INTERNATIONAL INTERLABORATORY COMPARISON ON THE SUBCHRONIC TOXICITY TEST WITH THE FRESHWATER OSTRACOD CRUSTACEAN *HETEROCYPRIS INCONGRUENS*

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INTRODUCTION

In aquatic ecosystems, chemicals not only pollute the water but accumulate in the sediments, and potentially can affect benthic biota.
Besides toxicity tests on water samples with pelagic test species, bioassays are therefore to date performed in parallel on sediment samples, to make an ecological meaningful estimation of the "toxic hazard" of the concerned aquatic environment.

Two types of toxicity tests are usually performed to determine the seriousness of the degree of pollution of sediments : bioassays with pelagic test species on the interstitial water fraction of the sediment (pore water), and "direct contact tests" (also called solid phase tests) with benthic test organisms.

Historically two types of test species have taken the lead for application of direct contact tests for freshwaters: the amphipod crustacean *Hyalella azteca* and the chironomids *Chironomus riparius* and *Chironomus tentans*.

Similarly to other toxicity tests with invertebrate test species, these assays are dependent on the year-round culturing and maintenance of live stocks of the organisms, with all inherent equipment, space and labour costs.

In order to bypass the latter (major) handicap, extensive research has been performed for more than 20 years at the Laboratory for Environmental Toxicology and Aquatic Ecology at the Ghent University in Belgium, to make toxicity tests "independent" of the culturing of stocks of the test organisms.

The goal of this research was to arrive at a controlled production of "dormant eggs" of the test species, which can be stored for long periods of time, and from which live test organisms can be hatched at the time of performance of the assays, without any need for stock culturing and maintenance.

The investigations have led to the development of "culture/maintenance free microbiotests" with various test species, also including a small epibenthic ostracod crustacean : *Heterocypris incongruens*.

A subchronic "direct contact" assay for sediments was eventually worked out with this ostracod, based on a lethal (mortality) and a sublethal (growth inhibition) effect criterion, after 6 days of exposure of the neonates hatched from the dormant eggs (cysts) to the whole sediment sample.

The availability of the dormant eggs of *H. incongruens* subsequently triggered research on the comparison of the sensitivity of the ostracod test species, in comparison to that of the "conventional" test biota used for direct contact tests.

These studies revealed that the sensitivity of *H. incongruens* is similar to that of the amphipod crustacean *H. azteca* and the midge larva *C. riparius* (Chial and Persoone, 2002; Chial *et al*., 2003a; 2003b; Blaise *et al*., 2004).

Over the last 7 years the Environmental Agency of Flanders in Belgium (VMM) has applied the ostracod contact test in parallel to the *H. azteca* whole sediment test on about 1000 samples originating from 600 different sites of rivers in Flanders.
Statistical analysis of the data pairs revealed that for the majority of the samples both test organisms also gave a similar indication on the subchronic impact of the sediments (De Cooman and Persoone, 2009).

A substantial number of studies have also been performed over the last few years in several countries on the use of this microbiotest to assess the toxicity of sludges and soils.

PROPOSAL TO THE INTERNATIONAL STANDARDISATION ORGANISATION (ISO) FOR A NEW WORK ITEM ON THE SUBCHRONIC *HETEROCYPRIS INCONGRUENS* MICROBIOTEST

The *H. incongruens* microbiotest is to date already used extensively in many countries.
This has triggered the request from various sides to propose this microbiotest to international organisations for endorsement as a "standard toxicity test", for specific applications in a regulatory framework.

A request has therefore been submitted in 2009 by the Belgian Bureau for Normalisation to the International Standardisation Organisation (ISO) to take the *Heterocypris incongruens* microbiotest in consideration as a new ISO standard ecotoxicological test.

A call related to a "New Work Item Proposal (NWIP)" for this specific test was issued by the ISO in July 2009, and was voted upon positively in September 2009 by the member countries.

A detailed description of the methodology for the proposed standard "Determination of fresh water sediment subchronic toxicity to *Heterocypris incongruens* (Crustacea, Ostracoda)" was worked out and submitted to the ISO. This NWIP was discussed in September 2009 in Vienna at the annual meeting of the ISO ecotoxicology working group for invertebrate tests, and was subsequently registered as a new project in the TC/SC work programme.

On the basis of the comments made at the annual ISO meeting, a revised document was worked out and sent to the ISO secretariat. A CD (Committee draft) of the subchronic *H. incongruens* assay was subsequently sent out in February 2010 by the ISO to the member countries for comments and voting.
This CD has received the approval from the ISO members and a DIS (Draft International Standard) version of this assay has been worked out, which took into account suggested improvements.
The DIS of the subchronic *H. incongruens* sediment test will be sent out by the ISO during the second half of 2010 for evaluation and final voting, prior to publishing as a new ISO standard.

ORGANISATION OF AN INTERNATIONAL INTERLABORATORY COMPARISON ON THE SUBCHRONIC TOXICITY TEST WITH THE FRESHWATER OSTRACOD CRUSTACEAN *HETEROCYPRIS INCONGRUENS*

During the discussions at the ISO meeting in Vienna in September 2009, it was suggested that with regard to the precision of the subchronic *H. incongruens* assay and in analogy to other ISO standards on toxicity tests, it would be appropriate to organise an interlaboratory comparison on this microbiotest.

It was agreed that since Belgium had proposed the new assay to the ISO, this country would organise the interlaboratory comparison, under the coordination and supervision of the Laboratory for Environmental Toxicology and Aquatic Ecology (LETAE) of the Ghent University.

In accordance to the ISO rules on the determination of accuracy of measurement methods and results (ISO 5725-2: 2002) between 8 and 15 laboratories should be participating in the exercise, each of which should already have experience with the test under analysis.

