

INTERNATIONAL INTERCALIBRATION EXERCISE ON THE ALGALTOXKIT MICROBIOTEST – COMPARISON WITH CONVENTIONAL ALGAL ASSAYS

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EXECUTIVE SUMMARY

The impact of toxicants on primary producers in freshwaters is mainly evaluated through the use of toxicity tests on micro-algae. So far, only a few interlaboratory comparisons have been made to evaluate the degree of standardisation of the 72h algal growth inhibition test.

In analogy to other cost-effective microbioassays which are independent of the year-round culturing of live test species, a miniaturised small-scale assay with micro-algae has been developed. The “Algaltoxkit” microbioassay adheres to the test procedure prescribed by the international organisations ISO and OECD for algal toxicity tests and is presently used in many laboratories worldwide.

In view of the scarcity of ringtests on micro-algae and in order to determine the interlaboratory variability of the Algaltoxkit microbioassay and to compare the precision of this test with that of “conventional” algal assays in glass vessels or in microplates, an “International Algaltoxkit Intercalibration exercise” was performed by the Laboratory for Environmental Toxicology and Aquatic Ecology of the Ghent University, Belgium..

Twenty eight laboratories from 14 countries participated in the ringtest and turned in 42 Algaltoxkit assay results using the reference chemical potassium dichromate. Five laboratories also provided EC50s for 5 tests in glass vessels and 8 for tests in microplates. Furthermore, 53 EC50s from quality control tests in glass vessels or in microplates were also received from 4 laboratories, as well as 76 “in house” quality control EC50s of Algaltoxkit assays from the company producing the kits.

From the information provided by the Algaltoxkit participants, it appeared that, except for a too high pH increase in the controls in some assays, all the results of the 42 tests fulfilled the validity criteria prescribed by ISO and OECD.

The EC50s for the Algaltoxkit assays were determined using 3 different calculation methods : the Area under the Growth Curve (AUGC) method (EbC50), the growth rate method (ErC50) and the yield method (EyC50).

Each calculation method resulted in a different mean EC50 with variation coefficients ranging from 31% to 40%. The mean EbC50 and EyC50 were, however, very similar.

The mean Algaltoxkit EbC50s and ErC50s were first compared to the mean EbC50 values obtained in the tests in glass vessels and in microplates submitted during the course of the ringtest, and subsequently also to those of the quality control tests provided by the 5 additional laboratories.

From these comparisons it appeared that a) the difference between the mean EbC50 and ErC50 of the Algaltoxkit and those of conventional algal tests was not larger than that between the mean EC50 of the laboratories that had provided individual data for the conventional tests or those from the laboratories with results for quality control tests and b) that the same conclusion could be drawn for the variation coefficients of the respective types of algal tests.

Statistical analysis of all the data using ANOVA analysis and a non parametric method revealed that Algaltoxkit results and results from conventional algal tests differed “either or not” significantly, depending on the method of statistical analysis. This was also the case for the comparison of the results between the different categories of conventional tests.

The conclusion of the International Algaltoxkit interlaboratory exercise is that this microbioassay is as reliable as algal tests performed in glass vessels and microplates and that its overall precision (repeatability and reproducibility) is as good as that of conventional algal testing procedures.

1. BACKGROUND AND RATIONALE

The acceptability of toxicity test methods - especially those used in regulatory frameworks - is highly dependent on their degree of standardisation. Many bioassays prescribed by national and international organisations have hence been the subject of “inter-laboratory” comparisons.

Concerning toxicity tests with freshwater micro-algae, 3 internationally accepted methods are currently in use :

- the 72h “Freshwater algal growth inhibition test with unicellular green algae”, described by the International Standardisation Organisation (ISO) for substances and mixtures contained in water, and for wastewater (ISO, 2004)
- the 72h “Algal Growth Inhibition Test” detailed in the Guidelines for Testing of Chemicals of the Organisation of Economic Cooperation and Development (OECD), and which is primarily used to assess chemicals (OECD, 2006)
- the 96h “Green alga *Selenastrum capricornutum* growth test” described in the “Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms” published by the United States Environmental Protection Agency (USEPA, 1994).

As far as “interlaboratory” standardisation of algal bioassays is concerned, to date only 3 major ringtests have been performed: in the early eighties the ISO organised two intercalibration exercises and in 1998 the USEPA assessed the interlaboratory variability study of 12 EPA acute and short-chronic whole effluent toxicity test methods including the 96h algal assay (USEPA, 2001).

In 2003, the need for a new interlaboratory exercise of the ISO freshwater algal bioassay was emphasized by the ISO working group on algae toxicity tests but no action has been taken so far.

The currently applied algal toxicity tests mentioned above are usually performed in Erlenmeyers or in glass containers as test vessels, and more recently also in microplates. For the sake of simplicity these tests will throughout this report be referred to as “conventional” algal tests.

Due to their complexity and high costs, the application of conventional algal bioassays is, however, limited and only performed by a small number of specialised laboratories. This is clearly illustrated by the participation degree for algal tests in the USEPA variability study of whole effluent toxicity test methods referred to above. In this study only 11 laboratories volunteered to perform this assay versus more than 40 laboratories for the tests with invertebrates or fish.

During the last two decades, efforts have been made to develop practical and cost-effective alternatives to “conventional” algal test procedures. One of the most popular techniques resulting from these endeavors is the algal assay in microplates. This micro-scale alternative of the tests in glass containers was endorsed by Environment Canada in 1992. The ISO, in its most recent revision of the freshwater algal growth inhibition test published in 2006, also allows the use microplates for rapid screening of wastewater.

Despite the latter progress, it should be recognised that the need for continuous culturing of live test organisms remains a major burden in ecotoxicology. This seriously limits routine application of bioassays.

The development of “culture-independent TOXKIT microbiotests” with micro-algae and invertebrates by LABRAP (the Laboratory for Biological Research in Aquatic Pollution at the Ghent University in Belgium, presently renamed Laboratory of Environmental Toxicology

and Aquatic Ecology LETAE), has therefore been acknowledged as a major breakthrough for practical and cost-effective toxicity testing.

A miniaturised algal assay, the Algaltoxkit, has been developed (Algaltoxkit, 1996), which basically adheres to the ISO 8692 method (ISO, 2004) and the OECD 201 Test Guideline for 72h algal growth inhibition tests (OECD, 2006). This microbiotest uses “algal beads” which can be stored for long periods of time, as the initial source of the algal cells. This micro-scale algal assay further uses disposable spectrophotometric cells of 10 cm path length as test containers which allow rapid direct scoring of the algal densities in a colorimeter or spectrophotometer without any additional manipulations.

