

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

Renato Baudo

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EXECUTIVE SUMMARY

In aquatic ecosystems there are only a few standard methods to assess the hazard of toxicants to higher plants.

The toxicity test with the duckweeds *Lemna minor* or *Lemna gibba* is presently the only standard method with a floating aquatic macrophyte and this assay is in regular use in the EU for the authorization of Plant protection products (herbicides and plant regulators).

Lemna bioassays are dependent on the culturing/maintenance of live stocks, they require substantial bench and incubation space, and they are hence quite costly.

In view of these handicaps, joint research was initiated about 5 years ago by the Laboratory of Ecology and Environmental Sciences at the Agricultural University of Athens in Greece, and the company MicroBioTests Inc. in Mariakerke/Ghent in Belgium to try to develop a practical and low cost microbiotest with an aquatic macrophyte.

This research led to the selection of *Spirodela polyrhiza* which is one of the very few duckweed species which produce "dormant vegetative buds (turions)".

Extensive experimental work has been performed on the biological and technical aspects for the production of turions of this species and on the storage medium and storage conditions for subsequent successful germination.

A methodology was then developed for a "stock culture free" microbiotest with *Spirodela polyrhiza*, departing from stored and germinated turions.

The test procedure consists of 3 days germination of the turions in a Petri dish, in Steinberg medium, and at 25°C with 6.000 lux continuous illumination, followed by transfer of the germinated turions in the cups of a 6 x 4 multiwell containing 5 toxicant concentrations prepared in Steinberg medium. The multiwell is incubated for 72h at 25°C with 6.000 lux continuous illumination, after which a photo is taken from the multiwell with a digital camera and transferred to a computer. The areas of the first fronds are then measured by Image Analysis and the % inhibition of the size of the first fronds in the toxicant concentrations is calculated versus their size in the controls. Data treatment and calculation of the 72h EC50 is performed on specific Excel sheets.

Sensitivity comparisons of the duckweed microbiotest with the conventional *Lemna* assay were made on the reference chemicals 3,5 DCP and KCl, and on 18 inorganic and organic chemicals, and revealed that the new duckweed test was as sensitive as the traditional *Lemna* assay.

In order to evaluate the ease of performance and the practicality of the *Spirodela* microbiotest and its interlaboratory precision, a preliminary ringtest has been organized with 6 laboratories on the reference chemical KCl. The outcome of this ringtest was very successful as shown by a mean 72h EC50 of 6.593 mg KCl/l and a CV of only 6%.

The methodology was, however, subsequently refined and improved by the use of 6 x 8 cup multiwells containing only one germinated turion per cup with extension of the number of replicates to 8 per test concentration. In addition, "growth" was selected as the effect criterion by measurement of the area of the first frond on a photo of the multiwell taken at the start of the test (= t0h) and a second measurement on a photo of the multiwell taken at the end of the assay (= t72h). Calculation of the growth of the first fronds during the toxicity test, i.e. the t72h-t0h size of the first fronds was found to substantially increase the sensitivity of the assay.

A second (extensive) International Interlaboratory Comparison with the same reference chemical KCl has been launched in early 2014 under the coordination of Dr. R. Baudo of the C.N.R (Istituto per lo Studio degli Ecosistemi).

Fifty six laboratories, institutes, organisations and companies from 22 countries indicated their interest in participating in this ringtest and were sent a test kit with detailed instructions for performance of the assay (and for a second test in case of problems with the first attempt).

All the participants turned in their result sheets within the prescribed 2 months period to the organizer who then made a detailed analysis of the findings and performed extensive statistical analysis.

The very first interesting finding of the International Interlaboratory Comparison was that all the participants obtained an excellent germination of the turions, exceeding the number of test organisms needed for the assay. An analysis has even been made if the “aging” of the turions, i.e. the time period between the shipment of the kit to the participants and the date of performance of the test might have had an influence on the EC50 results. The outcome of this analysis revealed that during a period of 75 days, the aging of the turions had no influence on the *Spirodela* test results.

The mean area of the germinated turions transferred to the cups at the start of the toxicity test was 3.83 mm², and 95% of them were > 1 mm².

A detailed look at the Excel sheets with the results revealed that some laboratories had performed the transfers of the germinated turions into the cups “by first taking those with the largest first fronds”. Since the size of the first fronds in that particular “population” in the Petri dish ranges from “larger” to “smaller”, this procedure goes against the rule of random distribution of the test material, and attention will be drawn on this point in the updated test protocol.

Attention was subsequently paid to the “validity criteria” which had been originally prescribed for the ringtest.

With regard to the first validity criterion, namely that the mean area of the first fronds in the controls had to be at least 15 mm², it appeared that this value had not been reached in all the laboratories.

Since the (refined) test procedure is based on “growth”, this parameter seemed to be a better one than the size of the first fronds. On the basis of the t72h - t0h data provided in the Excel Sheets, it was found that the mean growth of the first fronds in the controls was >10 mm² in more than 90% of the tests.

It was therefore decided to take 10 mm² growth of the first fronds in the controls as a good validity criterion for the *Spirodela* microbioassay.

The submitted results furthermore showed that the second original validity criterion, namely that the CV% of the mean area in the controls at the end of the test should be < 30%, was not obtained either by quite a number of laboratories.

The reason for this is that for quite a number of data sets, the size of the first fronds either covered a rather large range, or there were one or more turions which although they had germinated, did not produce first fronds which were growing as substantially as their fellows in other cups.

Since statistical analysis (see further on) revealed that EC50s calculated “with” or “without” outliers were not much different, it was decided to abandon this second validity criterion.

Although the third (original) validity criterion, namely a monotonic increase of the effect percentage was met in the results of all the participants, it was finally decided not to keep monotonicity as a validity criterion. The reason is that whereas monotonic effect increase was obtained for the reference chemical KCl, it is not excluded that for other toxicants of real samples monotonicity will not always be obtained.

Since, because of problems with the validity criteria, some laboratories had performed a second test (but had sent their results of the first test as well), and since some other laboratories “deliberately” performed two assays, eventually a total of 80 test results were received.

Yet, in order “to put the same weight” on the results of each participant for the EC50 estimate, it was decided to make the statistical analysis (and the discussions) on “only one” result from each participating laboratory, i.e. 52 data sets.

The Excel sheet programme provided to the participants automatically calculated the EC50 by linear regression of the mean % inhibition of the turions growth versus the logarithmic concentrations, with the aid of the built-in formulas of the Excel sheet.

Since this calculation does not give “confidence limits”, it was decided to make a recalculation by using all % inhibition data, instead of only the mean, and the logarithmic concentrations to estimate by “log regression” the 72h EC50 values. This approach also gives the upper and lower confidence limits. Comparison of the (original) Excel regression with the “log regression” data revealed that the EC50 values are in both cases very close to each other.

Since a statistical approach using linear regression may not be the best estimate of the EC50 and its 95% confidence limits for toxicity tests based on “growth inhibition”, an additional calculation has been made on all the 52 results with the aid of “the Hill model”, via a BMDS programme provided by the US EPA.

The Report contains a substantial number of Figures and Tables on the statistical analysis of the results with the 3 methods mentioned above.

One of the Tables shows that the mean EC50's calculated for either the 80 results received, or for 1 laboratory (= 52 data) with or without outliers, were not much different. The calculations made with the Hill model gave an EC50 very close to that calculated with the log regression analysis.

The mean EC50 calculated by log regression was 5932 mg KCl/l (with a CV% of 22.4) versus 6300 mg KCl/l (with a CV% of 18.8).

The higher CV% in the ringtest with 52 participants (22.4%) versus the CV% obtained in the preliminary ringtest with 6 participants (6.2%) can be explained by the fact that for the large ringtest different batches of turions (with a different biological variability) had to be mixed to obtain the >10 000 turions needed, whereas in the preliminary ringtest all the turions were coming from the same batch.

Detailed statistical analysis has also been made on the “intra” versus the “inter” laboratory variability, i.e. the h statistics for outliers and stragglers “between” labs (reproducibility) and the k statistics for the “within” labs variability (repeatability). The outcome of this analysis is that the log regression and the Hill model statistical calculations provide different values, but for both methods the intralab variability (as expressed by the s_p) is quite high, whereas the interlab variability (s_R) is negligible for the log regression calculations or rather low for the Hill model.

All by all this means that the 52 laboratories applied very successfully the *Spirodela* test, obtaining similar results, despite being “first time users” and working with turions coming from different batches.

Furthermore the ringtest revealed that the *Spirodela* test is sufficiently robust to withstand the lack of randomness and the amounts of outliers reported for this International Interlaboratory comparison.

In conclusion. the International Interlaboratory Comparison on the *Spirodela* test has undoubtedly contributed very positively to the improvement and refining of the test procedure.

The amendments will be incorporated in the updated version of the Standard Operational Procedure, which will be prepared shortly.

INTRODUCTION

In aquatic ecosystems there are only a few standard methods to assess the hazard of toxicants to higher plants.

For submerged rooted plants a test for determination of the toxic effect of sediment on the growth behaviour of the aquatic macrophyte *Myriophyllum aquaticum* has been developed over the last few years and was published in 2013 as an ISO standard (ISO 16191)¹. The method is now also proposed to OECD to become a guideline.

For floating plants, a standardized assay with duckweeds, in particular *Lemna minor* or *Lemna gibba* is already in practice for quite a number of years in many countries, and this test is in regular use in the EU for the authorization of plant protection products (herbicides and plant regulators - Reg (EC N°1107/2009).

Test procedures for Lemna tests have been published by many national and international organizations (OECD Guideline 221, ISO 20079, US EPA OPPTS 850-4400, Environment Canada EPS 1/RM/37, ASTM E1415-91, DIN (Germany), AFNOR (France), SIS (Sweden).

Irrespective of their merits, the two toxicity tests mentioned above have a number of handicaps not the least for application in routine.

Like the majority of toxicity tests, these assays are dependent on the culturing and the maintenance of live stocks of the test species, which de facto requires space, equipment and know how.

The tests furthermore need much bench space and incubation space and require substantial handlings and work.

Besides their complexity the standard toxicity tests with higher aquatic plants which are presently available are also quite costly.

In view of the former handicaps, research was initiated about 5 years ago as a joint effort of the Laboratory of Ecology and Environmental Sciences of the Agricultural University of Athens in Greece with the company MicroBioTests Inc. in Mariakerke/Ghent in Belgium, to try to develop a practical and low cost microbiotest with an aquatic macrophyte.

The efforts rapidly targeted on duckweeds from which it was known that a few species, besides their “vegetative” mode of reproduction (i.e. increase of the number of fronds and splitting into new colonies), also produce “dormant vegetative buds” under specific environmental conditions.

These so-called “turions” remain viable for months, and when brought into suitable environmental conditions they germinate into new duckweed colonies.