A list was therefore compiled of the laboratories, organisations, institutes and companies in different countries known to regularly apply the subchronic *H. incongruens* microbiotest for toxicity research or for toxicity monitoring, and an invitation was sent out in February 2010 by LETAE for participation in the International Interlaboratory Comparison.

It was indicated in the invitation that no subscription charge would be asked to the participants and that they only had to perform one test on a reference chemical.

All the materials needed for performing the assay would be included in a "testkit" which would be sent free of charge to the participants.

An "Operational Procedure" has been worked out by the organisers describing in detail the experimental procedure and the procedure to prepare the toxicant dilution series of the reference chemical.

The conditions for participation in this comparison are that the participants should strictly follow the Operational Procedure of the comparison assay (the text of which is included in the testkit) and that the detailed results of their toxicity test should be sent to the organising laboratory "within the stipulated deadline" (i.e. within two months after reception of the testkit).

A request was made by LETAE to the company MicroBioTests in Belgium to work out a model for the testkit and its contents, for performance of two complete toxicity tests (the second test being only needed in case of problems with the first assay).

The company MicroBioTests was subsequently asked to prepare all the testkits and to send them to the participants at the timing indicated by the organizers.

The following time schedule for the International Interlaboratory Comparison was eventually worked out:

- shipment of the materials to the participants: mid April 2010
- time to perform the intercomparison assay: mid April – mid June 2010
- sending of the results to the organiser: third week of June 2010 (at the latest).

NB : Due to the closure of many airports in Europe for several days because of the dust clouds from the volcanic eruption in Iceland, the shipment of the testkit had to be deferred to the first week of May, and the deadline for sending the results to end of June.

PARTICIPANTS IN THE INTERNATIONAL INTERLABORATORY COMPARISON ON THE SUBCHRONIC TOXICITY TEST WITH THE FRESHWATER OSTRACOD CRUSTACEAN *HETEROCYPRIS INCONGRUENS*

About 30 laboratories, organisations, institutes and companies in countries worldwide were contacted for participation in the *H. incongruens* interlaboratory comparison.

Twenty six laboratories, institutes, organisations and companies from 14 countries indicated their interest to participate in this intercomparison and sent their Participation Form to the organisers, 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 - MicroBioTests Inc. – Mariakerke-Gent - Vlaamse Milieumaatschappij VMM - Erembodegem
- Hungary	- National Insitute of Environmental Health – Dept. of Water Biology and Ecotoxicology - Budapest
- Poland	- Medical University of Gdansk – Interdepartmental Institute of Maritime and Tropical Medecine – Dept of Environmental Protection and Hygiene of Transport - Gdynia - International Institute of the Polish Academy of Sciences – European Regional Centre for Ecohydrology u/a UNESCO - Lodz - Department of Analytical Chemistry – Chemical Faculty – Gdansk University of Technology - Gdansk - Department of Environmental Health Sciences – Medical University of Warsaw - Warsaw - University of Opole – Department of land protection – Opole - Institute of soil and environmental management – University of Life Sciences in Lublin - Lublin
- Portugal	- Laboratorio de Referencia do Ambiente – Zambujal Amadore - Instituo di Mar – Department of Life Sciences – University of Coimbra - Coimbra
- Czech Republic	- Czech Technical University in Prague – Faculty of Civil Engineering Department of Sanitary and Ecological Engineering - Prague
- Italy	- EcoBioqual srl – Torino - Centro Regionale di Riferimento per le Attività Biologiche – Departimento di Pisa ARPAT – Pisa - ENI spA – Istituto eni Donegani – DHFIS – Novara
- Sweden	- HST – Mälardalen University – Vasteras
- France	- Université Paul Verlaine – Laboratoire LIEBE – UMR CNRS 7146 Campus Bridoux – Metz - INSAVALOR–POLDEN – Villeurbanne Cédex
- Germany	- TU Braunschweig – Institut für Okologische Chemie und Afballanalytik - Braunschweig

AMERICA	
- Canada	- Quebec Laboratory for Environmental Testing – Environment Canada Montreal
- Guatemala	- Servicios y Productos Ambientales, SEPRA – Guatemala City
ASIA	
- South Korea	- Department of Biology – University of Incheon – Incheon
- Japan	- Environmental Science Center – University of Tokyo – Tokyo - National Institute for Environmental Sciences – Research Center for Environmental Risk – Ibaraki
AFRICA	
- South Africa	- Golder Associates Research Laboratory – Gauteng

REFERENCE CHEMICAL

Copper sulphate was selected as the reference chemical for spiking of the sediment in the ostracod interlaboratory comparison.
This choice was based on the fact that copper sulphate is either the selected or one of the recommended chemicals prescribed by four national organisations (US EPA, ASTM, AFNOR and Environment Canada) for reference tests on the whole sediment assay with *H. azteca.*

A vial with copper sulphate (under the form of $CuSO_4.5H_2O$) in solid form has been included in each testkit for the preparation of the toxicant solutions.

The toxicant range which was chosen for the interlaboratory comparison and which is indicated in the Operational Procedure for preparation of the toxicant dilution series is 1-10 mg $CuSO_4.5H_2O$

DATA TREATMENT

Dr. R. Baudo from the CNR - Istituto per lo Studio degli Ecosystemi (I.S.E) in Italy, who has performed the statistical analysis of the data from the International Interlaboratory Comparison on the acute *Thamnocephalus platyurus* microbiotest, kindly agreed to also perform the statistical analysis and the calculation of the LC50 and the growth inhibition EC50 of the *H. incongruens* interlaboratory comparison.

Participants could, if they wished, calculate themselves the LC50 and the EC50 of their test, but they had anyhow to send to the organiser their two Result Sheets (Excel Sheets sent by email to all the participants), filled out with all the mortality data and the growth inhibition data.

