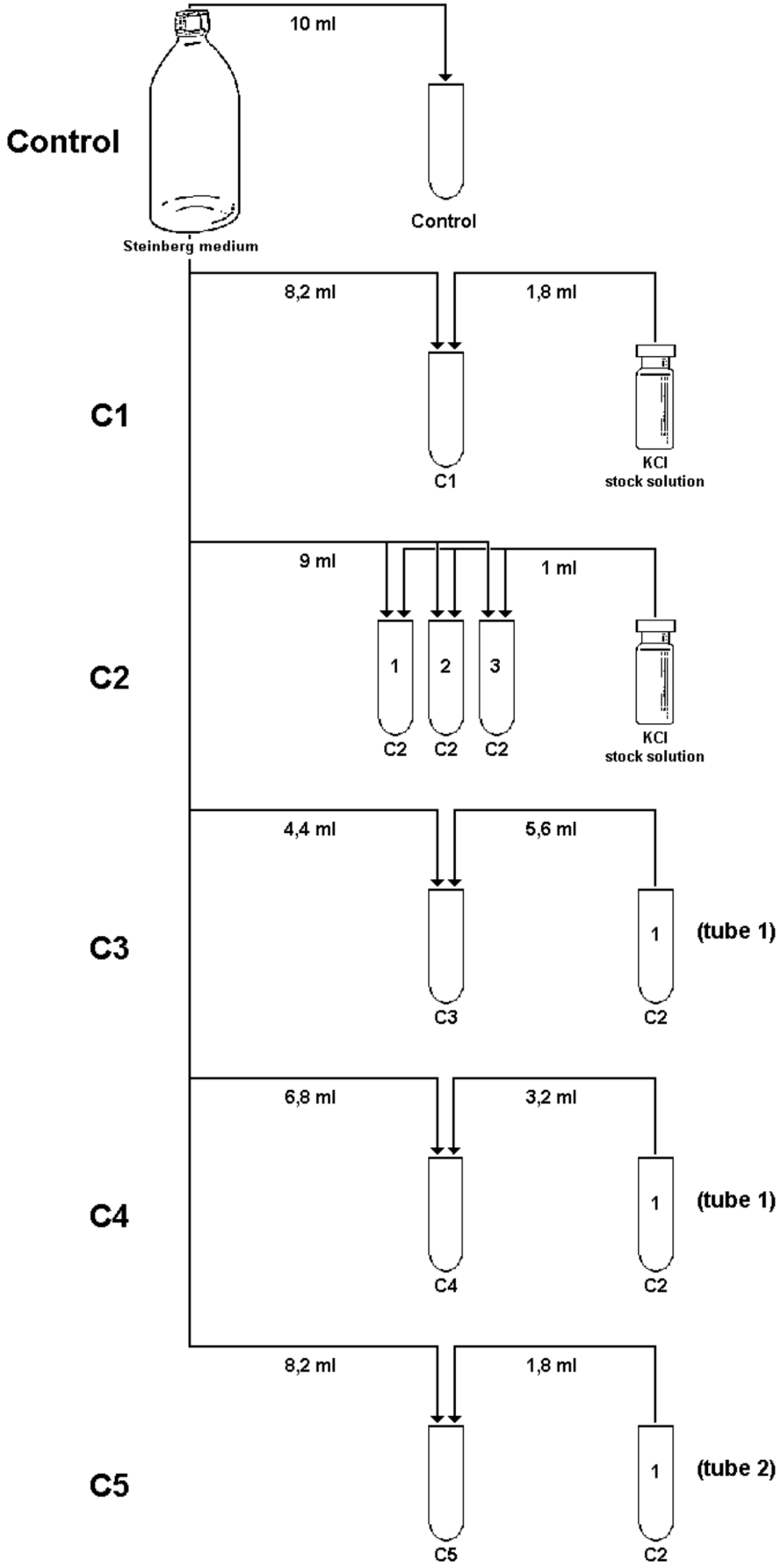
The interlaboratory comparison test will be carried out with the following 5 concentrations of KCl : 18 000 – 10 000 – 5 600 – 3 200 – 1 800 mg/l.