During recent years, scientists in several countries have already made their own intra-laboratory comparisons of the relative sensitivity and repeatability of the Algaltoxkit microbiotest with that of the ISO or OECD algal test procedures (Latif and Zach, 2000; Vandebroele et al., 2000; Van der Wielen and Halleux, 2000; Lucivjanska et al., 2000 and Daniel et al., 2004). The outcome of these investigations all show that Algaltoxkit data correlate very well with those obtained with conventional algal assays.

Three inter-laboratory comparisons aiming at evaluating the degree of precision and standardisation of Toxkit microbiotests have been performed under the supervision of LABRAP. Already in 1989 three ringtests were organised with the first Toxkits : the Artoxkit M with the brine shrimp *Artemia*, and the Rotoxkit F and Rotoxkit M with freshwater and marine rotifers of the genus *Brachionus*. The results of these successful round robin studies have been published by Persoone *et al.* (1993).

An inter-laboratory comparison of the *Daphnia magna* acute test which also involved the Daphtoxkit F *magna* microbiotest was organised in 2003 and 2005 by the Italian Agency for Environmental Protection (APAT). The results of the 2003 ringtest involving 60 Italian laboratories have been published (Baudo et al., 2004)) and a peer-reviewed paper on the outcome of both exercises (in which eventually 105 laboratories participated) is now in preparation.

Yet, no in-between laboratory comparison has been made so far with the Algaltoxkit microbiotest. As such LETAE decided to organise and coordinate an international ringtest with this practical and low cost small-scale assay.

2. OBJECTIVES OF THE ALGALTOXKIT INTERCALIBRATION EXERCISE

The objectives of the International Algaltoxkit intercalibration exercise were :

- a) to determine the interlaboratory variability of the Algaltoxkit microbiotest;
- b) to compare the precision of the Algaltoxkit microbiotest with that of “conventional” algal toxicity tests.

Like for the recent *Daphnia magna* ringtests, potassium dichromate ($K_2Cr_2O_7$), which is one of the reference compounds suggested by ISO, was selected for the Algaltoxkit intercalibration exercise.

To avoid incorrect application of the Algaltoxkit by laboratories not familiar with this assay, the organisers decided to limit participation to laboratories known to have some experience with this microbiotest.

3. IMPLEMENTATION OF THE INTERCALIBRATION EXERCISE

3.1. Invitation to participate and conditions for participation

A call for participation was sent out by LETAE in February 2006; i.e. as indicated above, to laboratories known to have some experience with the Algaltoxkit test procedure.

The conditions for participation were as follows:

1. The Algaltoxkit test has to be applied according to the Standard Operational Test Procedure of this microbiotest.
2. The test has to be performed in a concentration range of 0.1 mg/l to 1 mg/l potassium dichromate, with the following 5 test concentrations (+ the control): 0.1 mg/l – 0.18 mg/l – 0.32 mg/l – 0.56 mg/l – 1 mg/l.
3. The laboratories interested in participating have to send the “participation sheet” to the organisers prior to the stipulated deadline.
4. Each participant will then receive one Algaltoxkit and practical information for the ringtest.
5. In order to avoid custom problems with “hazardous chemicals”, the reference chemical will not be included in the Algaltoxkit package. Each laboratory has to use potassium dichromate obtained from their local supplier.
6. Participating laboratories have to provide the organisers with the detailed results of their Algaltoxkit test prior to the stipulated deadline.
7. All submitted results will be processed by the organisers and treated confidentially.
8. A copy of the final report will be sent to all participants.
9. It is the intention of the organisers to prepare a peer reviewed publication on this international Algaltoxkit intercalibration exercise.

To generate data for the Algaltoxkit vs. conventional algal test comparison, participating laboratories were invited to also perform conventional algal tests with the same reference chemical. A few laboratories known to perform conventional algal toxicity tests were also contacted to obtain additional EC50s for potassium dichromate with these traditional algal assays.

3.2. Practical implementation and participants

Originally 33 laboratories indicated their interest to perform an Algaltoxkit test and 5 laboratories volunteered to submit results from their own conventional algal assays on potassium dichromate.

At the end of the exercise in August 2006, Algaltoxkit results were received from 28 laboratories and results obtained with conventional tests from 5 laboratories. Several laboratories provided results of 2 or 3 repeated assays so that in total results for 42 Algaltoxkit tests, 5 for assays in glass containers and 8 for microplate tests were available

NB. The 42 Algaltoxkit results are strictly spoken not all “interlaboratory data” since some of them are from repeated tests in the same laboratory. Yet for the sake of simplicity the terminology “interlaboratory” will be used throughout this report.

The results submitted for the Algaltoxkit tests originate from the following 28 laboratories in 14 countries :

Belgium : BFB Oil Research, Ecover Belgium, Epas, Institut Provincial d'Hygiene et de Bactériologie, MicroBioTests, Umicore Research

Cyprus : State General Laboratory

England : AlControl Laboratory

France : SGS Multilab, Yves Rocher,

Germany : Technical University Braunschweig – Institute of Ecological Chemistry

Guatemala : SEPRA

Italy : APAT, ARPA Emilia Romagna, ARPA Puglia, Ecobioqual, Milan University
- Laboratory for Environmental Toxicology

Poland : Institute of Industrial Organic Chemistry Warsaw, Institute of Organic Industry
Pszczyna, Medical University of Warsaw – Department of Environmental Health Sciences,
University of Gdansk – Department of Marine Biology and Ecology

Portugal : Coimbra University - Instituto do Ambiente e Vida, New University of Lisboa –
Environmental Biotechnology Research Unit

South Africa : Ecosun Environmental Laboratory

Spain : Fundacion TEKNIKER

Sweden : Mälardenen University – Department of Public Technology

The Netherlands : Waternet

USA : North Carolina Division of Water Quality

The following 5 laboratories sent results obtained with tests in glass containers or microplates
:

Belgium : ISSEP, VITO

France : Université Paul Verlaine Metz

Italy : Arpa Umbria

Portugal : Coimbra University – Instituto do Ambiente e Vida

South Africa : Department of Water Affairs and Forestry

In view of the limited number of “conventional” algal assay data, and to allow a better comparison of Algaltoxkit microbiotests with traditional algal assays, additional data were requested from 4 laboratories. ISSEP, Lisec and VITO in Belgium and Aquasense in the Netherlands provided EC50s based on their own algal quality control tests with the reference chemical potassium dichromate. Thirty eight additional results were eventually obtained from these laboratories for tests in glass containers and 15 for assays in microplates.

This eventually resulted in a total of 43 “glass container” and 23 “microplate” results for comparison with the 42 Algaltoxkit EC50s.

4. ANALYSIS AND DATA TREATMENT OF THE ALGALTOXKIT RESULTS

All participants in the Algaltoxkit ringtest were requested to submit the sheets with the daily “optical density” measurements to allow the organisers to calculate the 72h EC50.