The participants were requested to type in their data on the Excel Result Sheets which calculate automatically the mortality and growth inhibition percentages, and to send these Sheets by email to the organiser.

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 the discussion of the results in this report.

It was furthermore agreed that on completion of the data treatment, each participant will receive a copy of the Final Report on the ostracod interlaboratory comparison and the LC50 and EC50 values of their own test.

RESULTS OF THE INTERNATIONAL INTERLABORATORY COMPARISON ON THE SUBCHRONIC TOXICITY TEST WITH THE FRESHWATER OSTRACOD CRUSTACEAN *HETEROCYPRIS INCONGRUENS*

All 26 participants performed the ostracod test and sent their results to the organisers. A few of them, however, did not obtain satisfactory results with their first assay and had to repeat the test.
From all the Result Sheets submitted, it eventually appeared that the results from 3 laboratories were unacceptable (probably by errors in the preparation of the toxicant dilutions) and could not be taken into account for the data processing.

The submitted results brought forward the following interesting facts:

1. Hatching success of the ostracod cysts
No complaints were received from the participants with regard to the hatching of the cysts and all the participants obtained a sufficient number of ostracods to perform the assay.

2. Mortality percentage in the controls at the end of the test
The Mortality Results Sheets showed that 15 participants reported 0 % mortality in the controls; the other laboratories had either 3 % or 6 % mortality in the controls (i.e., only 1 or 2 dead organisms from the 30 ostracods inoculated in the control test cups).
The validity criterion of “less than 20 % mortality in the controls” at the end of the test was hence achieved by all the participants.

3. Mortality percentages in the toxicant concentrations
From the 23 Mortality Results Sheets with “meaningful data” all except one showed mortality percentages above 50 % in the highest test concentration (C1). Only one laboratory obtained a mortality percentage of 46,67 % in the C1 concentration. Since this result is very near the 50 % value, the data of this participant have nevertheless also been taken into consideration, and an (extrapolated) LC50 was calculated. However, this extrapolated result was not included into the set used to calculate average and intra- and interlaboratory variabilities.

4. Length measurements of the ostracods at the start of the test
The majority of the participants have used the micrometer coverslip included in the testkit to make the length measurements.
Two laboratories have used a calibrated eyepiece or image analysis respectively to make the length measurements. From the data of these two laboratories, it appears that the size of the ostracods at the start of the test ranges from 190 µm to 240 µm, with a mean of 210-215 µm.

The subdivisions on the perpendicular micrometer on the coverslip are, however, only 50 µm and the measurements made with this micrometer are therefore “less precise” and from the data it can be seen that this has often lead to an “underestimation” of the real length of the ostracod neonates. Values of 150 µm have indeed been commonly reported (and in one case even 100 µm). As a result the mean values for the initial length reported by those which used the micrometer coverslip measurement technique encompass a range from 140 µm to 235 µm.

Calculation of the overall mean for the mean length data of neonate ostracods for all the tests, except those of the 2 laboratories which did not use the micrometer coverslip, resulted in a value of 205 µm.
NB: Taking into account the lower precision of the "micrometer coverslip" measurements, this overall mean is actually "not that far" from the mean length (210 – 215 µm) reported by the 2 laboratories which performed their length measurements with a more precise technique.
The variation coefficient for the overall mean length measurements of the neonate ostracods was 12 %.

5. Length measurements of the ostracods in the controls at the end of the test

The mean final lengths in the controls reported by the participants range from 482 µm up to 896 µm. The "overall mean" of all these means is 709 µm, with a variation coefficient of 14 %.

According to the instructions given in the Operational Procedure for the ostracod interlaboratory comparison, the length increase of the ostracods in the controls had to be at least 400 µm at the end of the test.
This target value was reached in the majority of the tests, but four laboratories reported data with a length increase lower than 400 µm (353 µm, 355 µm, 367 ml and 395 µm respectively).
For a few other laboratories the length increase was also only "borderline" above 400 µm.

The large spread between the lowest and the highest growths of the ostracods in the controls at the end of the assay can be attributed to either "environmental" factors, e.g. slight differences in the incubation temperature between the different laboratories, or to "biological" factors, such as the quantity of algal food provided at the start of the test or the nutritional quality of the algal food (which is known to decrease with the age of the algal beads).

It was therefore suggested by some participants not to impose a "strict mathematical increase value" of 400 µm for the controls as a validity criterion for the ostracod assay, but "a growth factor" to be applied on the mean length of the ostracods at the start of the test.
The latter approach is more relevant since it departs from and takes into account the mean length of the ostracod neonates at the start of the assay, which as reported above ranges from 140 µm to 235 µm.
Calculated on the latter two values, the original acceptability condition (growth of 400 µm) means that ostracod neonates with a mean length of 140 µm should grow to 540 µm in the controls, whereas for those with a mean length of 235 µm, their final length should be 635 µm, and this was not achieved in all the test.

It was therefore decided by the organisers to apply a factor of 1,5 for the length increase of the ostracods during the test (instead of 400 µm) as the acceptability criterion for growth of the test organisms during the assay.

Application of the 1,5 growth factor to the data reported by the laboratories in the interlaboratory comparison made all the results "acceptable".
Indeed, when applied to the former mean start lengths (140 µm and 235 µm), the corresponding mean end lengths are 350 µm and 587,5 µm respectively and this was achieved in all the tests.

NB : Based on this conclusion from the ostracod interlaboratory comparison, the 1,5 growth factor will be proposed instead of the 400 µm length increase, as the second validity criterion for the ostracod test in the ISO standard on the *Heterocypris incongruens* subchronic assay.