The first investigations revealed that from the very low number of duckweed species which produce vegetative buds, *Spirodela polyrrhiza* is the only one which can be easily triggered in the laboratory to produce turions, and the research was subsequently targeted on this duckweed species which is very similar to, but slightly larger than most *Lemna* species, and has a quite ubiquitous distribution.

Extensive experimental work has then been performed on a variety of biological and technical aspects such as e.g. the environmental factors inherent to the production of *Spirodela polyrrhiza* turions (temperature, illumination, growth medium), the controlled production of the turions, the storage medium and storage conditions, the shelf life of the turions, and their germination success after prolonged periods of storage.

¹ ISO 16191:2013. Water quality -- Determination of the toxic effect of sediment on the growth behaviour of *Myriophyllum aquaticum*

The investigations subsequently focused on the development of a practical microbiotest, based on and departing - as is the case with all the Toxkit microbiotests - from “the dormant stages” of the test organisms, *in casu* : the turions.



Fig. 1 – Left: picture of turions and fronds. Right: enlargement of a germinated turion with its first frond, in one cup of the 48 multiwell plate used for the test.

After trials with various types of materials and effect parameters, a simple and practical test procedure was gradually worked out 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.

SENSITIVITY OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

With the former test procedure a first sensitivity analysis of the new duckweed microbiotest was made with comparison with the 7 days ISO *Lemna* test.

As can be seen from the table, this comparison revealed that the 72h EC50 of the *Spirodela polyrrhiza* assay was “within the acceptability range” of the ISO *Lemna* test for the two reference chemicals 3,5 DCP and KCl.

Further sensitivity comparisons between these 2 tests were subsequently made on 16 inorganic and organic chemicals, including pesticides and the results (Fig. 2) showed that the *Spirodela* microbiotest is as sensitive as the “conventional” *Lemna* test ($R^2 = 0.97$).

Tab. 1 – Sensitivity comparison of the *Lemna* and *Spirodela* tests.

EC50	3,5 DCP	KCl
ISO <i>Lemna</i> test (acceptability range for the total number of fronds)	2,2 -3,8	5.000 – 10.000
<i>Spirodela</i> microbiotest (area of first frond)	3,3	5.200

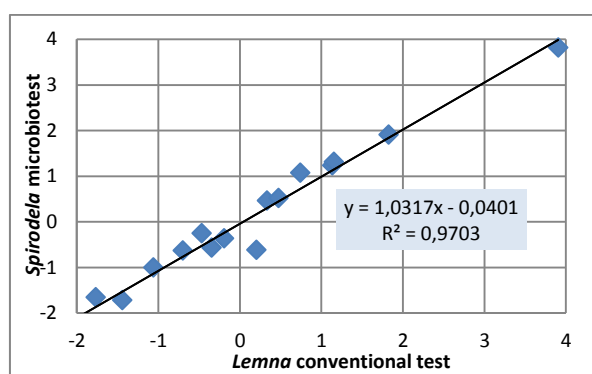


Fig. 2 – Sensitivities of the *Lemna* and *Spirodela* tests for 16 inorganic and organic chemicals.

ORGANIZATION OF A “PRELIMINARY” RINGTEST ON THE *SPIRODELA* DUCKWEED MICROBIOTEST

During the early stage of development of the *Spirodela* microbiotest the assay was performed in a 6 x 4 multiwell, with 2 germinated turions per cup, i.e. 6 replicates per test concentration, and measurement of the area of the first fronds at the end of the 3 days test as the effect criterion.

Based on this procedure, a “preliminary ringtest” has been organized in the summer of 2013 with a small number of laboratories, to obtain an indication of the ease of performance and the practicality of the assay, as well as a first idea of the interlaboratory precision of the new microbiotest.

Six laboratories from 6 different countries agreed to participate in this small ringtest, and performed the assay on the reference chemical KCl.

The outcome of the preliminary ringtest was very positive and revealed a mean 72h EC50 of 6.593 mg/l KCl, with a variation coefficient of only 6%.

The test procedure was then further refined with a shift to a 6 x 8 cups multiwell with 1 germinated turion per cup (i.e. 8 replicates per test concentration) + selection of a number of “validity criteria” for the assay.

ORGANIZATION OF AN INTERNATIONAL INTERLABORATORY COMPARISON ON THE *SPIRODELA* DUCKWEED MICROBIOTEST

After extensive “in house” checking of the reliability and the intralaboratory precision of the adapted test procedure, it was decided to launch an International Interlaboratory Comparison “at large scale” to which laboratories from many countries would be invited to participate.

A call for participation was issued in December 2013 to 60 laboratories in various countries worldwide, with information on the conditions for participation and a Registration Form.

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 2 months of reception of the kit.

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

An “Operational Procedure” was 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 testkits for all the participants and to send them at the timing indicated by the I.S.E.

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

Fifty six laboratories, institutes, organisations and companies from 22 countries indicated their interest and returned the Registration Form to I.S.E, with their agreement to abide by the conditions for participation.

The names of the participating laboratories, institutes, organisations and companies are given hereunder, per country.

EUROPE

Belgium

- Institut Scientifique de Service Public ISSEP – Liège
- Vlaamse Milieumaatschappij VMM – Erembodegem
- University of Antwerp – Dept. Biology, Systemic Physiology and Ecotoxicological Research – Antwerp
- Ghent University – Laboratory for Environmental Toxicology and Aquatic Ecology – Ghent
- Vito ABS – Mol
- Laboratorium ECCA – Merelbeke
- MicroBioTests Inc. – Mariakerke (Ghent)

Czech Republic

- Czech University of Life Science, Faculty of Environmental Sciences, Dept. of Applied Ecology – Prague

Estonia

- National Institute of Chemical Physics and Biophysics – Tallinn

France

- Université de Lorraine – Laboratoire Interdisciplinaire des Environnements Continentaux (LIEC), Campus Bridoux – Metz
- Université de Lyon 1/CNRS, Ecologie des Hydrosystèmes Naturels et Anthropisés - ENTPE/LEHNA-IPE – Vaulx en Velin
- INSAVALOR-POLDEN – Villeurbanne Cedex
- INERIS – Verneuil en Halatte
- Vigicell – Villejuif
- Groupe CARSO, Laboratoire Ecotoxicologie– Lyon Cedex 07

Germany

- Federal Environment Agency (Umweltbundesamt) – Berlin

Greece

- Agricultural University of Athens, Laboratory of Ecology and Environmental Sciences – Athens

	<ul style="list-style-type: none"> Aristotle University of Thessaloniki, Laboratory of Environmental Engineering and Planning, Division of Hydraulics and Environmental Engineering, Dept. of Civil Engineering – Thessaloniki
Hungary	<ul style="list-style-type: none"> National Institute of Environmental Health, Dept. of Water Hygiene – Budapest
Italy	<ul style="list-style-type: none"> ISPRA, Servizio AMB LAB – Roma Istituto Superiore di Sanita, Dipartimento Ambiente e Prevenzione Primaria Roma Ecobioqual – Torino
Lithuania	<ul style="list-style-type: none"> Institute of Botany at Nature Research Centre – Vilnius
Poland	<ul style="list-style-type: none"> Wroclaw University of Technology, Environmental Engineering Institute – Wroclaw University of Agriculture in Krakow, Dept. of Agricultural and Environmental Chemistry – Krakow Medical University of Gdansk, Dept. of Environmental Toxicology – Gdansk University of Opole, Dept. of Land Protection – Opole The Silesian University of Technology, Environmental Biotechnology Dept. – Gliwice Gdansk University of Technology, Faculty of Chemistry, Dept. of Analytical Chemistry – Gdansk Polish Academy of Sciences, European Regional Centre for Ecohydrology, Dept. of Ecohydrological Biotechnology – Lodz Medical University of Warsaw, Dept. of Environmental Health Sciences, Faculty of Pharmacy – Warsaw University of Maria Curie-Sklodowska, Dept. of Environmental Chemistry, Faculty of Chemistry – Lublin Wroclaw University of Environmental and Life Sciences, Dept. of Environment, Animal Hygiene and Welfare – Wroclaw The John Paul II Catholic University of Lublin, Dept. of Physiology and Ecotoxicology, Institute of Biotechnology – Lublin University of Life Sciences in Lublin, Dept. of Hydrobiology – Lublin Laboratorium Badawcze SORBO, Research Laboratory SORBO – Poznan
Portugal	<ul style="list-style-type: none"> University of Coimbra, Dept. of Life Sciences, IMAR – Instituto do Mar – Coimbra
Romania	<ul style="list-style-type: none"> Ovidius University of Constanta, Dept. of Natural Sciences, Faculty of Natural and Agricultural Sciences – Constanta
Russian Federation	<ul style="list-style-type: none"> Tomsk State University, Research Center bioassay safety of nanomaterials and nanotechnology – Tomsk Kazan Federal University, Institute of Ecology and Geography – Kazan
Spain	<ul style="list-style-type: none"> Technical University of Catalonia (UPC), CRIT Centre for Research and Innovation in Toxicology – Terrassa University of Valencia, Laboratory for Aquatic Toxicology – Burgassot, Valencia Xenobiotics, S.L. University of Valencia Science Park (PCUV) – Paterna, Valencia
Sweden	<ul style="list-style-type: none"> Mälardalen University, ACWA Research Group Future Energy Centre, School of Business, Society and Engineering – Västerås
The Netherlands	<ul style="list-style-type: none"> Grontmij Nederland B.V. , W&E-Ecology – Amsterdam
United Kingdom	<ul style="list-style-type: none"> Opus Ltd – Stromness, Orkney
AFRICA	
Cameroon	<ul style="list-style-type: none"> University of Bamenda, Ecotoxicology Laboratory, College of Technology – Bamili

South Africa

- University of Jannesburg, Department of Zoology – Johannesburg
- Council for Scientific and Industrial Research (CSIR), Natural Resources and the Environment – Pretoria
- Clean Stream Biological Services – Pretoria
- Golder Associates Research Laboratory – Johannesburg
- ToxSolutions, Kits and Services – Brackenhurst
- Department of Water Affairs, Resource Quality Services, Pretoria

AMERICA

Canada

- Environment Canada, Laboratory for Environmental Testing – Quebec, Montréal

Guatemala

- SEPRA – Guatemala
- Montana Exploradora de Guatemala S.A./Marlin Laboratory – San Miguel Ixtahuacan, San Marcos

ASIA

Thailand

- Kasetsart University, Chemistry Department, Faculty of Science – Bangkok

TEST PROTOCOL OF THE 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 testkit 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 the last week of January 2014 and sent to the 56 participants the first week of February 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 the end of the first week of April 2014 “at the latest”.

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

During the course of February and March, all the 56 participants actually performed the prescribed *Spirodela* test and sent their results.

Taking into account that the *Spirodela* test procedure was “totally new” to virtually all the participants, the results of several laboratories did not abide by one or several of the validity criteria which had been indicated, and these participants (kindly) performed a second test with the materials provided in the test kit.