Procedure (see Figure 2)

1. Take the 8 test tubes and label them as C1, C2 (3 tubes), C3, C4, C5 and Control
2. Put 10 ml Steinberg medium in the Control tube.
3. 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 Steinberg medium
4. Concentration C2 (= 10 000 mg/l) : Put 1 ml KCl stock solution in the 3 tubes labelled C2 and add 9 ml Steinberg medium to each of them
5. 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 labelled C3, C4 and C5 :

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

FIGURE 2 : PREPARATION OF TOXICANT DILUTIONS



FILLING OF THE TEST PLATE WITH THE TOXICANT DILUTIONS

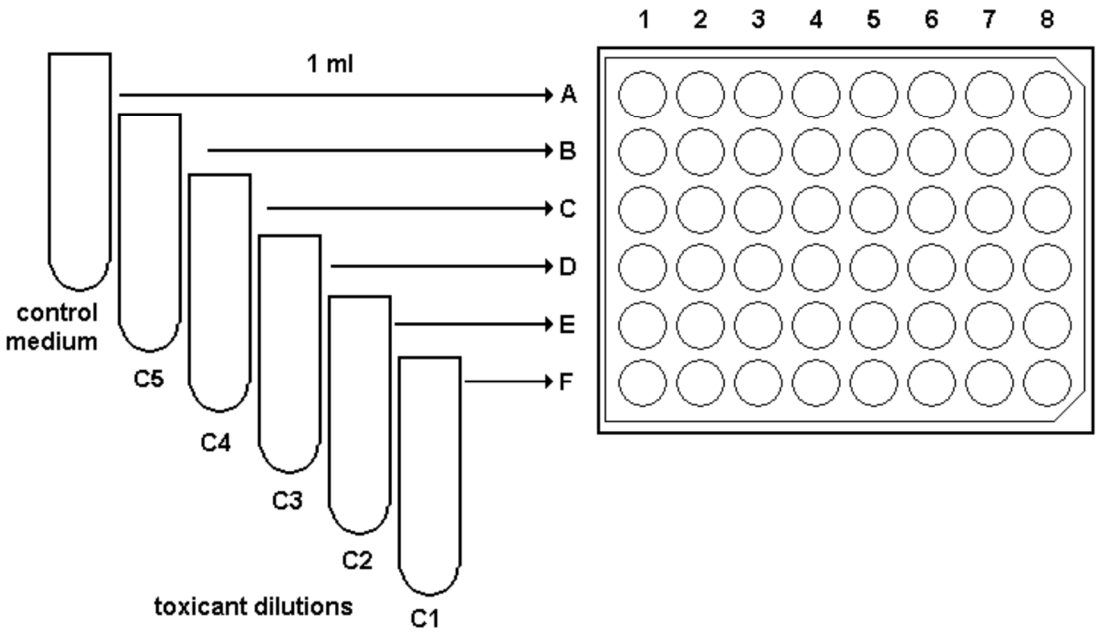
Each toxicant dilution has to be transferred into all the 8 cups of one row in the multiwell plate. The rows are labelled from A to F and the cups from 1 to 8.

The distribution of the test solutions must 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 toxicant concentration in the bottom row (= row F).

Procedure (see Figure 3)

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 3 : FILLING OF THE TEST PLATE WITH THE TOXICANT DILUTIONS



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.

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

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) up to the row with the highest test concentration (C1 in row F).

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).