6. Length measurements of the ostracods in the different test concentrations

The ISO standard on the subchronic toxicity test with *H. incongruens* specifies that the "sublethal" effect criterion (growth inhibition) has only to be measured (and is only meaningful) in case the percentage

mortality in the highest concentration of the test sample is “below 30 %”. It does indeed not make much sense to calculate a growth inhibition for tests in which the majority of the organisms have died.

The toxicant range selected by the organisers for the ostracod interlaboratory comparison was deliberately selected such that it would give more than 50 % mortality in the highest test concentration, in order to allow for the calculation and the comparison of the 6 d LC50’s.

It was therefore in fact not necessary for this specific interlaboratory comparison to also apply the “growth inhibition criterion” to the surviving test organisms, since the dilution series used already showed that the sample indeed induced a substantial toxic effect (mortality) to the test organisms.

The organisers nevertheless decided to include the length measurements of the ostracods in the different test concentrations in this interlaboratory comparison in order to make (wherever possible) EC50 calculations on the surviving organisms for comparison of the results of the individual laboratories.

The data provided by the participants in their “Growth inhibition Results Sheet” allowed to formulate the following interesting statements:

1. All the laboratories except one reported “meaningful” (i.e. logic) length data in the different test concentrations
2. The decrease in the growth of the ostracods in the increasing test concentrations was more or less linear in most tests, and also clearly shows the (sublethal) impact of the toxicant on the growth of the surviving organisms
3. The percentage growth inhibition of the surviving organisms in the highest test concentration was in 8 cases below 50 %, so no EC50 could be calculated for these assays.
4. In 7 tests there was 100 % mortality in the highest test concentration (C1), so there were “no survivors” on which length measurements could be made. Therefore the data for the second highest test concentration were taken into consideration for these 7 assays.
5. The mean “final” length of the ostracods in the highest test concentration (C1) (and by default in the second highest test concentration) ranges from 267 µm to 700 µm, with an overall mean value for all the laboratories, of 485 µm and a variation coefficient of 23 %.
NB: clearly the “highest” of these values in the C1 and C2 concentrations are coming from the laboratories which also reported the “highest” growth in the controls !

DATA TREATMENT

All the data have been analysed with the US EPA Benchmark Dose Software (BMDS), Version 2.1.

Calculation of the 6 d LC50

For each laboratory the LC50 and its 95 % confidence limits have been calculated using the log-probit model. Figure 1 shows the LC50 of the individual laboratories (numbered from 1 to 23), along with the “overall” LC50 (horizontal broken line).

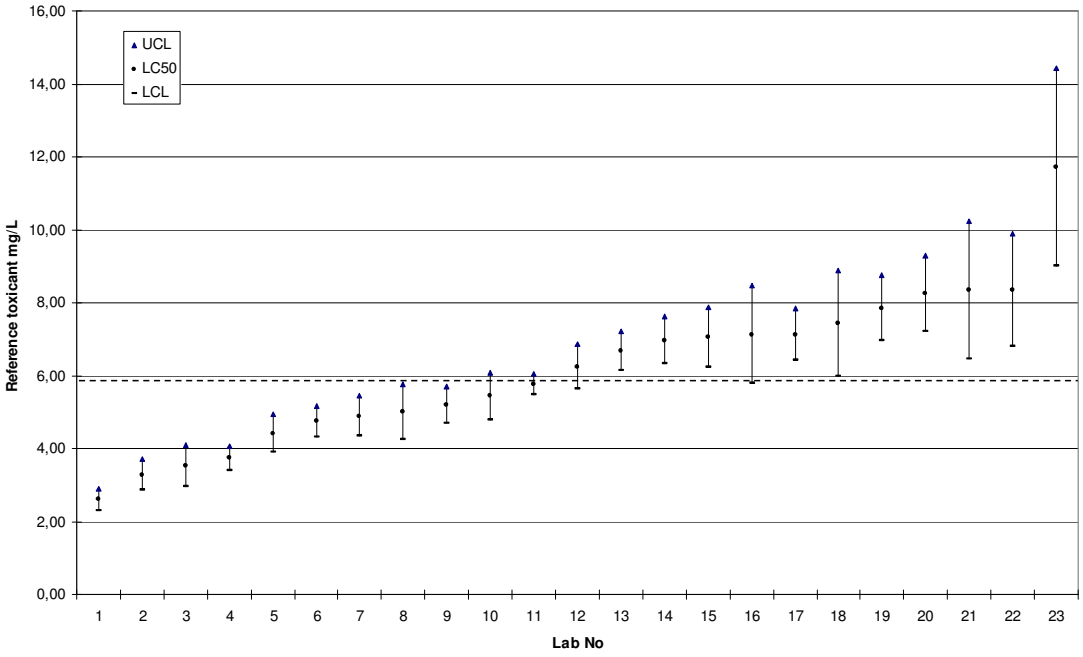

Fig. 1 – LC50's plus upper 95% confidence limit UCL, and lower 95% confidence limit LCL for each participating laboratory. The horizontal broken line indicates the overall LC50.

The repeatability and reproducibility of the interlaboratory comparison have been calculated according to the ISO 5725-2 (2002) procedure, with the following criteria:

s_L^2 the estimate of the between-laboratory variance;
s_W^2 the estimate of the within-laboratory variance;
s_r^2 the arithmetic mean of the within-laboratory variances (after outliers have been excluded);
s_R^2 the estimate of the reproducibility variance: $s_R^2 = s_L^2 + s_r^2$.

To check the consistency of the data, the Mandel's h and k statistics have been used: the first (h) provides the between-laboratory consistency statistic, and the second (k) the within-laboratory consistency statistic.
The Grubb's test has then been applied to identify "stragglers" (if the test statistic is greater than its 5 % critical value and less than or equal to its 1 % critical value, the item tested is called a straggler and is indicated by a single asterisk), and "outliers" (if the test statistic is greater than its 1 % critical value, the item is called a statistical outlier and is indicated by a double asterisk).
Figures 2 and 3 show the results of the Grubb's test applied to the h and k statistics.