In addition, and although the results of their first test were totally acceptable, a number of laboratories have performed a second test and also sent these results to the organizer.

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.

Eventually a total of 80 data sets have been received, but the analysis of these data sets revealed that despite performance of a second test the results of 4 laboratories were not acceptable.

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. 52 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 0 h) and after 72 h exposure. The results have to be filled out on specific Excel Result Sheets, and sent by e-mail to the organiser.

A copy of the Excel Result Sheets is given in Annex 2.

Since the Image J program was new for most of the participants, some of the area data given in the Excel Sheets were not in mm² because of wrong setting of the scale values.

Yet, since the measurements are done on a recorded image of the multiwell plate, it was possible to remake them at any time. Therefore, the participants were asked to repeat the measurements or, alternatively, to send the pictures to the company MicroBioTests, where the frond areas were re-measured.

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 robust statistics have been computed according to the Standard ISO 13528³.

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”.

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)⁴, provides practically identical results for the EC50 values as those of the Excel regression, as proved by figure 3, but in addition gives the upper and lower 95 % confidence limits.

³ ISO 13528:2005. Statistical methods for use in proficiency testing by interlaboratory comparisons

⁴ R.R. Sokal and F.J. Rohlf. 2009. Introduction to biostatistics, 2nd Edition. Dover ed.

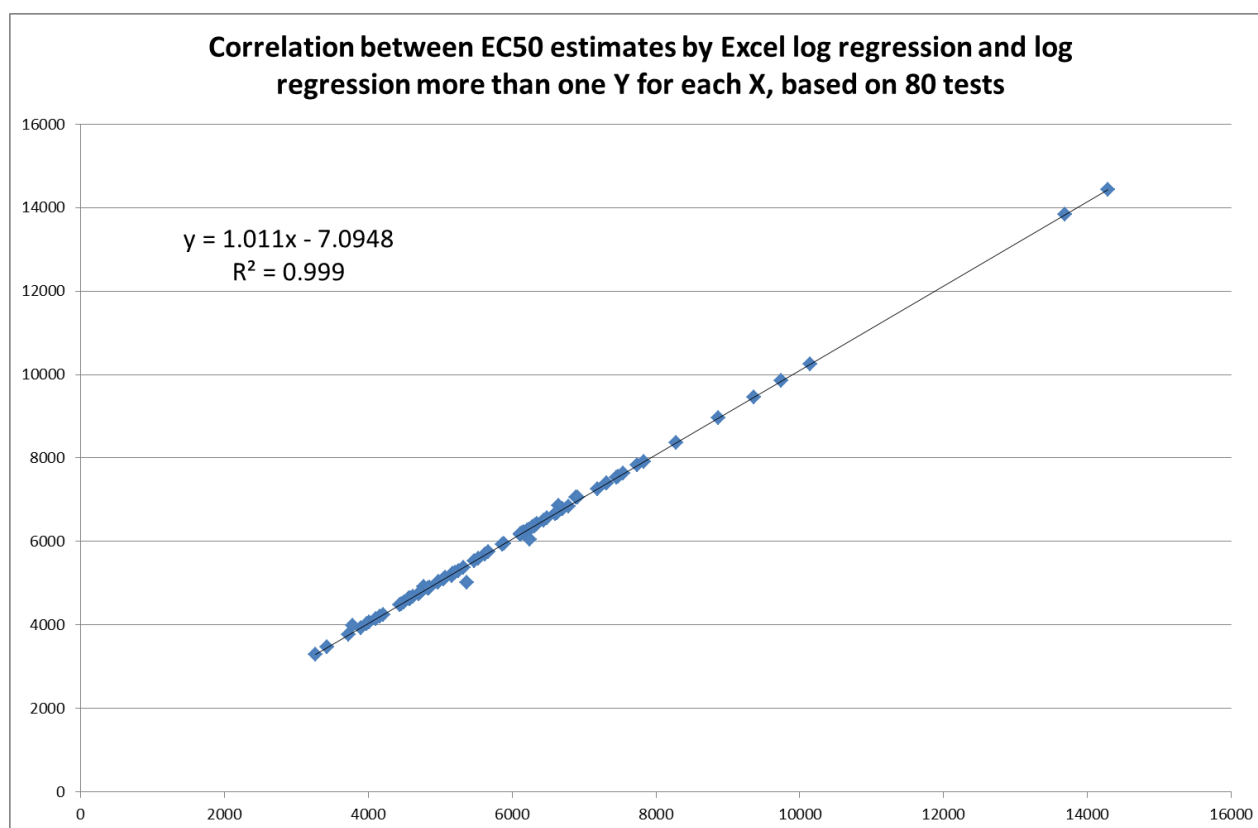


Fig. 3 – Correlation between EC50 estimates by Excel log regression and log regression using the more than one Y for each X procedure. The determination coefficient is reported (the correlation explains 99.9 % of the variability).

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 20281⁵. 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/>.

The statistical analysis furthermore produced the repeatability and reproducibility of this International Interlaboratory Comparison, according to the ISO 5725-2⁶ procedure. The data treatment is reported in Annex 3.

⁵ ISO/TS 20281: 2006, Water quality – Guidance document on the statistical analysis of ecotoxicity data

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

Turions germination

Out of the 56 participant laboratories, only one lab completely failed to obtain germination of the turions, and another lab obtained an insufficient number of germinated turions to execute a full test, with 8 repeats each for the negative control and the 5 test concentrations.

Two other laboratories were unable to perform the tests for technical reasons (frozen turions, or equipment malfunctioning).

All the other 52 labs succeeded in obtaining a very satisfactory turions germination, to the point that in all the results of 80 tests have been provided.

Taking into account the failed germination, the overall turion germination (for the toxicity test) amounts to 98.4 %. This is actually even an underestimate of the true germination success, since not all the germinated turions were needed for the toxicity test.

According to the participants data, the area at time 0 measured on a total of 3840 turions, provides a mean value of 3.83 mm², with a CV % of 65.8, and 95 % of the area data are ≥ 1.00 mm².

Turions aging

Since the 80 results from the 52 labs were obtained at different times after the shipment of the turions, it is possible to verify whether the delay in performing the tests, corresponding to the aging of the turions, reflects in the area of the first fronds.

This does not seem to be the case, since the regression of the fronds area *versus* the days of delay (after shipment) in performing the test (Fig. 4) is not statistically significant. Actually, looking at the plot it seems that the fronds areas show a trend toward a slight decrease with time, but in statistical term the turions aging explains only 9.1 % of the frond area variability over a delay time of 75 days (after shipment of the turions, which were already a few days old when prepared for shipments).

Analogously, the turions aging, over this time span, has no statistically significant influence on the final results, that is the EC50 calculation: in fact, aging accounts for 2.4 % of the variability in the EC50 estimates by Excel, for 2.3 % for the log regression, and for 6.3 % for the Hill model.

Therefore it can be concluded that, at least for 75 days, the aging of the turions has no influence on the *Spirodela* test performance.

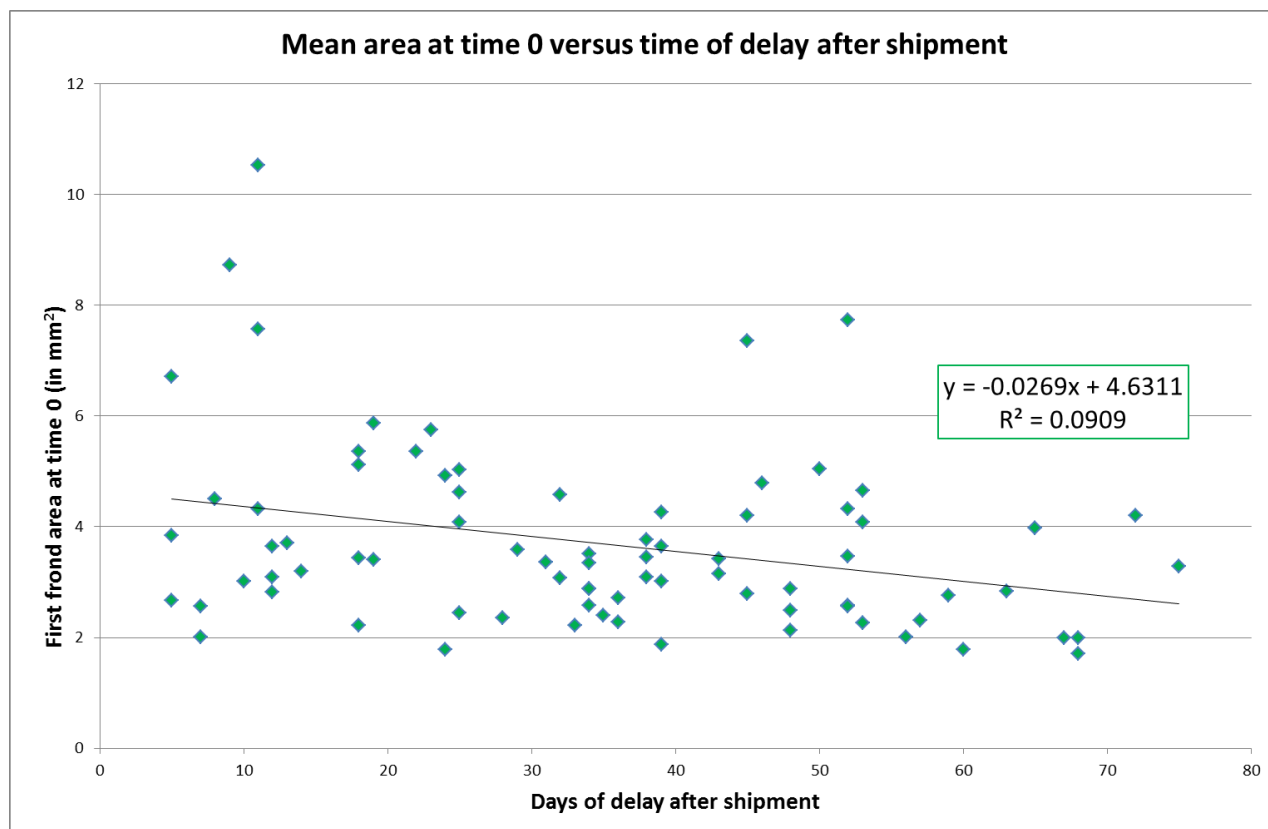


Fig. 4 – Mean areas of the first frond (n = 80) versus the days of delay in performing the tests after the date of shipment of the *Spirodela* kits.

VALIDITY CRITERIA FOR THE REFERENCE TEST WITH KCl

For the reference test to be valid, the following 3 criteria were originally proposed:

1. 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 $\geq 15 \text{ mm}^2$.
2. The variation coefficient between the individual first frond area values in the negative controls at the end of the test (t72h) must be $\leq 30 \%$.
3. The % inhibition values of the growth of the first fronds in the test concentrations must follow a “monotonic” increase (i.e. from low to high) in the increasing test concentrations.