In addition the participants also had to submit a “Test Conditions Sheet” specifying the following :

- the type of spectrophotometer used for the optical density measurements in the long cells;
- the “water” temperature inside the incubator during the test period;
- the type of illumination (lateral or bottom) of the long cells in the transparent box;
- the light intensity at the surface of the long cells during the test;
- the optical density of the algal suspension in the algal growth medium in the 25 ml calibrated flask, prior to dilution;

- the pH of the algal growth medium at the start of the test;
- the pH of the algal suspension in the control long cells at the end of the test.

4.1. Data analysis of the environmental conditions for the Algaltoxkit assays

1. Measurement of the optical density : From the 28 participating laboratories, 15 reported to have used a Jenway 6300 spectrophotometer for the optical density measurements in the long cells. The other laboratories performed the measurements in 9 other types of spectrophotometers equipped with a holder for 10 cm cells (Perkin Elmer, Shimadzu, Jasco, Genesys, Hewlett-Packard, Varian, Unicam, Hitachi, AquaMate).
2. Incubation temperature : According to the information received, all the tests were performed in the temperature range 21-25°C, with a maximum variation of 2°C during the exposure period.
3. Illumination : 10 laboratories indicated that the transparent box with the long cells received light from below and 15 laboratories reported lateral illumination. Three laboratories did not specify the illumination.
4. Light intensity : The illumination intensity varied substantially from one laboratory to the other. For bottom illumination, light intensities ranging from 3,000 to 10,000 lux were reported, and from 4,000 lux to 1,000 lux for lateral illumination.
5. Optical density (OD) of the algal suspension prior to dilution : After de-immobilisation from the algal beads followed by centrifugation and rinsing, the optical density of the algal suspension in the 25 ml flasks was mostly in the range 1.0 to 1.4 with min-max values of 0.6 and 1.6.
6. pH of the algal suspension in the 25 ml flasks prior to dilution : The majority of the pH values (which basically correspond to the pH in the controls at the start of the bioassay) was between 7.8 and 8.1. Only two lower values (7.2 and 7.5) were reported.
7. pH of the algal suspension in the controls at the end of the test : The range between the minimum and the maximum pH in the controls after the 72h incubation period was quite large. From the 35 reported pH values (two participants did not report their final pH value) 20 were in the pH range 8.8 – 9.3, 9 in the 9.4 – 9.9 range and 5 in the 8.0 – 8.6 range.

4.2. Algal growth and pH increase in the controls

Exponential growth of the algae in the controls and a maximum increase value of the pH at the end of the exposure period are two criteria which are used by both the ISO and the OECD to determine the validity of the bioassay.

Until the recent revisions of the ISO and OECD guidelines both organisations prescribed that the cell concentration in the controls “should have increased by a factor of at least 16 within 3 days”. The recent ISO 2004 document, however, indicates that an average control growth rate of at least 1.4 day⁻¹ should be obtained, which corresponds to an increase in cell density by a factor 67.

In order to calculate the increase in algal density in the Algaltoxkit tests, the 72h OD values reported by the participants for the controls were transformed into cell numbers by application of the OD/N regression formula, which is “algal batch specific”. For the Algaltoxkit ringtest,

for which algal batch SC080306 was used, the optical density/cell number (OD/N) regression was : $N = 1700481 \cdot OD - 68237$, with N=number of cells.

The outcome of the calculations revealed that in all the Algaltoxkit assays the increase in algal density in the controls at the end of the test was higher than the factor 16 indicated by the OECD and only in 3 assays was the algal multiplication below the factor 67 presently prescribed by ISO.

With regard to the increase of pH in the controls at the end of the test, both the ISO and the OECD, in their recent revisions, indicate a maximum increase of 1.5 pH units for the validity of the bioassays.

From the pH increase values calculated for 36 tests in the Algaltoxkit ringtest (as indicated above some participants did not provide the pH values), 25 were below 1.5 units. For 5 assays, the increase was less than 0.1 pH unit above the 1.5 threshold, but for 6 others the pH increase was ≥ 1.7 units.

Since in quite a number of the Algaltoxkit assays, the increase in cell numbers was higher than a factor 100 (even up to 150 and 180) the substantial algal growth is probably the cause of the quite high pH increases in the controls in some assays. All the “high” pH values (9.4 to 9.9) in the controls at the end of the test were indeed from tests in which the algal numbers had increased by a factor 100 or more.

The ISO 2004 and OECD 2006 versions of the algal growth inhibition test also indicate that the variation coefficient of the growth rates in the control replicates should not exceed 5% or 7%, respectively.

A detailed analysis of the growth rates in the 3 replicates of the controls revealed variation coefficients below 5 % for all 42 Algaltoxkit tests.

NB : Algaltoxkit tests are normally performed with 3 replicates for the controls and for the toxicant dilutions. The ISO, however, prescribes 6 replicates for the controls, whereas the OECD only “advise” to use 6 control vessels...

The Algaltoxkit ringtest results demonstrate the uniformity of the algal growth in the 3 control replicates. The 5% variation coefficient recently prescribed by ISO was indeed not exceeded in any of the 42 Algaltoxkit tests performed by the 28 participating laboratories.

4.3. Calculation of the 72h EC50

As mentioned above, the participants had to send their detailed results to the organisers for calculation of the 72h EC50.

In order to make the best use of the data generated in this ringtest, the organisers decided to derive EC50s with 3 different calculation methods : the area under the growth curve (AUGC) method, the growth rate method and the yield method. The variability of the EC50s obtained with each of these 3 methods was compared.

The AUGC calculates the 72h EbC50 from the reduction of the biomass integral. A specific computer programme was developed in the early nineties by LABRAP for the Algaltoxkit microbiotest which calculates the EbC50 directly from the optical density values of the algal

suspensions in the long cells. The AUGC is one of the two methods originally prescribed by both the ISO and the OECD for EC50 calculation.

The second data treatment procedure, namely the growth rate method, calculates percentage inhibition of the growth rate and results in the 72h ErC50.

Both ISO and OECD have, in their recent revisions, decided not to recommend the AUGC calculation method anymore. Details on the rationale of this decision are given in the September 2006 ISO review “Water Quality – Scientific and technical aspects of batch algal growth inhibition tests (ISO 2006).

In its March 2006 revision of the alga growth inhibition test, the OECD also includes a second data treatment method, based on the “yield” of the algal populations. Yield is the biomass at the end of the exposure period minus the biomass at the start in each of the treatments. The difference in yield in the toxicant treatment and in the control allows calculation of the 72h EyC50. Like the AUGC method, biomass can be expressed as cell numbers (which in the case of the Algaltoxkit microbiotest can be derived from optical densities).