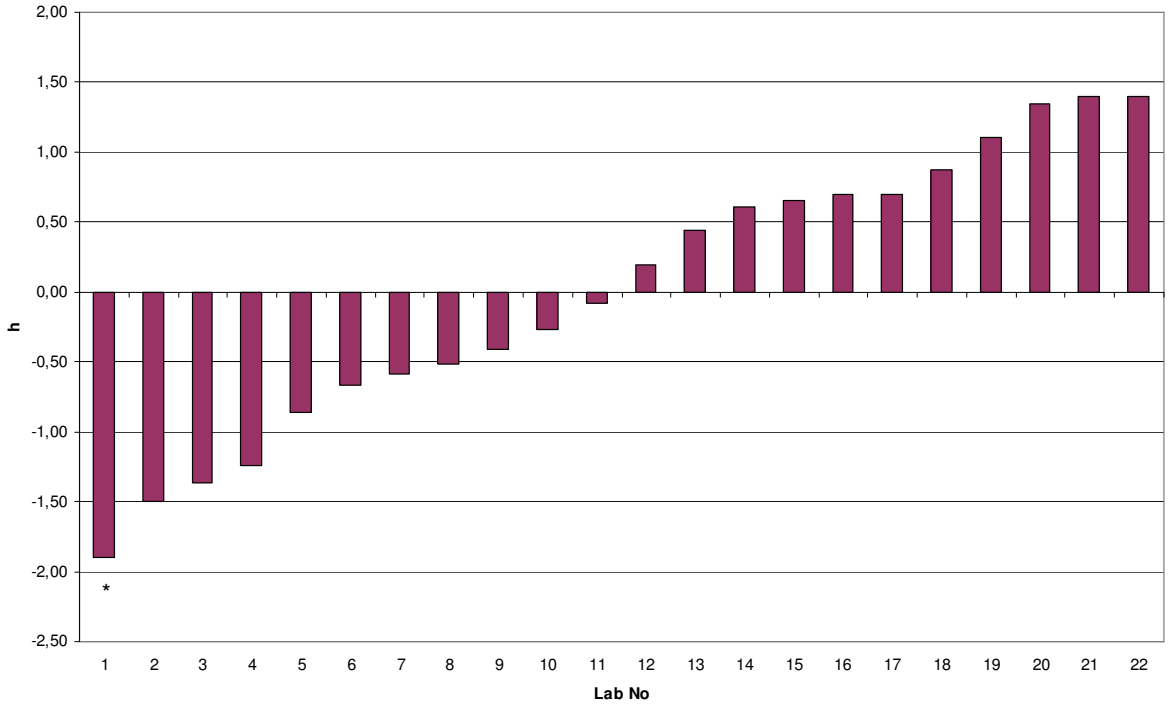

Fig. 2 – Mandel's between-laboratory consistency statistic, h, grouped by laboratories.

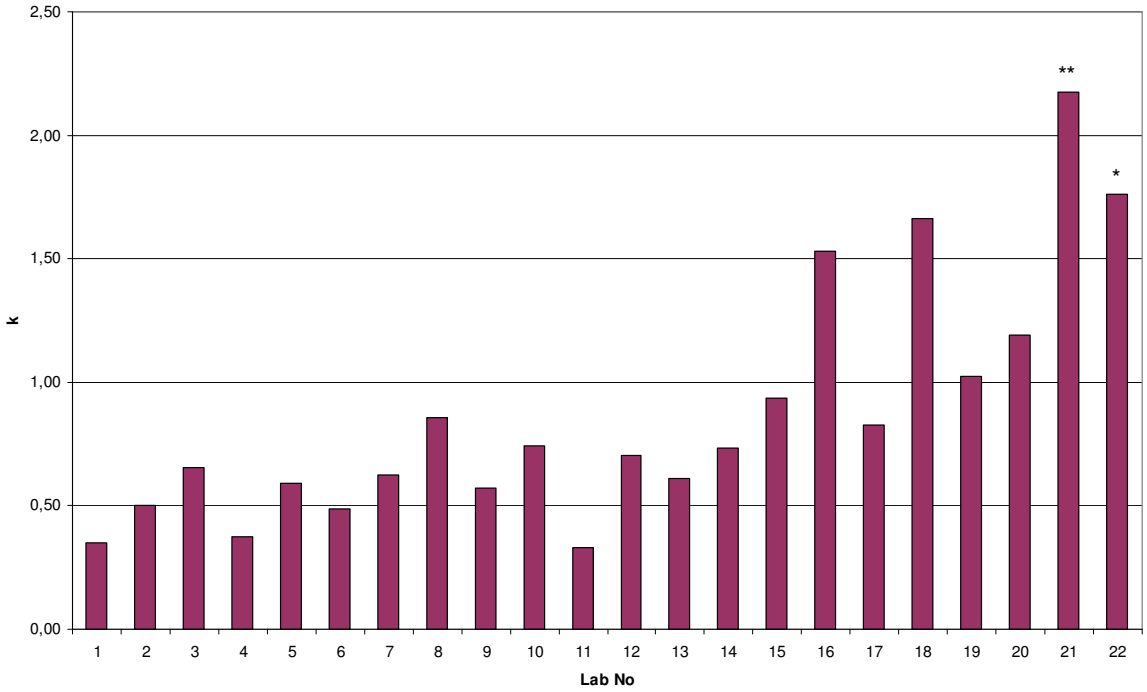

Fig. 3 – Mandel's within-laboratory consistency statistic, k, grouped by laboratories.

The final results for the mortality criterion are summarized in Table 1.