In case these 3 validity conditions were not fulfilled, a second test had to be performed (with the second set of materials provided in the kit).

During the course of the International Interlaboratory Comparison it became clear that the original validity criteria which had been set need to be “reconsidered” for the future of the *Spirodela* microbiotest.

In fact, 21 labs performed only one test (abiding the three above mentioned criteria), 27 labs two tests, and 4 labs three tests. On a total of 80 tests, 22 and 23 failed criterion 1 and criterion 2, respectively.

Actually, considering all 80 tests, the robust statistics estimates a mean area at 72 h of 17.2 mm^2 (95 % confidence limits: 15.9 - 18.6), with 90 % of the results $> 12.4 \text{ mm}^2$. And taking into accounts 52 results (1 per lab), the robust statistics estimates a mean area at 72 h of 17.9 mm^2 (95 % confidence limits: 16.4 - 19.4).

However, since the selected effect criterion for the assay is “growth”, it eventually seemed more logical to take “the growth” of the first fronds during the 3 days test into consideration as a validity criterion, that is the

first fronds area at 72 h minus the corresponding area at time 0, instead of “the mean size” of the first fronds in the negative controls at the end of the assay.

A calculation has therefore been made of “the growth” of the first fronds in the 52 data sets and the mean growth data in the controls are shown in figure 5.

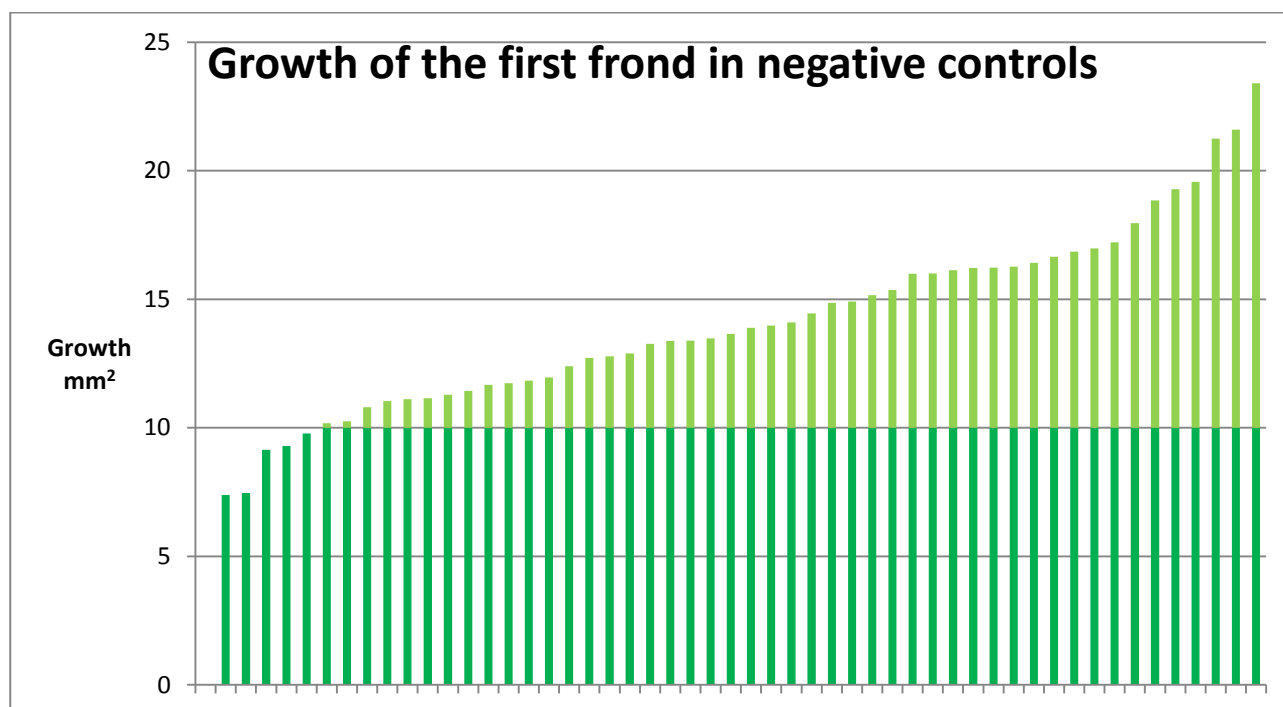


Fig. 5 – Growth of the first frond in negative controls from 52 labs. All the values $>10 \text{ mm}^2$ are shown in lighter colour.

The robust statistics for the 52 results (1 per lab) estimates a mean growth at 72 h of 14.0 mm^2 (95 % confidence limits: 12.6 - 15.4). The smallest growth was 7.4 mm^2 , and only 5 data were lower than the 90° percentile. Therefore, a new validity criteria is proposed herewith instead of the (original) 15 mm^2 for the mean area of the first frond, namely that the first fronds growth in the negative control after 72 h must be $\geq 10 \text{ mm}^2$.

The second original validity criterion, namely that the CV% for the mean area size in the control row at t72h must be below 30% also appeared to be too stringent, since only 23 out of 80 tests presented a variation coefficient between the individual first frond area values in the negative controls at the end of the test (t72h) $\leq 30 \%$.

Even when considering only 1 result per lab (Fig. 6), 19 of the 52 results were higher than 30 %, so it was decided that this validity criterion should not be maintained.

For what regards the third validity criterion (monotonicity), all tests resulted valid. This means that monotonicity is OK for the reference toxicant KCl, but it cannot be excluded that for other toxicants, or real samples, monotonicity will not always be obtained (for instance, some chemical may display hermetic effects).

Therefore, it has been decided that even this third validity criterion will not be maintained, except for reference tests with KCl.

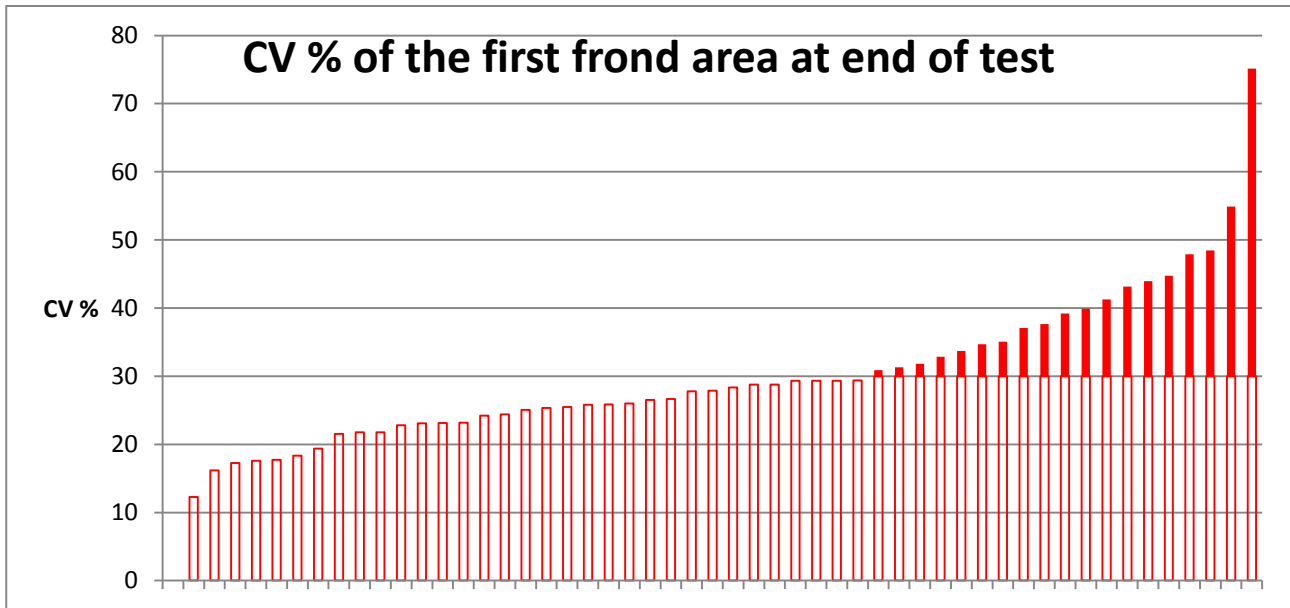


Fig. 6 – Variation coefficient of the first frond in negative controls after 72 h from 52 labs (results above the formerly proposed validity criteria of 30 % in darker colour).

Further statistical analysis of the original data

For quite a number of data sets, the size of the first fronds either covered a rather large range, or there were one or more turions which although they had germinated, did not produce first fronds which were growing as substantially as their fellows in the other cups.

A specific statistical analysis has therefore been made on the results of all 80 reported tests.

First, it has been checked whether there are statistically significant differences among column (treatments), or among rows (repeats) in the first frond areas at time 0, suggesting that unawares selection of the turions leads to a non – random distribution among the wells in the multiwell plates.

Second, it has been verified (by the Tukey's rule) whether there are statistically outliers in the data of first frond areas at time 0, at time 72 h, and in the first fronds growth at the end of the tests.

Then, the EC50s have been computed (both with the log regression model and the Hill model) for the following sets of data:

1. all 80 tests (original data);
2. 39 tests with no statistical differences among column or rows (random)
3. 52 tests, after deletion of the outliers (without outliers)
4. original data of 52 tests (1 per lab).

The analysis (quite interestingly) revealed that the 72h EC50s did not change that much in all cases (Tab. 2).

This means that the *Spirodela* test is sufficiently robust to withstand the lack of randomness and the amounts of outliers reported for this International Interlaboratory comparison.

However, it is strongly recommended that future users place a great care in trying to achieve a random selection of the turions, avoiding their selection (according to size or other criteria) when partitioning them among the different treatments.

In other words, attention has to be paid “not” to start the transfer of the germinated turions in the cups with those having the “largest” first fronds !

Tab. 2 – Comparison of the EC50s computed for different sets of data.

	N of tests	EC50 Excel	CV %	EC50 log regression	CV %	EC50 Hill model	CV %
All	80	6025	31.8	6061	31.8	6472	19.48
Random	39	6125	31.5	6190	31.5	6635	19.95
without outliers	52	6011	20.8	5832	24.6	6194	18.42
1 per lab	52	5879	22.3	5932	22.4	6320	18.79

Considering that this type of statistical analysis is rather laborious and cannot be easily done on a routine basis, for the sake of this International Interlaboratory Comparison it has been therefore decided that the subsequent statistical treatment, to assess the intra- and interlaboratory variability, will be done using 52 data sets (1 per lab) formerly selected.

It can be noted that this interlaboratory comparison obtained a mean EC50 value quite close to that of the 1st ringtest (Tab. 3), albeit with a somewhat increased interlaboratory variability (22.3 % CV *versus* 6.2 % CV, when only the average values for each lab is taken into account).

However, this increase is easily explained, since the new comparison involves many more labs (52 *versus* 6), and moreover, for the huge amount of turions needed (> 10 000), it has to start from turions coming from different batches, hence exploring the full range of the biological variability among turions, whereas the 1st ringtest was based on turions collected all at the same time, from one batch only.