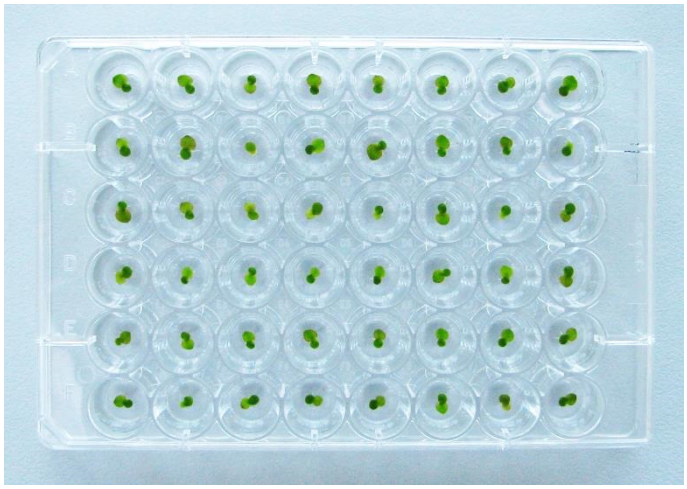


Photo of a multiwell plate with the germinated turions and their small first fronds (at t0h)

NB : 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 !

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

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” !

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

A digital photo of the multiwell plate containing the grown first fronds has to be taken again at the end of the 3 days toxicity test.

Procedure

1. Take the multiwell plate out of the incubator and remove the lid.
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.

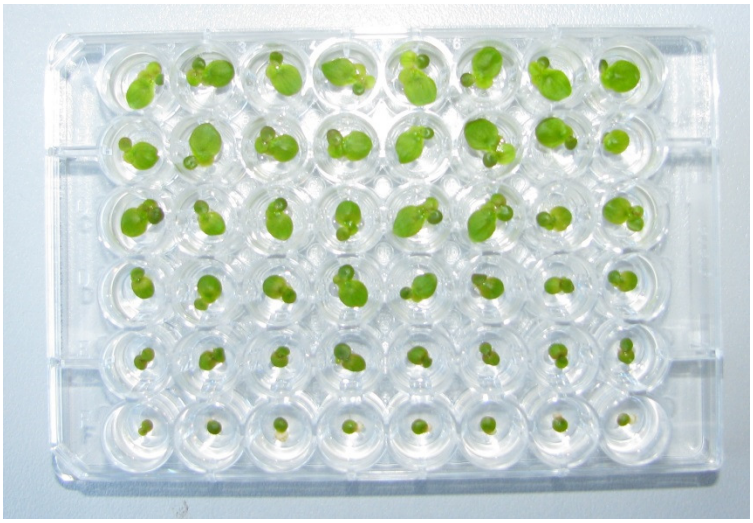


Photo of the multiwell plate with the grown first fronds, after 3 days incubation (t72h).

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 “Image Analysis” programme, 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”.

N.B : The Image J programme and the Excel programme for the data treatment are provided on the USB stick which is included in the kit. Both these programmes have to be transferred to a file on your own computer for their subsequent use.

IMPORTANT REMARK

The Excel programme which will process the results of the area measurements will only work properly if the area data of the first fronds (as measured with the Image J programme) are transferred to this Excel programme with a “decimal point” i.e. “a dot” (.) and not as a “comma” (,).

Depending of the computer, the configuration for the decimal has been set either with a dot or with a comma ! In case the decimal setting in your computer is “with a comma”, you have first to change yourself the comma setting to the dot setting, via the control panel of your computer.

For Windows XP

- Click on “Start”
- Click on “Control Panel”
- Click on “Country settings”
- Click on “Adapt”
- Click on “Numbers” and make sure that the decimal symbol is set as a “dot”(.)
- Click on “Execute”
- Click on “OK”

For Windows Vista, Windows 7, etc.