4.3.1. Data treatment using the Algaltoxkit computer programme and calculation of the EbC50

The EbC50s of the 42 Algaltoxkit tests are represented graphically in Figure 1. The mean value (0.52 mg/l) \pm one standard deviation (0.21 mg/l) are indicated by the lines. The variation coefficient calculated from these data is 40 %.

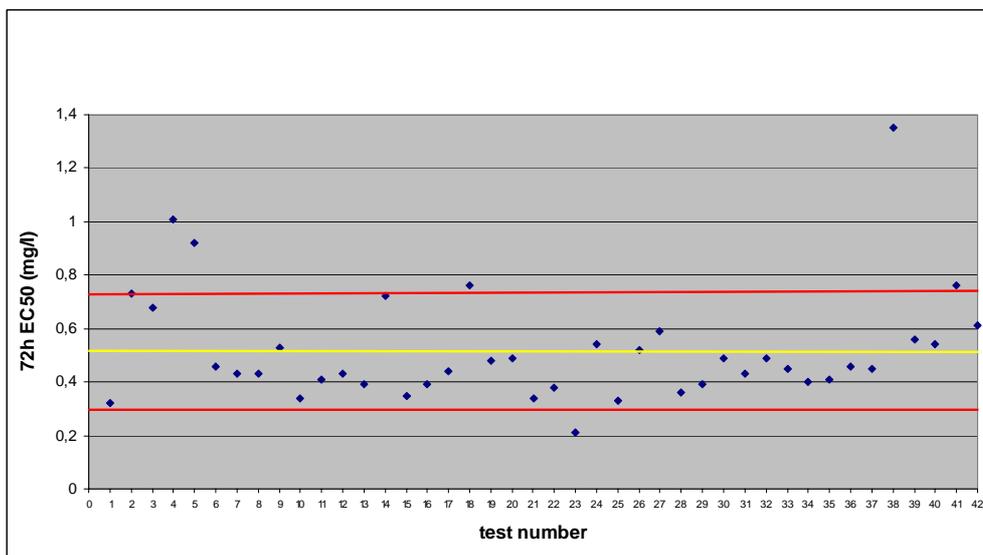


Figure 1. EbC50s of the 42 Algaltoxkit tests, with the mean \pm 1 S.D.

These results indicate that 85 % of the EbC50s are situated within one standard deviation of the mean (i.e. in the range 0.31-0.73 mg/l). Only 6 data points are outside of this range, 5 are above 0.73 mg/l and one is below 0.31 mg/l.

4.3.2. Data treatment using the growth rate method and calculation of the ErC50

The mean OD values for the controls and the toxicant concentrations of the 42 assays were first transformed into cell numbers with the OD/N regression given above, after which average specific growth rates were calculated with the following formula indicated in the ISO and OECD guidelines : $\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0}$

with N_L = cell density at the end of the test, N_0 = cell density at the start of the test and $t_L - t_0$ = test duration in days.

Inhibition percentages of the growth rates in the toxicant concentrations in comparison to the growth rate of the control were subsequently calculated to determine the ErC50.

These calculations, however, revealed that for a number of tests the percentage inhibition at the highest test concentration (1 mg/l) was < 50%, and that as a result, the EC50 was beyond the range of test concentrations used for the Algaltoxkit ringtest.

For the latter tests, the logistic model described in the OECD Guideline for the *Daphnia* reproduction test (OECD 1998) has been used to calculate the EC50 through extrapolation. This was done by fitting growth rate responses to a logistic model using the least squares method with the aid of the Statistica software package 6.0.

For reasons of uniformity, it was eventually decided to also recalculate all the other ErC50s for the 42 Algaltoxkit tests using this method.

The data presented in Figure 2 correspond to a mean ErC50 of 0.84 mg/l with a standard deviation of 0.27 mg/l and a variation coefficient of 31%.

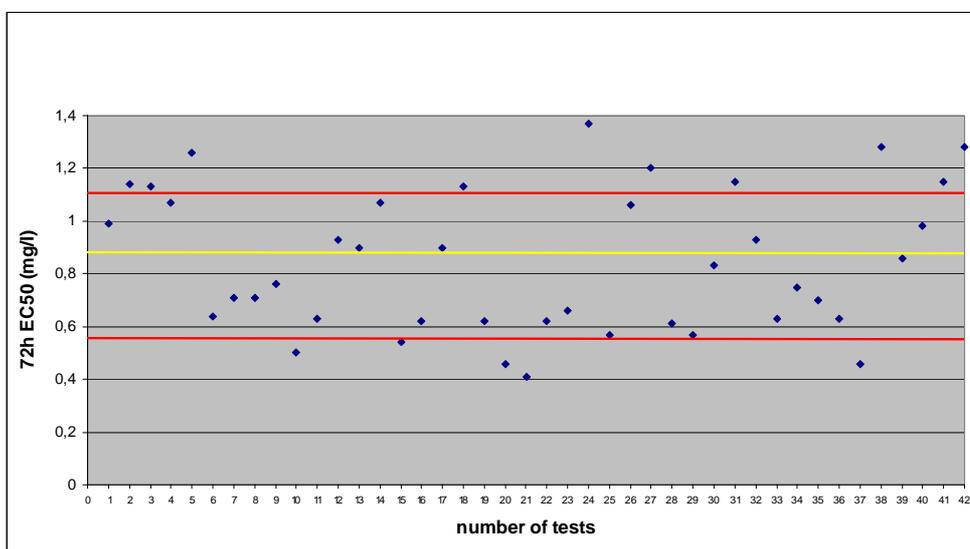


Figure 2. ErC50s for the 42 Algaltoxkit tests with the mean \pm 1 S.D.

Figure 2 shows that 64% of the ErC50s - of which 13 values were “extrapolated” EC50s - were situated within one standard deviation of the mean (versus 85% for the EbC50 calculations).

NB. The organisers are aware that the procedure of extrapolating EC50s can be criticised and that it would have been much better if the range of test concentrations chosen for the ringtest had been from 0.18 mg/l to 1.8 mg/l instead of from 0.1 mg/l to 1 mg/l.

The reason for the at first sight unfortunate range selection is due to the fact that Algaltoxkit results are normally processed with the specific Algaltoxkit data treatment programme which calculates the 72h EbC50...

With the latter data treatment method, the effect level in quality control tests with potassium dichromate is always > 50 % at 1 mg/l and therefore the 0.1 - 1 mg/l range was (logically) selected for the Algaltoxkit ringtest...

4.3.3. Data treatment using the yield method and calculation of the EyC50

Two EyC50 values were calculated: the first was based on the OD values of the algal suspensions in the long cells after the 72h exposure, the second was obtained after transformation of the OD's into cell numbers. Both EyC50's were calculated using the moving average method.