Tab. 3 – Comparison between 1st and 2nd ringtests.

	N of labs	EC50 Excel	CV %
1st ringtest	6	6593	6.2
This interlaboratories comparison	52	5879	22.3

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.

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.

Log-regression EC50

The EC50 data calculated with this model for the 52 labs have been summarized in figure 7, as histograms ordered from the lowest to the highest average of each laboratory, and the data on repeatability and reproducibility are reported in table 4.

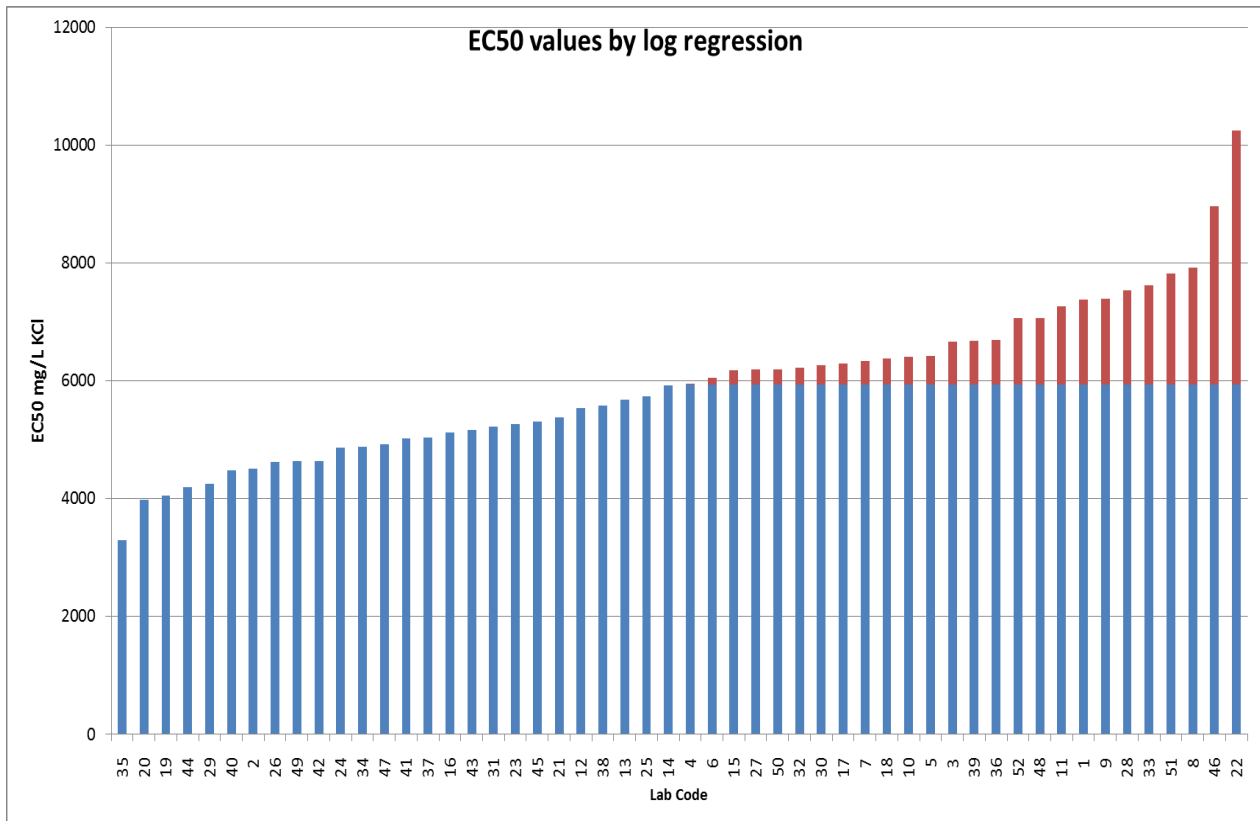


Fig. 7 – EC50 (log regression) from 52 labs, in increasing order (results above the overall mean in different colour)

Tab. 4 – Mean EC50 values estimated by log regression and their 95 % upper (UCL) and lower confidence limits (LCL).

	All data	Without h and k outliers	Without h and k stragglers
n lab	52	49	41
Mean	5932	5798	5806
s_r	4886	4807	4434
CV%	82.4	82.9	76.4
s_R	4886	4806.6	4434
CV%	82.4	82.9	76.4
h straggler	3	1	0
h outlier	1	0	0
k straggler	4	3	0
k outlier	1	0	0
95 % UCL	15509	15219	14496
95 % LCL	0	0	0

The h statistics points out outliers and stragglers between labs (reproducibility), while the k statistics refers to the within labs variability (repeatability). In other words, the h statistics considers the differences in the labs averages, and the k statistics highlights the labs showing the highest variability (CV %).

The first run identified as h outliers Lab 22 and as k outlier Lab 43; as h straggler Lab 46, and as k stragglers Labs 20, 35, 39. After removing those data, also Lab 46 becomes an h outlier, and Labs 8, 11, 40, 49, and 51 became stragglers.

This statistical analysis is shown in figures 8 (h statistics) and 9 (k statistics).

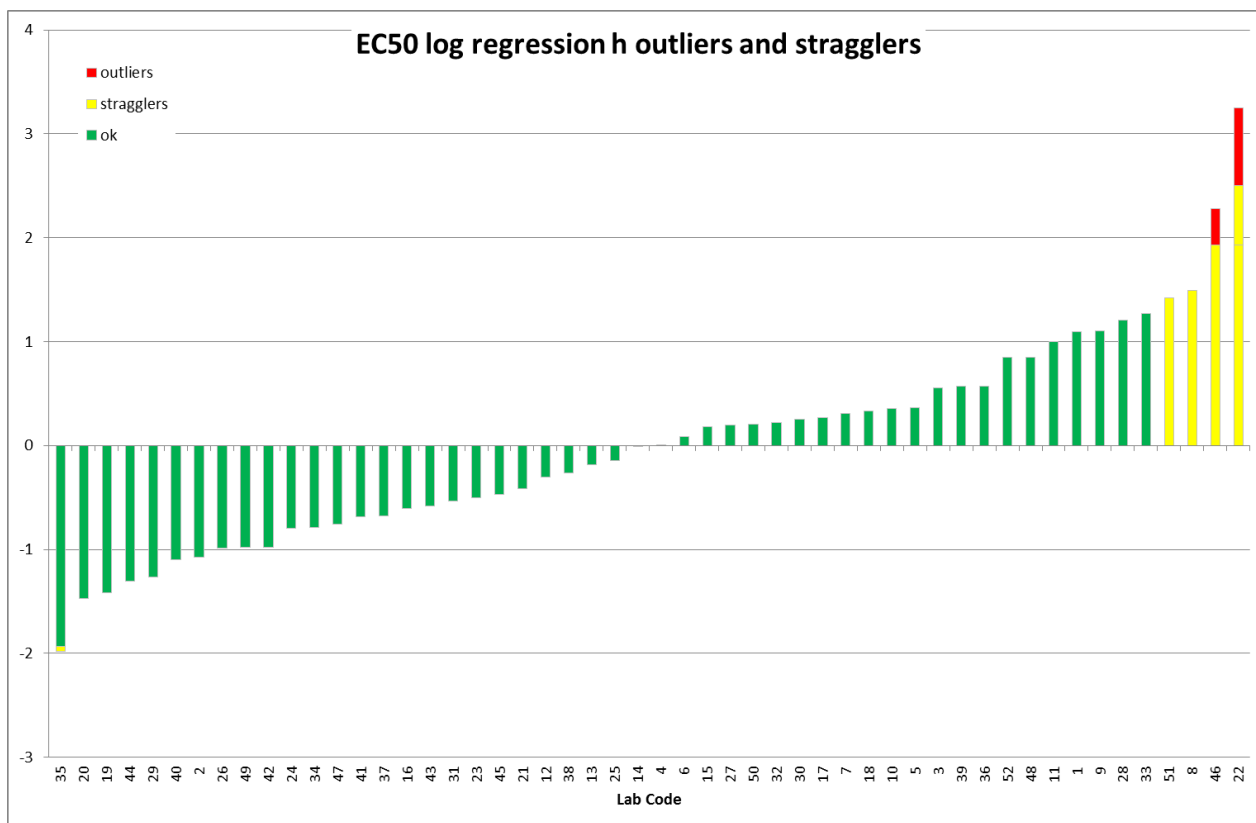


Fig. 8 - h statistics for the EC50 log regression.

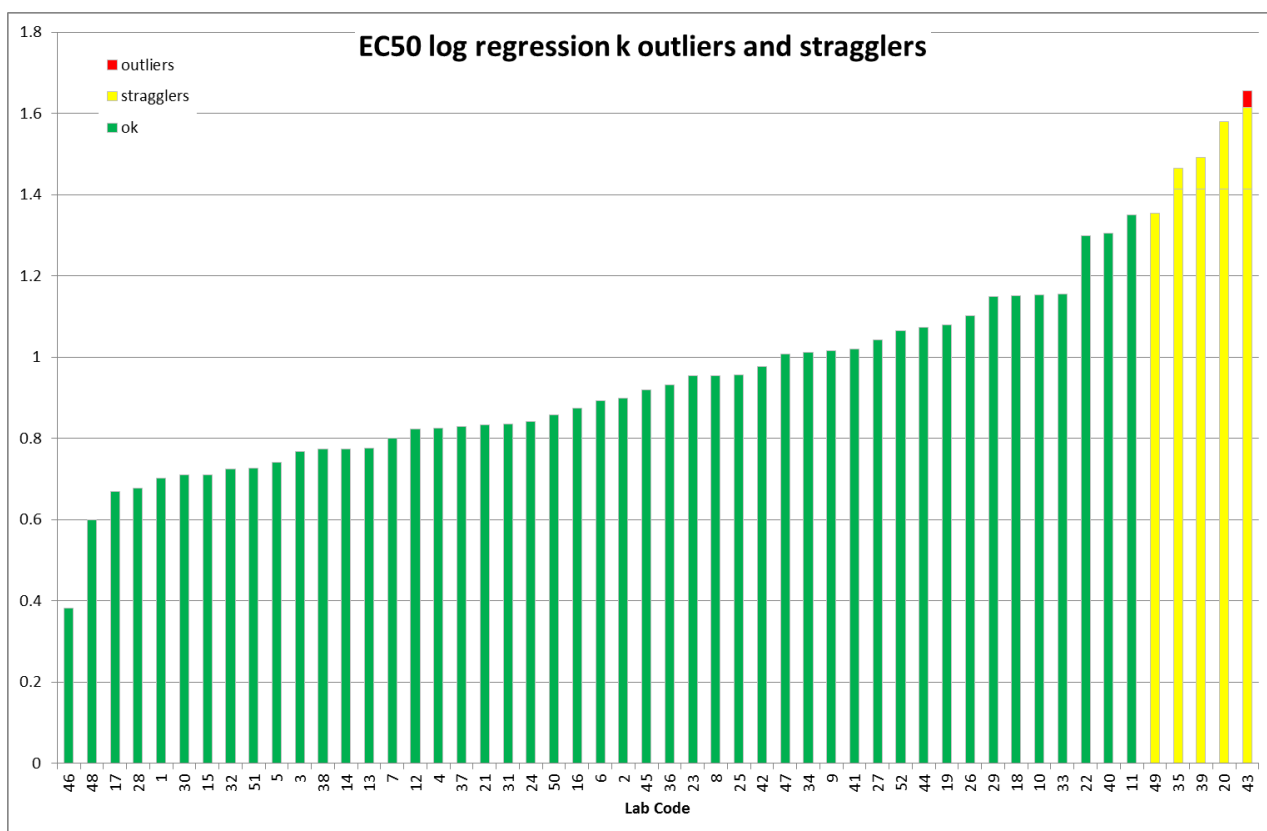


Fig. 9 - k statistics for the EC50 log regression.