Table 1. – Computed values for the LC50 data

	All data	**Without h and k outlier**	**Without h and k straggler**
n labs	22	21	18
Mean	5,910	5,794	5,487
s_r	0,761	0,690	0,540
CV%	12,87	11,91	9,85
s_R	1,85	1,789	1,685
CV%	31,32	30,88	30,71
h straggler	1	0	0
h outlier	0	0	0
k straggler	1	2	0
k outlier	1	0	0
95 % UCL	6,948	6,823	6,538
95 % LCL	4,872	4,766	4,436

From this table it appears that, after having excluded 1 outlier (Mandel's statistics), the mean LC50 value is 5,79 mg/l $CuSO_4.5H_2O$ (95% confidence limits: 4,76 – 6,82) with a repeatability standard deviation S_r (within-laboratory variability) of 0,69 (11,91 as CV%) and a reproducibility standard deviation S_R (between-laboratory variability) of 1,79 (30,88 CV%).

Calculation of the 6 d EC50

For the statistical analysis of the growth inhibition data, the selected model for continuous data was either the linear or power model (depending on the statistical test performed by the BMDS software), resulting in the calculation of an EC50, with its 95 % confidence limits, for each of the laboratories which submitted acceptable length data.
Figure 4 shows the EC50 of the individual laboratories (numbered from 1 to 23), along with the "overall" EC50 (horizontal broken line).

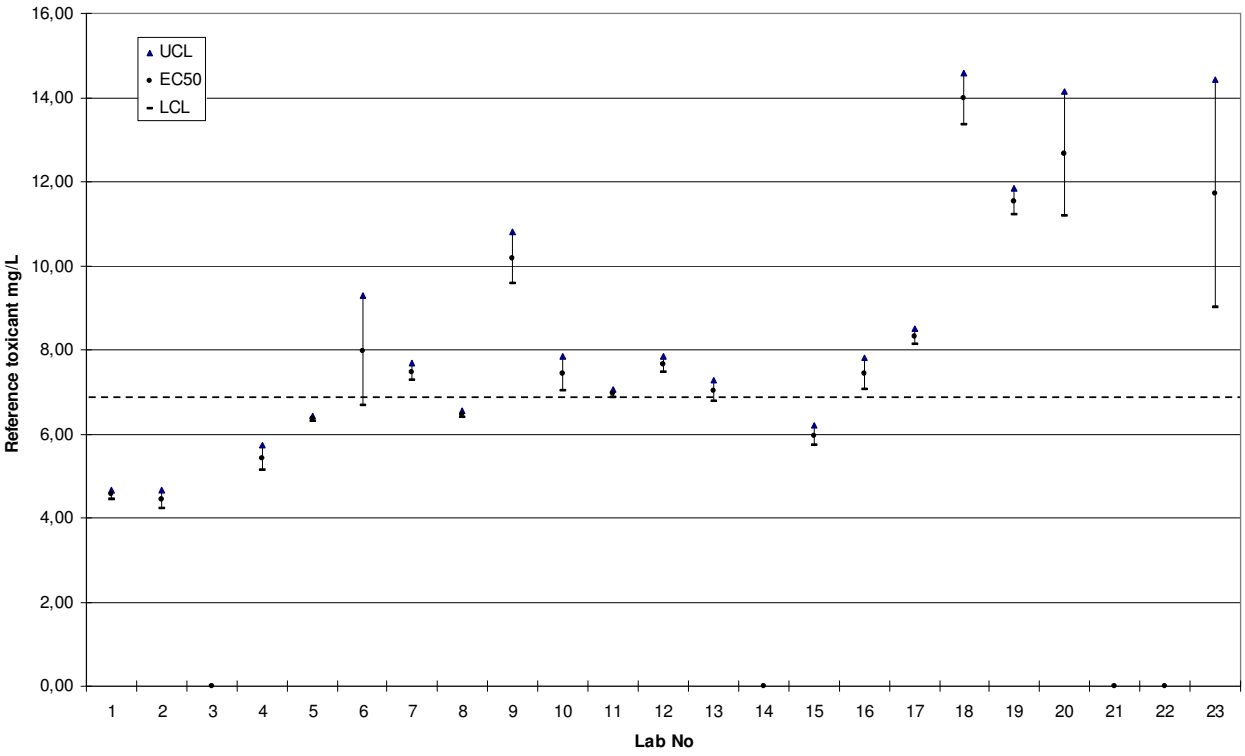

Fig. 4 – EC50's plus upper 95% confidence limit UCL, and lower 95% confidence limit LCL for each participating laboratory. The horizontal broken line indicates the overall EC50.

The repeatability and reproducibility of the interlaboratory comparison for the growth inhibition criterion have also been calculated according to the ISO 5725-2 (2002) procedure mentioned above for the mortality criterion. The results are shown in Figures 5 and 6.