The mean EyC50 based on OD values was 0.50 mg/l with a standard deviation of 0.18 mg/l and a variation coefficient of 36 %. All the EyC50s were within the 0.1- 1 mg/l range selected for the Algaltoxkit ringtest and 81% were situated within one standard deviation from the mean. Seven data points were above 0.68 mg/l and 1 EyC50 was below 0.32 mg/l.

Based on cell numbers, the mean EyC50 was 0.47 mg/l with a standard deviation of 0.16 mg/l and a variation coefficient of 34 %. Seventy six % of the EyC50s were within one standard deviation from the mean. Seven data points were above 0.63 mg/l and 3 below 0.31 mg/l .

Both calculations showed that irrespective of the use of OD's or cell numbers, the mean EyC50s were nearly identical.

Finally, as represented in Figure 3, the two mean EyC50 values (0.50 mg/l and 0.47 mg/l) are also very similar to the mean EbC50 (0.52 mg/l) indicating that the AUGC and the yield data treatment methods (which are both based on "biomass") give very similar results.

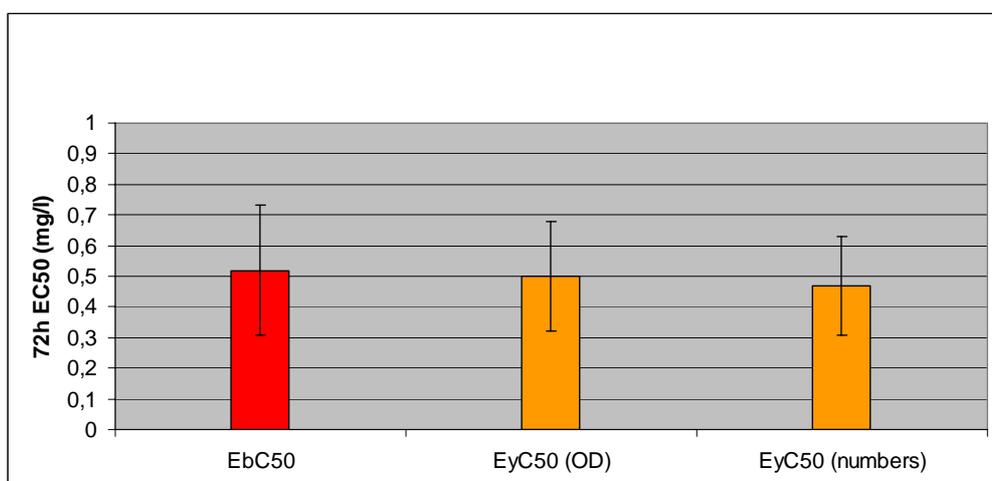


Figure 3. Mean EC50 of the Algaltoxkit tests calculated as EbC50 (AUGC), EyC50 (OD values) and EyC50 (numbers)

5. ANALYSIS AND DATA TREATMENT OF THE RESULTS OF ALGAL TESTS PERFORMED BY CONVENTIONAL METHODS

5. 1. Data analysis of the the tests in glass containers and in microplates performed in the framework of the Algaltoxkit ringtest

As indicated in the section *Practical Implementation and Participants*, only 5 results were received for tests in glass vessels and 8 for assays in microplates. All these assays were also performed according to either the ISO or the OECD procedures for algal tests.

The laboratories providing the data on conventional algal assays had been asked to calculate themselves the 72h EC50 using both the AUGC method (EbC50) and the growth rate method (ErC50).

From these data the organisers calculated the mean EbC50 and ErC50 (with the corresponding standard deviation and variation coefficient) in order to compare them to the mean Algaltoxkit EC50s.

Figure 4 shows that the mean EbC50 for the microplate tests (0.53 mg/l) is virtually identical to that of the Algaltoxkit assays (0.52 mg/l). The standard deviation for the 8 microplate tests is 0.17 mg/l and the variation coefficient 33 %.

The mean EbC50 for the 5 tests in glass vessels is 0.66 mg/l, with a standard deviation of 0.17 mg/l and a variation coefficient of 29 %.

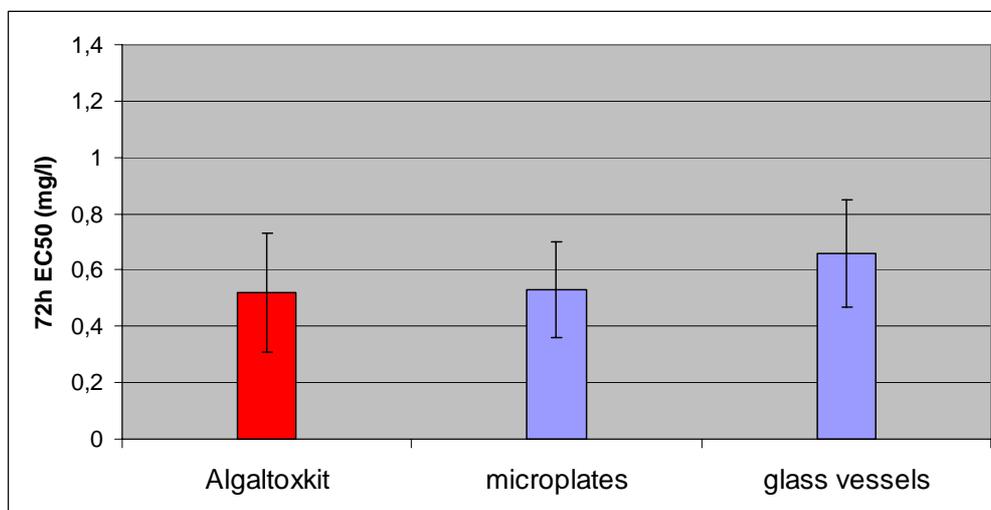


Figure 4. Mean EbC50 of the Algaltoxkit tests and the tests in microplates and in glass vessels from individual laboratories.

Figure 5 shows the same comparison for the ErC50 values, i.e. a mean value of 1.16 mg/l for the assays in microplates, with a standard deviation of 0.40 mg/l and a variation coefficient of 34 %. For the tests in glass containers the mean ErC50 is 1.55 mg/l with a standard deviation of 0.75 mg/l and a variation coefficient of 48 %.