Hill model EC50

The EC50 data calculated with this model for the 52 labs have been summarized in figure 10, as histograms ordered from the lowest to the highest average of each laboratory, and the data on repeatability and reproducibility are reported in table 5.

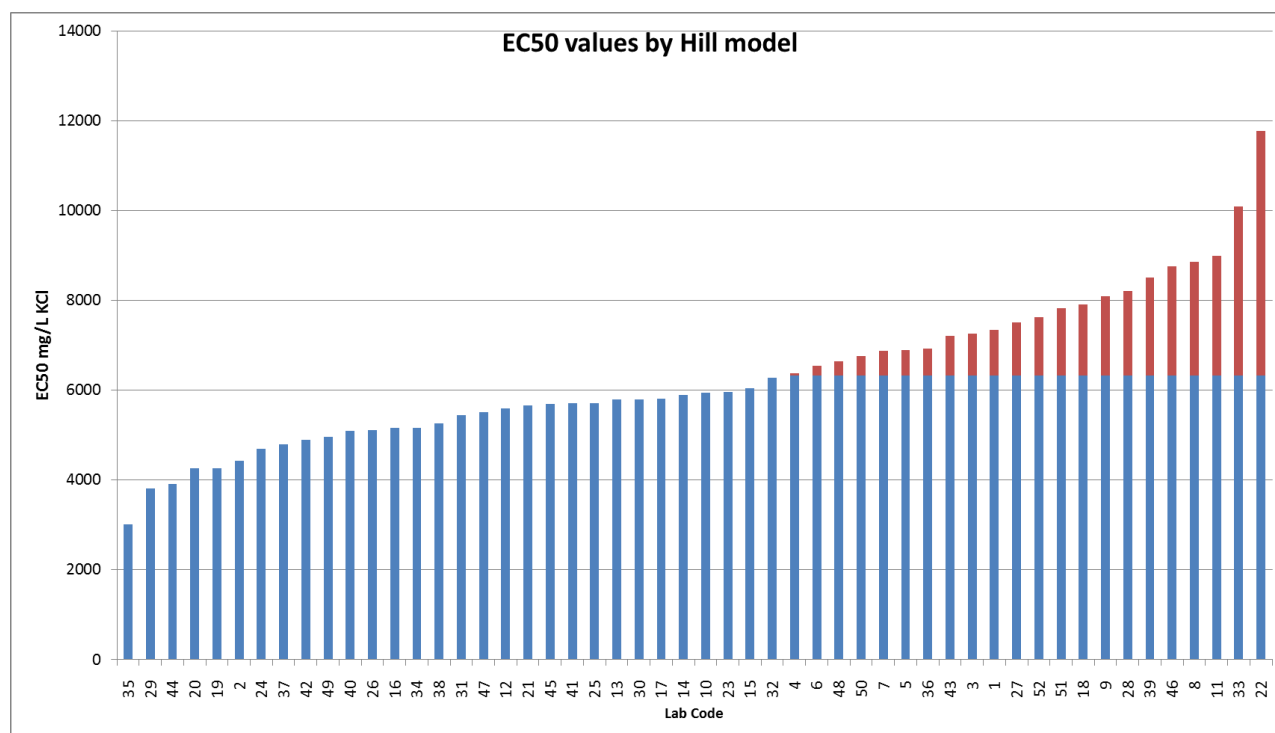


Fig. 10– EC50 (Hill model) from 52 labs, in increasing order (results above the overall mean in a different colour)

Tab. 5 – Mean EC50 values estimated by the Hill model and their 95 % upper (UCL) and lower confidence limits (LCL).

	All data	Without h and k outliers	Without h and k stragglers
n lab	52	46	27
Mean	6320	6051	5531
s_r	1297	1108	910
CV%	20.5	18.3	16.5
s_R	2066	1747	1145
CV%	32.7	28.9	20.7
h straggler	3	3	0
h outlier	1	0	0
k straggler	5	3	0
k outlier	4	0	0
95 % UCL	10369	9475	7775
95 % LCL	2271	2626	3287

The h and k elaboration identified as h outliers Lab 22 and as k outliers Labs 43, 36, 22, and 17; as h stragglers Labs 46, 8, 11, 33, and 35; as k stragglers Labs 39, 23, 18, and 48..

This statistical analysis is shown in figures 11 (h statistics) and 12 (k statistics).

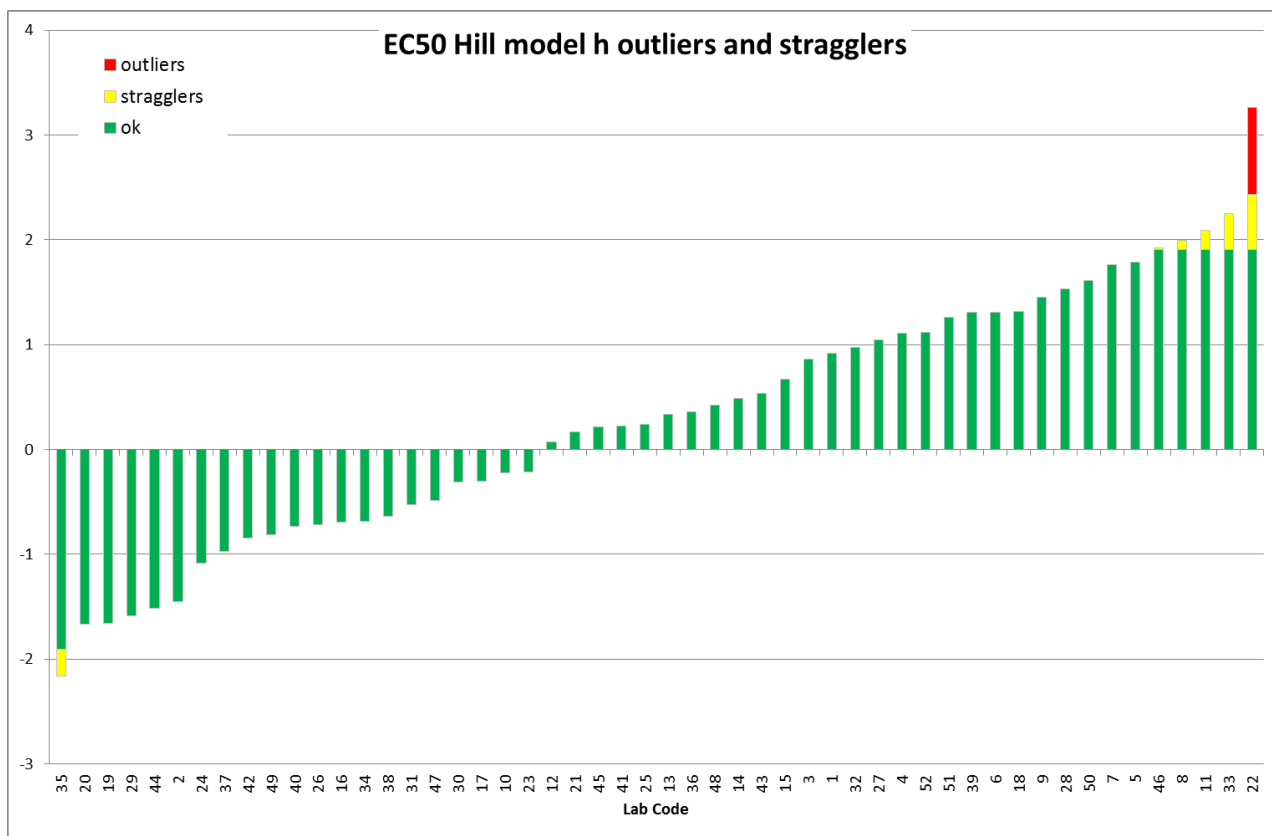


Fig. 11 - h statistics for the EC50 Hill model

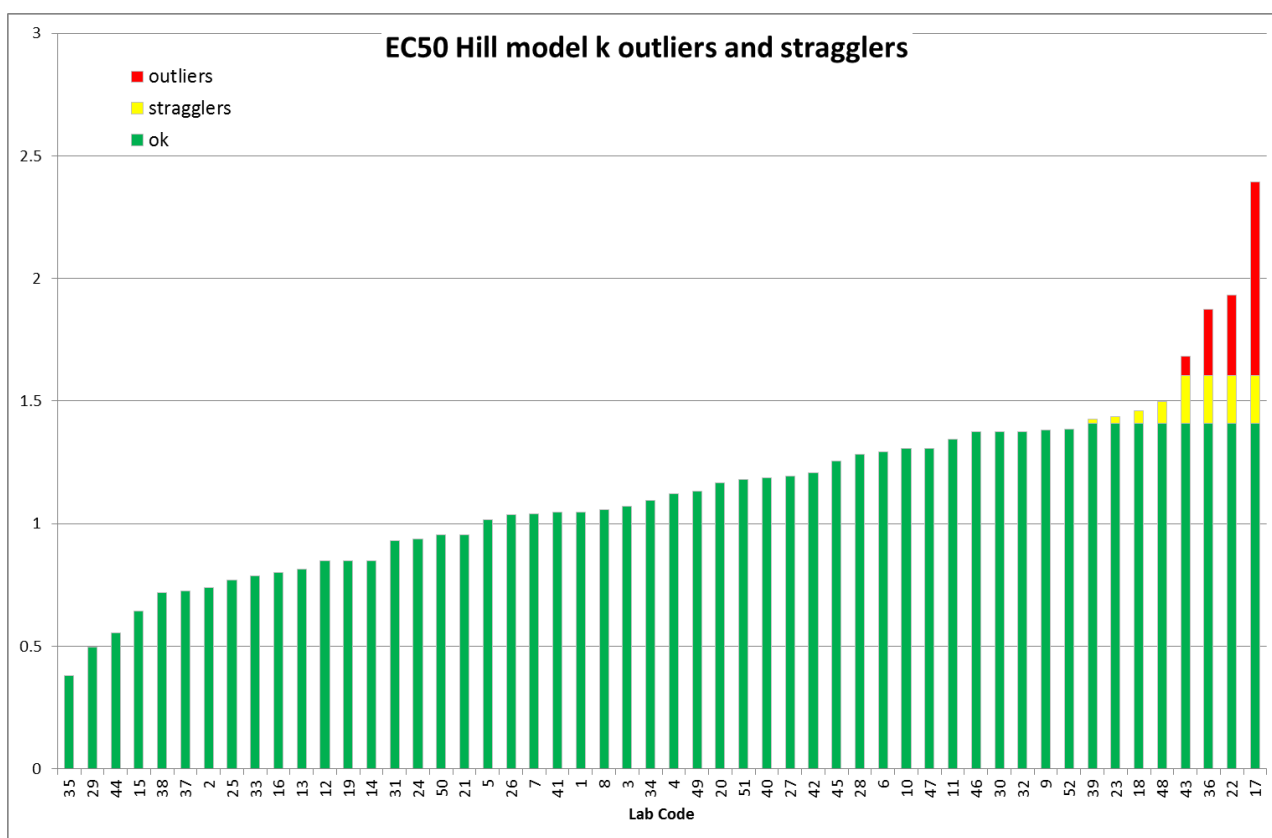


Fig. 12 - k statistics for the EC50 Hill model

Summarizing this data elaboration (Tab. 6), it is evident that the two EC50 estimates provide values very close: the log regression has a larger variability, that in turn means that less results are statistically outliers, but with a correspondingly more variable repeatability and reproducibility.