- Click on “Start”
- Click on “Control panel”

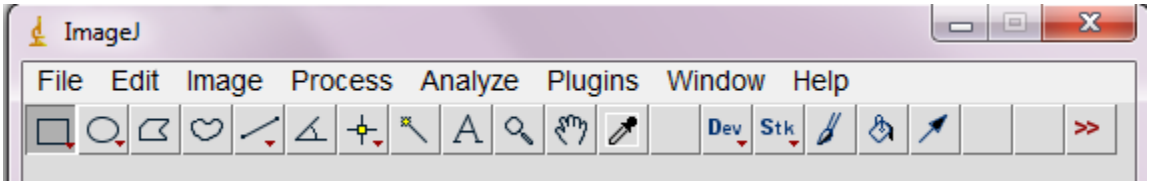
- Click on “Clock, Language and Region”
- Click on “Additional settings”
- Click on “Numbers” and make sure that the decimal symbol is set as a “dot”(.)
- Click on “Apply”
- Click on “OK”

IMAGE J Procedure for measurement of the area of the first frond

As indicated above, these 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” fronds at the end of the 3 days toxicity test.(t72h)

Access to the photo of the multiwell plate

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



2. Go to File and click on Open image
3. Select the Directory and click on the file with the saved photo of the multiwell plate.

Calibration of the measurements

1. Click on 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 on Analyze and on 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 on OK (this will subsequently automatically make the calculation of the areas in mm^2).

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).

Measurement of the “contour” of the first frond in the cups

1. Click on 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 on 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 performed “at the end of the test” one will see that in some cups a second frond has also already developed from the germinated turion. Only the largest of these 2 fronds shall be measured.

3. Click simultaneously on Ctrl and on 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 the first frond.
5. Save the result by clicking on Ctrl and M.
6. Proceed further with the measurements of the first fronds in the other 6 cups of the control row.

Saving of the area measurements

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 the control row), and click on all the values (which will then show up on a black background).
2. Click right on the mouse and click on Copy.
3. Open the Excel file named “*Spirodela* microbiotest” and go to the first page “Area measurements : Initial area first fronds (t0h)”.
4. Click on “Paste here” in the box “Control” to transfer the 8 data to this box.
5. Go back to the Results box in the Image J programme (showing the data on the black background)
6. Click on Edit and on 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 box C5 (1 800 mg/l KCl) of the Excel sheet “Area Measurements : initial area first fronds (t0h)”
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 box of the Excel sheet “Area measurements : Initial area first fronds (t0h).
9. Save the Excel file with the area data, and give it a name (e.g. ringtest *Spirodela* KCl test)

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

The procedure for the area measurements is similar to that of the area measurements at the start of the toxicity test.

For the saving of the measured areas, open the (saved) Excel file, go to “page 2” (Area measurements : Final area first frond (t72h) and paste the area data for each row in the corresponding boxes.

The file has then to be saved again (under the same name).

Important 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 to this high concentration of the toxicant, and that they have lost their green colour and are “whitish”.

The areas of the first fronds in the highest test concentration must nevertheless be measured and saved in the corresponding box.

Data treatment

1. Open the saved Excel file with the measured areas at the start and at the end of the toxicity test and open page 3 : “Data treatment”.
2. Fill out on the top of the sheet the name of the operator, the Institute, laboratory or company and the date of performance of the test.

The Data Treatment page will contain in Table 1 and in Table 2 the data saved and shown in page 1 and page 2 of the Result sheets (initial versus final areas of the first fronds), and will show the calculated mean value for the 8 replicates in each row, with the Standard Deviation (STDEV) and Variation Coefficient (CV%).

The Data treatment page will show in Table 3 for each test cup “the growth” of the first fronds in the 6 rows of the multiwell plate. 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 displays “the mean growth” (in mm2) of the first fronds for the 8 replicates in the control row and in the rows with the 5 toxicant concentrations, with the Standard Deviation (STDEV) and the variation coefficient (CV%) for the individual area measurements.

In addition Table 3 also shows the calculated % inhibition of the growth of the first fronds in the 5 test concentrations versus the control.

Table 4 displays the % inhibition of the growth of the first fronds in the test concentrations (in mg/l) and in log values.

Calculation of the 72h EC50

This calculation is performed with the aid of a specific programme which is included in the Excel “*Spirodela* microbiotest” file, but which must be opened and operated using the Data Analysis command available in Excel.