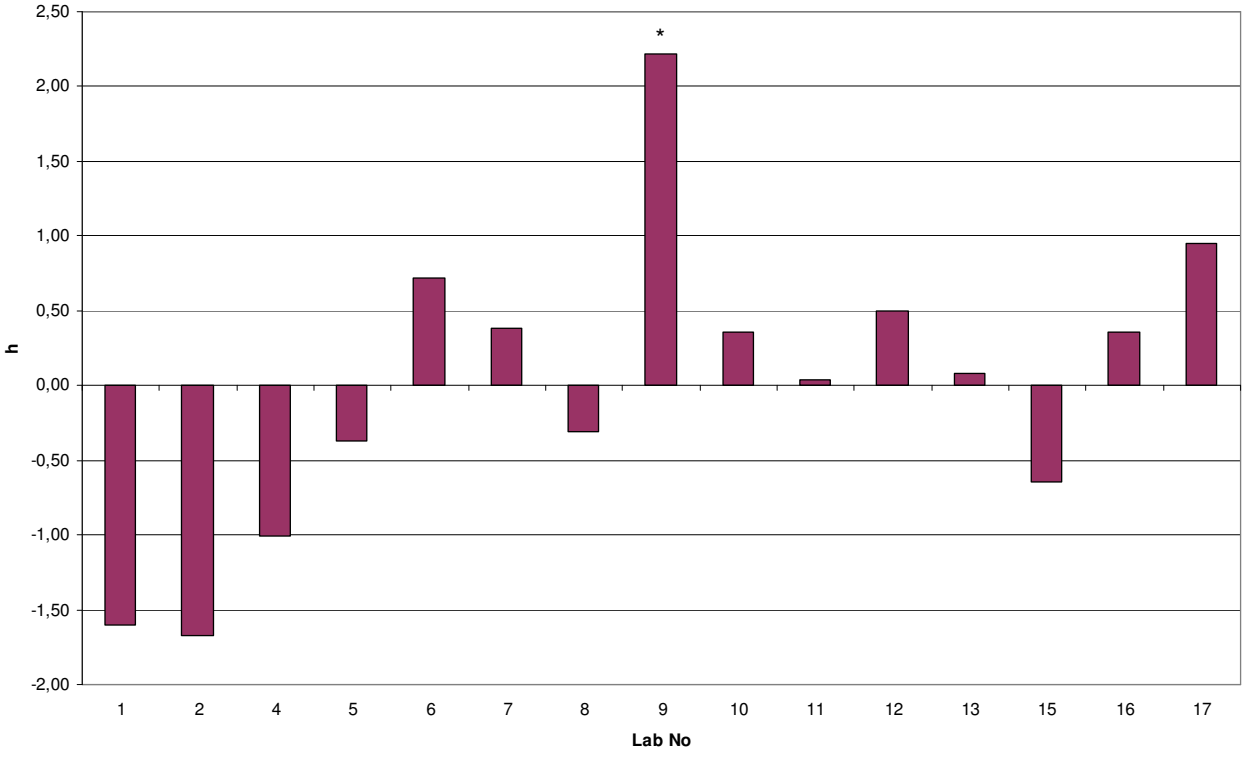

Fig. 5 – Mandel's between-laboratory consistency statistic, h, grouped by laboratories.

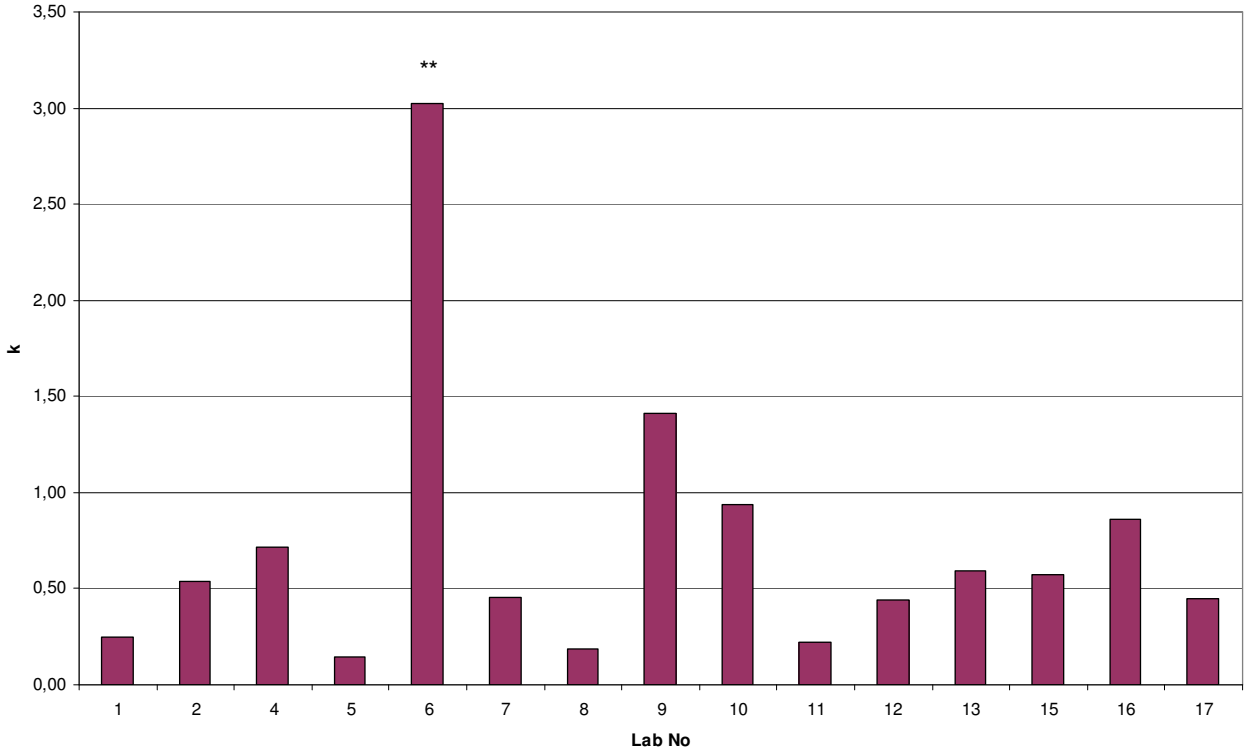

Fig. 6 – Mandel's within-laboratory consistency statistic, k, grouped by laboratories.

The final results for the growth inhibition criterion are summarized in Table 2.