Conversely, the Hill model consistently produces less variable estimates, with reduced intra- and interlaboratory variabilities.

Tab. 6 – 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	52	3	5798	0	15219	4807	82.9	4807	82.9
Hill model	52	1	6050	2626	9475	1108	18.3	1747	28.9

The overall variability obviously depends also on the turions biological variability, as indicated by the intralab s_r . However, considering that for this interlaboratory comparison a huge amount (> 10 000) of turions have been produced, coming from different batches, hence exploring the full range of the biological variability among turions, this variability seems to be acceptable (< 20 % for the EC50 estimated by the Hill model).

On the other hand, the interlab variability is negligible (log regression), or rather low (Hill model), considering that the variability s_R contains the variability s_r .

This means that the 52 labs applied very successfully the *Spirodela* test, obtaining similar results, despite being first time users of this test.

Conclusions

Despite being based on the attempt of “first time users”, this interlaboratory comparison has been very useful in pointing out the strengths and the weak points of the *Spirodela* test, which can be summarized as follows.

Positive findings:

1. The germination of the turions is excellent, and has not been influenced by delivery conditions;
2. There was almost no influence of the “aging” of the turions (at least for up to 75 days) on the size of the first frond (so the shelf life of the turions is several months);
3. The *Spirodela* test is “user friendly” (once familiarized with the procedure, all the labs easily applied the test);
4. The formerly proposed validity criteria have been reconsidered and only one solid new validity criteria is proposed, namely that the growth of the first fronds in the negative control after 72 h must be $\geq 10 \text{ mm}^2$.

Improvements which have to be considered :

1. Turions from different batches should not be pooled since this can lead to a substantial biological variability;
2. The distribution of the germinated turions in the wells of the multiplates must be randomized (although the ringtest has shown that the *Spirodela* test is sufficiently robust to withstand the lack of randomness and the amounts of outliers reported for this International Interlaboratory comparison);
3. The Image J settings (especially the scale) have to be respected (this is actually not very demanding, once the user takes confidence with the programme for measuring the frond areas).

In conclusion, the International Interlaboratory Comparison on the *Spirodela* test has undoubtedly contributed very positively to the improvement and refining of the test procedure.

The amendments will be incorporated in the updated version of the Standard Operational Procedure, which will be prepared shortly.

INTERLABORATORY COMPARISON OF THE *SPIRODELA* DUCKWEED MICROBIOTEST

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

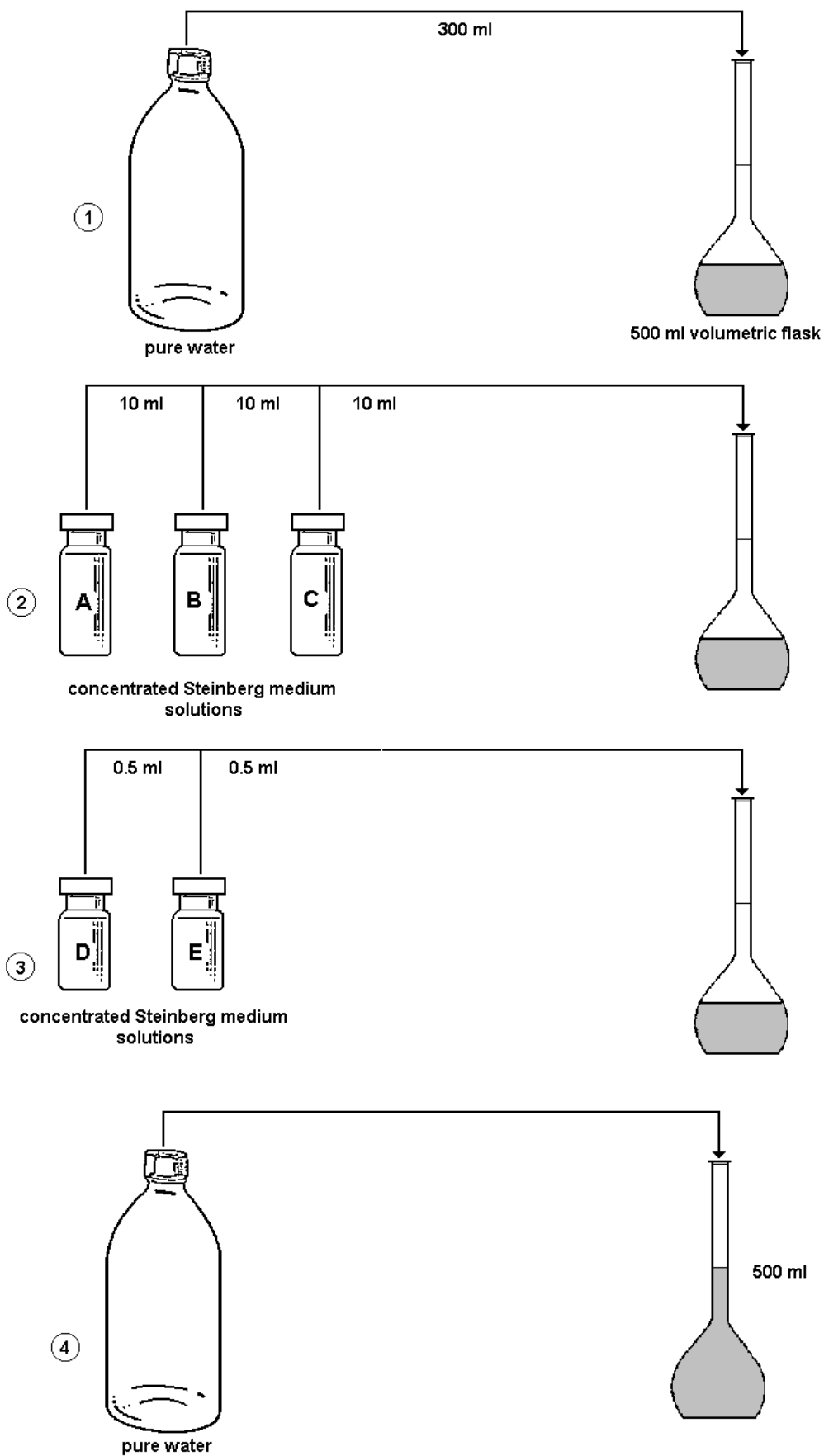
This medium will be used for the germination of the *Spirodela* turions and as growth medium for the duckweeds and dilution medium for the toxicants in the toxicity test. The composition of this medium is that of the “Steinberg medium” prescribed by ISO for Lemna toxicity tests (ISO 20079).

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)

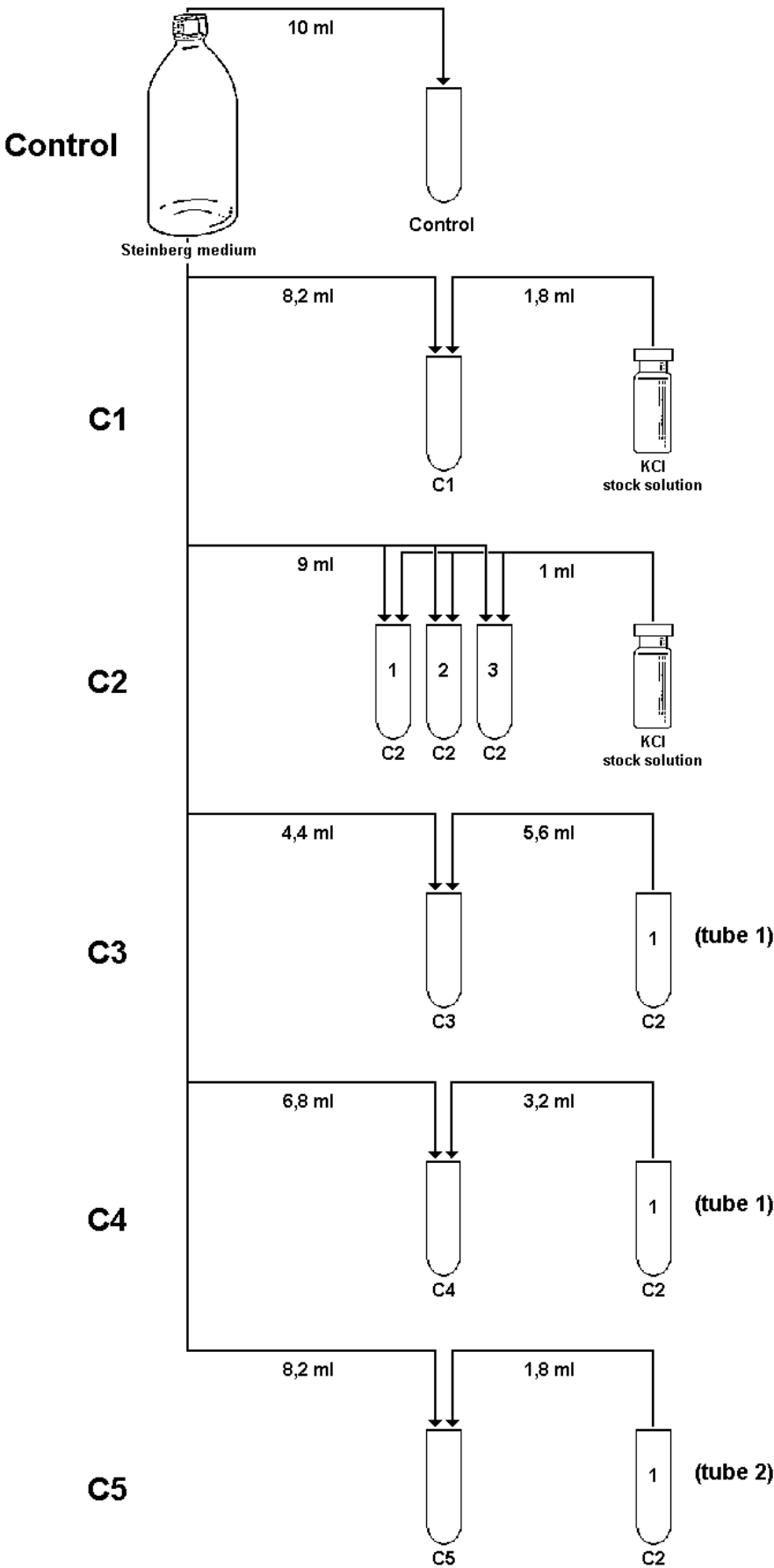
1. Take the 8 test tubes and label them as C1, C2 (3 tubes), C3, C4, C5 and Control
2. Put 10 ml Steinberg medium in the Control tube.
3. Concentration C1 (= 18 000 mg/l) : Put 1,8 ml KCl stock solution (= 100 000 mg/l) in test tube C1 and add 8,2 ml Steinberg medium
4. Concentration C2 (= 10 000 mg/l) : Put 1 ml KCl stock solution in the 3 tubes labeled 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 labeled 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