1. Click on the icon “Data” (or Tools) on top of the Excel sheet
2. Click on the icon “Data Analysis” to open a “Data analysis” box Data Analysis (Analysis Toolpack) is an add-in for Microsoft Excel which is disabled by default. To enable it, follow the instructions of the version of Excel you are using. For Excel 2010, you can visit :http://www.addictivetips.com/windows-tips/excel-2010-data-analysis/).
3. In the Data analysis box, look for and click on “Regression”
4. Click on OK – This will open a “Regression box”.
5. Go to Table 4 and click on all the inhibition (I%) percentages values to fill the “input Y range (D68:D72) in the Regression box.
6. Go to the “input X range” in the Regression box and click on the log concentration values in Table 4 to fill the “input X range” (C68:C72) in the Regression box.
7. Go to “Summary Output” in the Regression box and type in the Output range value A90 indicated on the right side of Table 4.
8. Click on OK.

The Excel programme will calculate the 72h EC50 and show this value in Table 5.

N.B The Excel programme calculates the EC50 according regression of the % inhibition versus the log concentrations. The organizer of the ring test will, however, also apply other EC50 calculation methods to the data submitted by the participants.

VALIDITY CRITERION FOR THE REFERENCE TEST WITH KCl

For the reference test to be valid, the following criterion must be fulfilled:

The mean growth of the first fronds in the cups of the control row after 3 days incubation must be $\geq$ 10 mm^2.

In case this validity condition is not fulfilled, a second test must be performed (with the second set of materials provided in the kit).

The Excel sheet with the test results has to be sent to Dr Renato Baudo (r.baudo@ise.cnr.it) who will perform the statistical analysis of the data of all the participants in the *Spirodela* ringtest.

Important remark : even if a second test has to be performed, the sheets with the data of the first test shall also be sent to the organizer. This can indeed still be helpful for the final evaluation of the ringtest.

Annex 2
STATISTICAL ANALYSIS

It was agreed with the participants that their results would be treated confidentially without mentioning names of the participating laboratories, organisations, institutes and companies in the presentation and discussion of the results. Therefore, in the following each laboratory is identified only by a randomly given code (the same for all data set).

The repeatability and reproducibility of the interlaboratory comparison have been calculated according to the ISO 5725-2 (2002)[6] procedure, providing the following results:

s_L^2 the estimate of the between-laboratory variance;

s_W^2 the estimate of the within-laboratory variance;

s_r^2 the arithmetic mean of the within-laboratory variances (after outliers have been excluded);

s_R^2 the estimate of the reproducibility variance: $s_R^2 = s_L^2 + s_r^2$.

To check the consistency of the data, the Mandel's h and k statistics have been used: the first (h) provides the between-laboratory consistency statistic, and the second (k) the within-laboratory consistency statistic.

The Grubb's test has then been applied to identify stragglers (if the test statistic is greater than its 5 % critical value and less than or equal to its 1 % critical value, the item tested is called a straggler and is indicated by a single asterisk), and outliers (if the test statistic is greater than its 1 % critical value, the item is called a statistical outlier and is indicated by a double asterisk).

If some straggler and/or outlier can be explained by a technical error, for example in transcribing a test result, after the proper correction the data is retained and the Grubb's test repeated. If it proves impossible to replace the suspect test result, then it should be discarded as a "genuine" outlier, while stragglers are retained as correct items. Therefore, the final statistics of this interlaboratory comparison, to be used in conclusions, include stragglers, but not outliers.

The tables show the statistical analysis, for all data and the same without h or k outliers (data higher than overall mean + 3 times the interlaboratory standard deviation s_R, or lower than overall mean - 3 times the interlaboratory standard deviation s_R), and without h or k stragglers (data higher than overall mean + 2 times the interlaboratory standard deviation s_R, or lower than overall mean - 2 times the interlaboratory standard deviation s_R).

[6] ISO 5725-2:1994/Cor 1:2002. Accuracy (trueness and precision) of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method