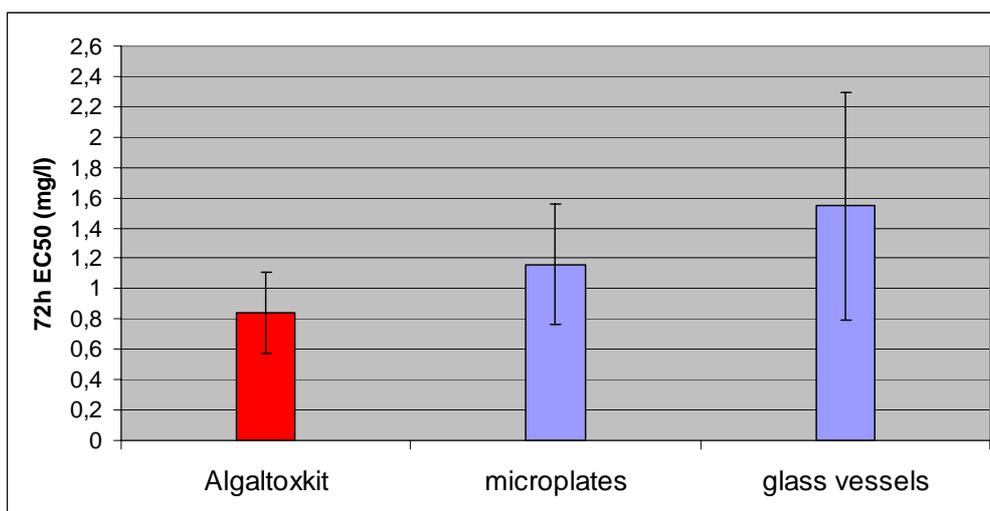


Figure 5. Mean ErC50 of the Algaltoxit tests and the tests in microplates and in glass vessels from individual laboratories.

5.2. Data analysis of the tests in glass vessels and in microplates: results submitted subsequent to the Algaltoxit ringtest

As already mentioned above, subsequent to the Algaltoxit ringtest 4 laboratories kindly sent EC50 data for their “in house” quality control tests with potassium dichromate, either as EbC50 or as ErC50. These 4 laboratories will be referred to hereunder as laboratories A, B, C and D.

Unfortunately not all 4 labs could provide their results calculated as both EbC50s and ErC50s.

Mean EC50s with standard deviation and variation coefficients have been calculated for these complementary data and are given in Table 1. This table also contains the EC50s from tests in glass flasks and in microplates obtained earlier.

Table 1. EC50s from conventional algal tests (in glass vessels and in microplates)

Tests in glass vessels

Laboratory	Number of tests	Mean EbC50 (mg/l)	Standard deviation	Variation coefficient (%)
Individual labs	5	0.66	0.19	29
Lab A	11	0.58	0.18	31
Lab B	22	0.87	0.09	10
Labs C and D	No data			
Laboratory	Number of tests	Mean ErC50 (mg/l)	Standard deviation	Variation coefficient (%)
Individual labs	5	1.55	0.75	48
Lab A	11	1.02	0.30	29
Lab B	22	1.53	0.24	16
Lab C	No data			
Lab D	5	1.06	0.29	28

Tests in microplates

Laboratory	Number of tests	Mean EbC50 (mg/l)	Standard deviation	Variation coefficient (%)
Individual labs	8	0.53	0.17	33
Labs A,B, C, D	No data			
Laboratory	Number of tests	Mean ErC50 (mg/l)	Standard deviation	Variation coefficient (%)
Individual labs	8	1.16	0.40	35
Labs A and B	No data			
Lab C	8	1.53	0.20	13
Lab D	7	1.00	0.09	9

Individual laboratories : *results provided during the ringtest by individual laboratories*
 Laboraties A, B, C, D : *results of quality control tests from 4 different laboratories*

From this table, the following observations be made :

1. The inter- and intralaboratory variability of the conventional algal assays is (approximately) 10 to 35%, irrespective of the method of calculation or the type of test container (glass vessels or microplates).
2. A substantially higher CV was, however, noted for the 5 ErC50s of the tests performed in glass vessels (48%).

Comparison of these data with those of the Algaltoxkit shows that the variation coefficients of the EC50s of the 42 Algaltoxkit (31% for the ErC50 and 40% for the EbC50) are similar to the overall range of CVs obtained with conventional algal tests.

6. COMPARISON OF THE ALGALTOXKIT RINGTEST RESULTS WITH THOSE OF QUALITY CONTROL TESTS PROVIDED BY MICROBIOTESTS INC

The Algaltoxkit EC50s calculated from the data of the 28 participating laboratories were also compared with those from quality control tests performed by the company MicroBioTests Inc which manufactured and shipped the Algaltoxkits to the participants of this ringtest.

Table 2 gives the mean EbC50s (with standard deviation and variation coefficient) for the 42 Algaltoxkit tests of the ringtest, and 76 quality control tests performed by MicroBioTests from 2002 to 2006 with 16 different batches of algal beads which allows to make an interesting comparison of “inter” versus “intra”laboratory

Table 2. Comparison of the mean EbC50 of the Algaltoxkit ringtest with the mean EbC50 of quality control tests performed by MicroBioTests Inc

	Number of tests	Mean EbC50 (mg/l)	Standard deviation	Variation coefficient (%)
Algaltoxkit ringtest	42	0.52	0.21	40
MicroBioTests Inc.	76	0.46	0.10	22

From this table it appears that the two mean EC50s only differ by about 10%. The “in house” repeatability of the Algaltoxkit microbiotest over a period of several years and with algae from different batches of algal beads is around 20% versus 40% variability for the “interlaboratory” results of the present intercalibration exercise.

7. GENERAL DISCUSSION OF THE ALGALTOXKIT RINGTEST RESULTS

A number of comments and some interpretations of the data have already been given in the different sections of this report and will not be repeated in this general discussion.

7.1. Overall number of data

For the variability analysis of Algaltoxkit microbiotests, 42 results are available from tests performed in 28 laboratories.

For the comparison of Algaltoxkit data with those of conventional algal tests, 43 data were obtained for tests in glass vessels and 23 for tests in microplates. These data were provided by 12 laboratories.

7.2. Variability of Algaltoxkit test results

The quite low variation coefficient (22%) of all the quality control tests performed with algae from different algal bead batches by MicroBioTests over a period of several years, demonstrates that the biological quality and the sensitivity of the algal test species used for the preparation of the algal beads is quite stable.

Depending on the method of data treatment, the variation coefficients for the 42 Algaltoxkit tests performed by the 28 laboratories range from 40% (EbC50 calculation method) to 31% (ErC50 determination) and 34-36% (EyC50 treatment).

These figures are higher than the “in house” variability for the quality control tests of MicroBioTests, but this should be no surprise. Because of the higher degree of uniformity, “intra” laboratory testing conditions are indeed more uniform than those in different laboratories.

The percentage of EC50s situated within 1 standard deviation from the mean varies from 85 % to 64% to 76-81% for the 3 data treatment methods, respectively. These data clearly indicate the importance of the selected methodology not only for the calculated EC50, but also for the variation coefficients.