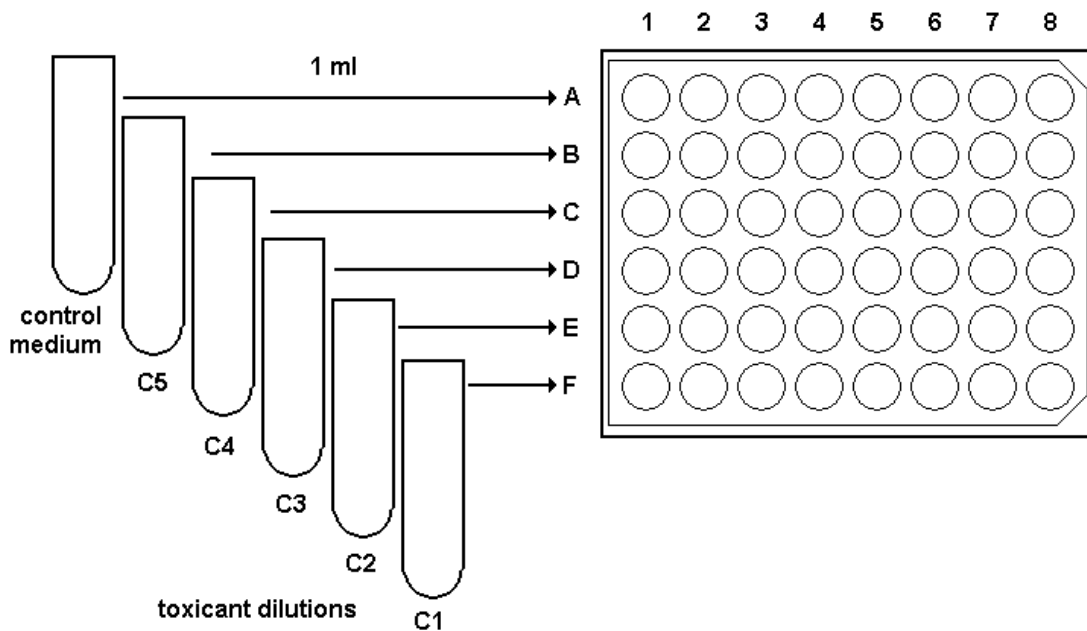
Each toxicant dilution has to be transferred into all the 8 cups of one row in the multiwell plate. The rows are labeled from A to F and the cups from 1 to 8.

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).

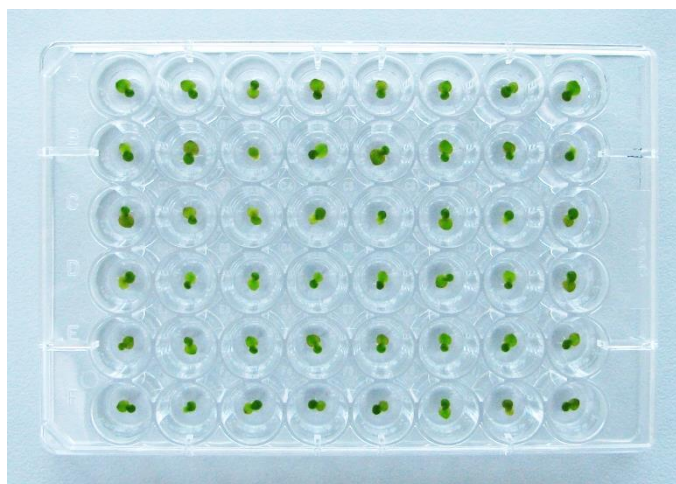


Photo of a multiwell plate with the germinated turions and their small first fronds (at t0h)

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.

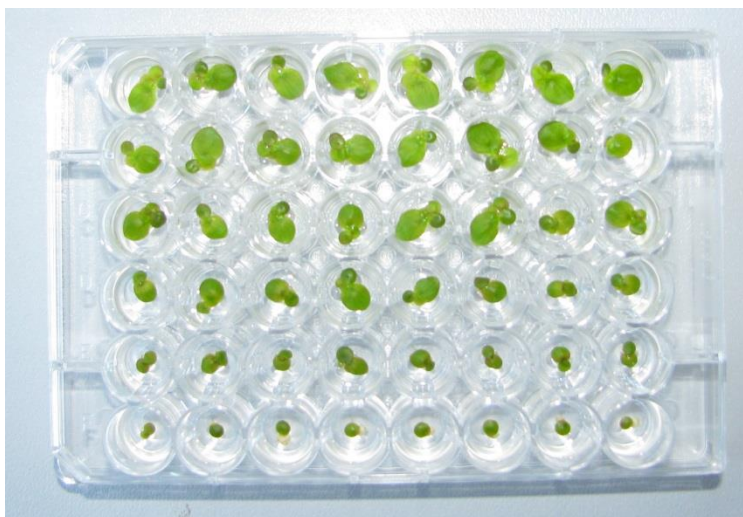


Photo of the multiwell plate with the grown first fronds, after 3 days incubation (t72h).

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”

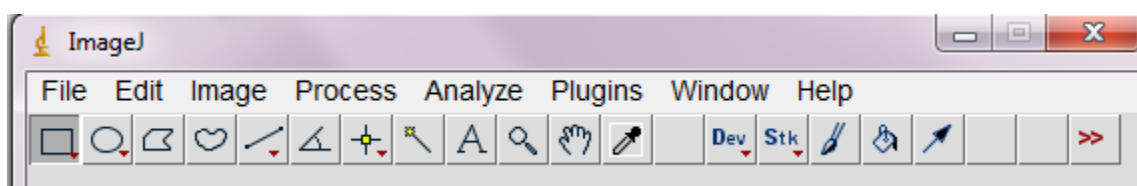
- Click on “Clock, Language and Region”
- Click on “Additional settings”
- Click on “Numbers” and make sure that the decimal symbol is set as a “dot”(.)
- Click on “Apply”
- Click on “OK”

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 mm²).

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 color 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 Toolpak) 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 to evaluate if they may not be better suited.

VALIDITY CRITERIA FOR THE REFERENCE TEST WITH KCI

For the reference test to be valid, the following 3 criteria must be fulfilled :

1. 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, should be at least 15 mm² (The mean area of the first fronds in the control row at t72h is shown in the box at the bottom of the first column in Table 2 of the Data Treatment sheet)
2. The variation coefficient between the individual first frond area values in the control row at the end of the test (t72h) should not be higher than 30% (The CV% value for the first fronds in the control row is also shown in the box at the bottom of the first column in Table 2 of the Data Treatment sheet)
3. The % inhibition values of the growth of the first fronds in the test concentrations should follow a “monotonic” increase (i.e. from low to high) in the increasing test concentrations (This can be evaluated by looking at the data in the column “I%” in Table 4 of the Data Treatment sheet)

In case these 3 validity conditions are 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 RESULT SHEETS

Measurement of the fronds area is performed with the Image J programme according to the instructions given in the corresponding section of Annex 1 .

As per the instructions given in the same section, the data are subsequently transferred to the Excel sheets “Area Measurements : initial area first fronds (t0h)” and “Area measurements : Final area first frond (t72h)”, copy of which is given below.

The Excel programme will automatically process the figures and calculate the statistics for the area measurements, shown in the Excel sheet “data treatment”.

RESULTS INTERCALIBRATION SPIRODELA DUCKWEED MICROBIOTEST

Area measurements first frond t 0h

control

paste here

1800

paste here

3200

paste here

5600

paste here

10000

paste here

18000

paste here

Area measurements first frond t 72h

control

paste here

1800

paste here

3200

paste here

5600

paste here

10000

paste here

18000

paste here

RESULTS INTERCALIBRATION SPIRODELA DUCKWEED MICROBIOTEST

Name of operator

Institute / Laboratory / Company

Date of test performance

Table 1. Area measurements first frond t0h

Replicate	CONCENTRATION (mg/l)					
	Control Area (mm²)	1800 Area (mm²)	3200 Area (mm²)	5600 Area (mm²)	10000 Area (mm²)	18000 Area (mm²)
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
Mean	0	0	0	0	0	0
STDEV	0	0	0	0	0	0
CV%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Table 2. Area measurements first frond t72h

Replicate	CONCENTRATION (mg/l)					
	control Area (mm²)	1800 Area (mm²)	3200 Area (mm²)	5600 Area (mm²)	10000 Area (mm²)	18000 Area (mm²)
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
Mean	0	0	0	0	0	0
STDEV	0	0	0	0	0	0
CV%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Table 3. Area measurements first frond (t72h - t0h)

Replicate	CONCENTRATION (mg/l)					
	control Area (mm²)	1800 Area (mm²)	3200 Area (mm²)	5600 Area (mm²)	10000 Area (mm²)	18000 Area (mm²)
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
Mean	0	0	0	0	0	0
STDEV	0	0	0	0	0	0
CV%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
% inhibition		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Table 4. Logarithmic concentration vs. percent inhibition

Conc.	Log(conc)	I%
1800	3.26	#DIV/0!
3200	3.51	#DIV/0!
5600	3.75	#DIV/0!
10000	4.00	#DIV/0!
18000	4.26	#DIV/0!

OUTPUT RANGE: A90

Table 5. Effect concentration value

72h EC50 =	#DIV/0!
log x =	#DIV/0!

Annex 3 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) 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 have 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, for each endpoint and seed include stragglers, but not outliers.

Two figures show the results of the Grubb's test for h and k statistics, followed by 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).