Although it is certainly an interesting subject for an in depth discussion, it is, however, beyond the scope of this Algaltoxkit ringtest to analyse the advantages or the weaknesses of different data treatment methods for algal tests.

Let it just be mentioned that the Algaltoxkit intercalibration exercise clearly revealed that data treatment by the OECD “yield” method, virtually gives the same outcome as the EbC50 calculation by the (now rejected) AUGC data treatment procedure...

The variability outcome of the Algaltoxkit ringtest can only be compared with that of the 2 other algal interlaboratory exercises referred to in the section “Background and Rationale”, namely the ISO and the USEPA algal growth inhibition ringtests.

In the ISO ringtest a variation coefficient of 23% is reported for the ErC50s for assays on potassium dichromate with *Pseudokirchneriella subcapitata*. This figure is, however, based

on only 4 test results. From the 9 ErC50 data which were submitted for this ISO ringtest, 5 were rejected because these participants apparently had been using different growth media.

The Final Report of the Interlaboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods (USEPA 2001) mentioned in the Introduction of this report, indicates that a substantial number of the results obtained for assays on blank water samples, on effluents, on receiving waters and on a reference toxicant (KCl) were considered “invalid” or “inconclusive”. The percentage of algal assays performed in this ringtest considered valid was around 60% and variation coefficients for these assays ranged from 9% to 60%.

From the results of these two round robin exercises, it can be concluded that the inter- as well as the intra-laboratory variability of the present Algaltoxkit microbiotests is similar to lower to that obtained with conventional algal tests. The 30-40% variability obtained in Algaltoxkit ringtest is, like for all interlaboratory comparisons, related to slightly different environmental and experimental conditions in the participating laboratories.

The ringtest also revealed that the variability of the growth rate in the controls was very low in all the Algaltoxkit results reported by the participants. Consequently it does not seem necessary to increase the number of control replicates to 6.

Finally the Algaltoxkit intercalibration exercise also revealed that the growth of the algae released from algal beads is exponential during the 72h test period.

7.3. Relationship pH increases and EC50s

The “water surface area” of the long cells is smaller than that in microplate cups, and probably also smaller than that in glass vessels, which means a lower “surface to volume” ratio and hence also a lower CO₂ transfer rate from the air into the algal suspension. This may possibly lead to larger pH increases in Algaltoxkit microbiotests than in conventional algal tests.

As indicated in the section “Algal growth and pH increase in the controls after 72h incubation”, a substantial increase in pH in the controls has been reported for a number of Algaltoxkit tests; 6 values were above the 1.5 pH units threshold increase, and 5 others values were “at the limit”.

The rationale of ISO and OECD in prescribing thresholds for the increase of the pH in algal tests is inspired by the possible impact of pH increase on speciation or ionisation of some chemicals, or on other chemical reactions in mixed samples.

This concern, however, does not seem to apply to the present ringtest with potassium dichromate as shown by the very low r^2 values which were calculated for the regression between the EC50s and the pH increases in the controls on one hand, and between the EC50's and the pH values at the end of the exposure period on the other.

The r^2 values for the calculated regressions are as follows :

EbC50/pH increase in the controls : $r^2 = 0.030$

ErC50/pH increase in the controls : $r^2 = 0.008$

EbC50/final pH in the controls : $r^2 = 0.019$

ErC50/final pH in the controls : $r^2 = 0.000$

This does, however, not mean that the problem of the high pH increases noted for many Algaltoxkit tests should not be addressed. As suggested in the ISO guideline for algal toxicity tests, an increase of the shaking frequency or intensity of the long cells will probably increase the CO₂ transfer from the air and contribute to a lower pH increase.

7.4 Variability of the results of conventional algal tests

The interlaboratory variation coefficients of the conventional algal tests given in Table 1, indicate that the variability is as high as that of the Algaltoxkit assays. Furthermore the intralaboratory variation coefficients of the quality control tests from 4 laboratories also range from quite low (9-10%) to >30%.

The variation coefficient of all the EC50 data (intra- and interlaboratory) received for conventional algal toxicity tests is discussed further below. For the glass vessels, the CV is 29% for the EbC50 and 33% for the ErC50; for the microplates, these figures are 33% and 28%, respectively.

These results again clearly indicate that the precision of the Algaltoxkit technology is as good as that of conventional algal tests in other types of containers.

7.5. Correspondence between Algaltoxkit results and results of conventional algal tests

In order to allow for an easy visual comparison of the mean EC50 data from the different “types” of tests (Algaltoxkit tests, microplate assays, glass flask tests from individual laboratories and quality control tests from both the 4 laboratories and the company MicroBioTests), two summary figures were made. Figure 6 shows all mean EbC50s and Figure 7 all ErC50s, with their respective standard deviation and indication of the number of assays on which the mean EC50s were calculated.

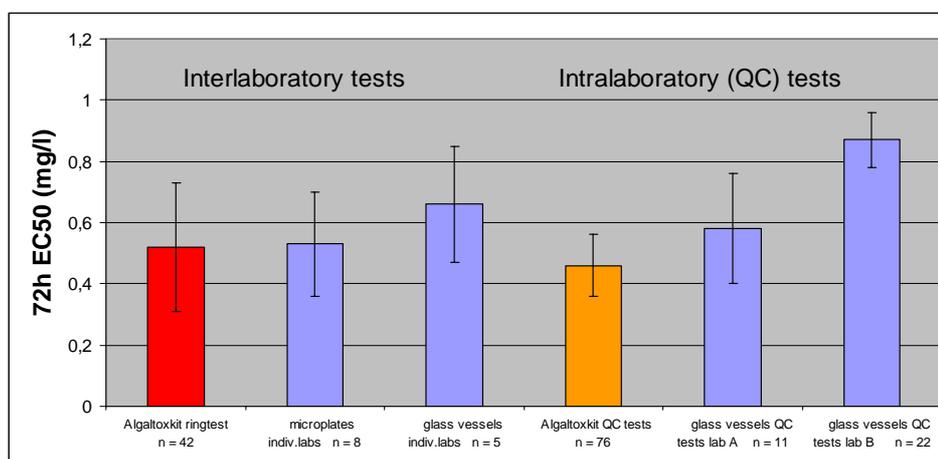


Figure 6. Mean EbC50s for all the “interlaboratory” and “intralaboratory (quality control)” results

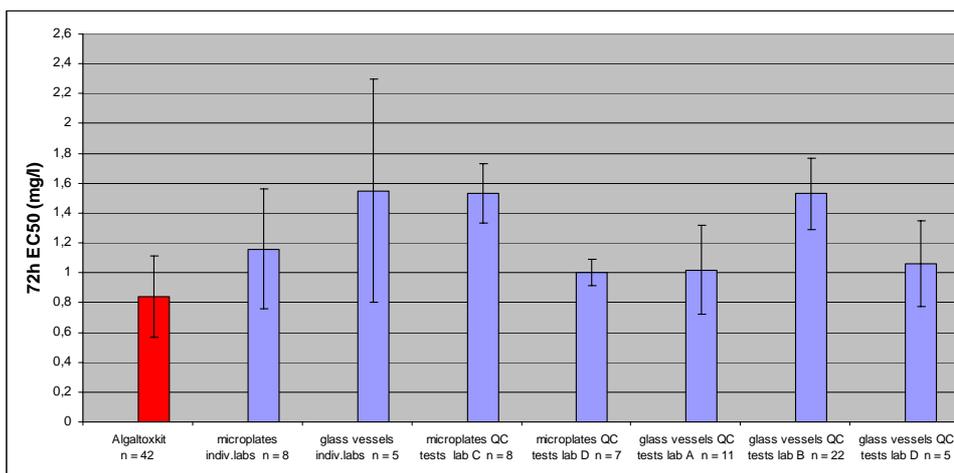


Figure 7. Mean ErC50s for all the “interlaboratory” and “intralaboratory (quality control)” results.

From Figure 6 and Table 2, it appears that the mean EbC50 value for the 42 Algaltoxkit tests (0.52 mg/l) is very close to the mean EbC50 (0.46 mg/l) of the 76 quality control Algaltoxkit tests performed in the company MicroBioTests. This finding is evidence of the high degree of standardisation of the Algaltoxkit microbiotest.

All mean EbC50s in Figure 6 for the different types of tests are situated in a rather narrow range (0.46 - 0.66 mg/l) except for the quality control tests of laboratory B in glass flasks, for which the mean EbC50 is 0.87 mg/l.

The mean ErC50s given in Table 1 and Figure 7 in turn show a larger variability between the different test types: the range between the lowest and the highest mean values obtained in “conventional” algal quality control tests range from 1 mg/l to 1.5 mg/l, whereas for the Algaltoxkit tests, the mean EC50 (0.84 mg/l) is about 20% lower than the lowest mean ErC50 value obtained with conventional tests.

As already emphasized above, these data clearly indicate the effect of the data treatment procedure on the EC50s, and as a result also on the degree of correspondence of the EC50s for the different types of algal assays.

It may be mentioned that neither low nor high EC50s can be regarded as “outliers” or “non acceptable”. Indeed, contrary to the acute toxicity test for *Daphnia magna*, for which the ISO stipulates a “validity range” for quality control tests, this is not the case (neither from ISO nor from OECD) for algal toxicity tests.

The mean ErC50 of the Algaltoxkit tests (0.84 mg/l) is lower than the mean value (1.19 mg/l) reported by ISO for the algal ringtest performed in 1981 (but which is based on only 5 tests performed in 5 laboratories). This difference, however, should be evaluated in the context of the other results in the present exercise. Indeed comparison of the mean ErC50 of the interlaboratory tests in glass vessels (1.55 mg/l) and the range of ErC50s for the quality control tests provided by 4 laboratories (1.02 mg/l to 1.53 mg/l) indicates that the above indicated ‘difference’ is negligible.

We have, however, performed statistical significance testing to determine whether or not the various mean EC50s obtained with the different testing technologies were significantly different.

Two types of analysis were performed on both the EbC50s and the ErC50s: i.e. analysis of variance (ANOVA) and/or, where required, a non parametric method.

In total, 36 statistical comparisons were made; the main findings of which can be summarized as follows :

- a) there were significant differences between the mean EC50s between some types of tests, but not between others;
- b) significant differences were found between the Algaltoxkit data and those of conventional tests for some comparisons, but not for others
- c) the same finding was noted for the statistical comparison of the results of the conventional tests, either between the results from individual laboratories and the quality control tests, or between the quality control tests submitted by the laboratories.

Since significant differences were found for a number of comparisons between tests in different types of test containers, it cannot be concluded that the Algaltoxkit data differ more from those of the conventional algal tests, than the latter data differ from each other.

8. CONCLUSIONS OF THE ALGALTOXKIT INTERCALIBRATION EXERCISE

This ringtest can be considered as a successful exercise from many points of view.

The large participation (28 laboratories for the Algaltoxkit assay, and 12 laboratories for conventional algal tests) made it the largest intercalibration exercise on an algal toxicity tests ever. This ringtest indeed generated a data base of 42 Algaltoxkit results, 66 EC50s of conventional algal tests and 76 EC50s for quality control Algaltoxkit tests from the company that had provided the kits for the ringtest.

All the Algaltoxkit tests were performed according to the test procedure prescribed by the international organisations ISO and OECD, which allowed for comparison of the Algaltoxkit data with those of conventional algal tests (also performed according to the same procedure).

Analysis of the individual Algaltoxkit data showed that (with a few exceptions) the assay results met the validity criteria indicated by ISO and OECD with regard to the growth rate in the controls and the variation coefficient of the average control growth rates.

The criterion which was not met by about 30% of all Algaltoxkit tests was pH increase in the controls of maximum 1.5 pH units as prescribed by the ISO and the OECD documents. For the present ringtest with potassium dichromate, there was, however, no impact of the high pH values on the EC50s.

The analysis of the individual Algaltoxkit results revealed that the mean EC50 and the variation coefficient were dependent of the data treatment method. The interlaboratory variability of the Algaltoxkit tests was comparable to that obtained by the laboratories that provided results from conventional algal assays. The intralaboratory variation coefficient of the (large number of) quality control tests performed by the company that had provided the Algaltoxkits for the ringtest was also in the range of the variation coefficients from quality control tests using conventional algal testing procedures in glass vessels.

Statistical analysis of the correspondence of the Algaltokit results with those from conventional algal tests revealed significant differences for some comparisons, but not for others. Similar findings were made for comparison of the data of quality control tests performed in different laboratories. Finally the absence or presence of significant differences were in some cases also dependent of the data treatment method and of the method of statistical analysis.

From this extensive inter- and intralaboratory exercise it can be concluded that the Algaltokit microbiotest is as reliable as 'conventional' algal tests in glass vessels or in microplates.. As shown by the detailed analysis of the Algaltokit results, the overall precision (repeatability and reproducibility) of this microbiotest is as good as that of conventional algal test procedures.

9. ACKNOWLEDGMENTS

The organisers herewith extend their appreciation and sincere thanks to all those who have actively participated in this ringtest by providing Algaltokit data, and/or data on conventional algal tests. This final report is only a modest reflection of the substantial amount of time and effort dedicated to the ringtest by each participating laboratory.

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