	All data	**Without h and k outlier**	**Without h and k straggler**
n labs	15	13	11
Mean	6,913	6,580	6,423
s_r	0,376	0,205	0,170
CV%	5,43	3,11	2,64
s_R	1,51	1,209	1,251
CV%	21,81	18,38	19,48
h straggler	1	0	0
h outlier	0	0	0
k straggler	0	1	0
k outlier	1	0	0
95 % UCL	7,949	7,477	7,440
95 % LCL	5,878	5,682	5,406

From this table it appears that, after having excluded 2 outliers (Mandel's statistics), the mean EC50 value is 6,58 mg/l $CuSO_4.5H_2O$ (95% confidence limits: 5,68 – 7,48) with a repeatability standard deviation S_r (within-laboratory variability) of 0,20 (3,11 as CV%) and a reproducibility standard deviation S_R (between-laboratory variability) of 1,21 (18,38 CV%).

Correlation LC50 with EC50 data

As emphasized in part 6. Length measurements of the ostracods in the different test concentrations in the section Results of the International Interlaboratory Comparison on the ostracod microbiotest, the growth inhibition criterion should (in principle) not have been calculated since the mortality percentage in the highest test concentration was above 30 %.

Still, as shown above, the EC50's calculated with the length data provided by the participants gave interesting information on the repeatability and reproducibility of the assay for this sublethal criterion.

The calculations furthermore revealed that the overall EC50 value (6,58 mg/l) was actually quite close to the overall LC50 (5,79 mg/l).

For the sake of "scientific curiosity" a correlation analysis has therefore been made between the LC50 and EC50 data pairs for the individual laboratories.

The outcome of this analysis which is shown in Figure 7 indicates that the correlation between mortality and growth inhibition for the 15 laboratories which produced both (acceptable) mortality and growth inhibition data is statistically highly significant ($P < 0,005$) and explains 50 % of the variability.

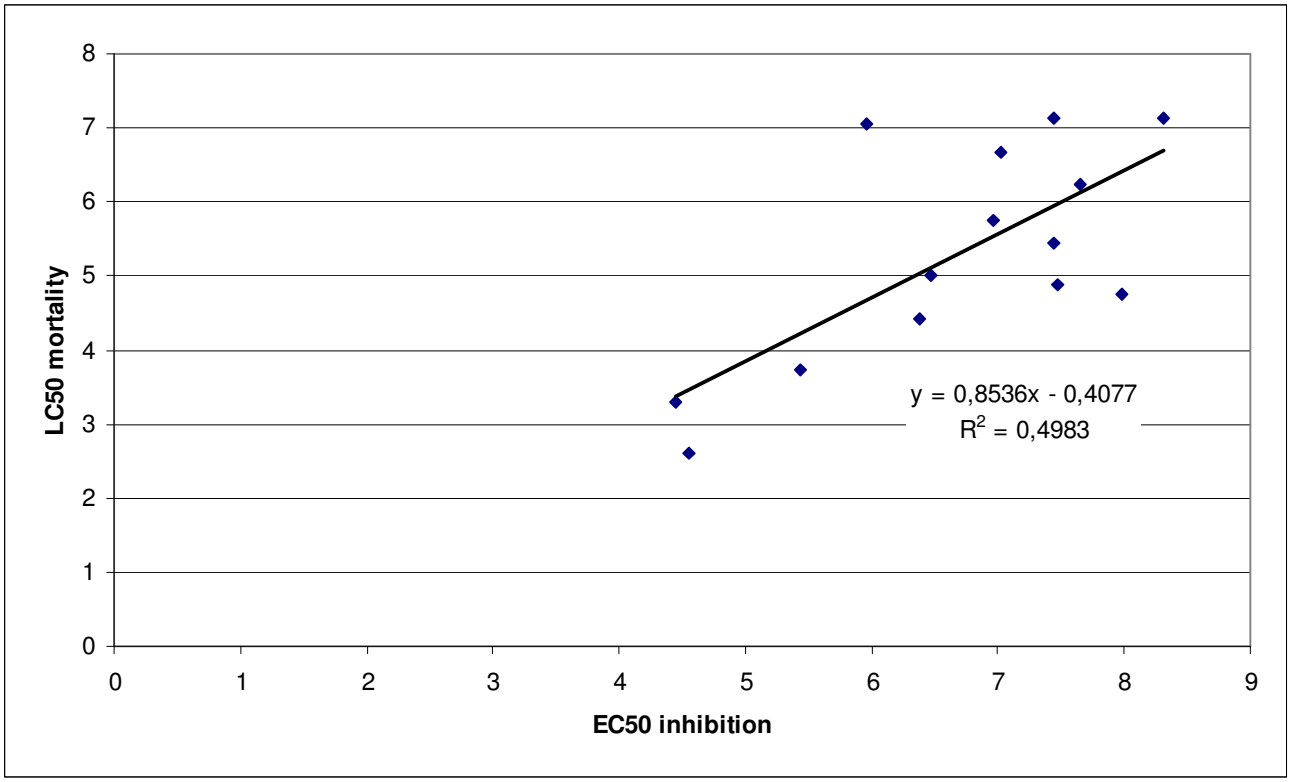

Figure 7. – Correlation analysis of the LC50/EC50 data pairs

CONCLUSIONS

The International Interlaboratory Comparison on the determination of the subchronic toxicity to *Heterocypris incongruens* (Crustacea, Ostracoda), following the method outlined by the ISO/CD 14371 (2010), has allowed to draw the following conclusions:

- the hatching of the *Heterocypris incongruens* cysts from the vial contained in the testkit in all cases provided enough living organisms to perform the test;
- twenty-three from the 26 participating laboratories were able to satisfactorily perform the test, some of whom, however, only after a repetition of their first assay;
- the validity criterion that the percentage mortality in the controls at the end of the test should not be higher than 20 % was achieved by all the participating laboratories;
- the requested "growth increment by 400 µm" of the test organisms in the controls, in comparison to their initial length was not achieved in a number of tests;

- as suggested by some participants, this “second validity criterion” of the ostracod microbiotest has therefore been changed to “an increase by a factor of 1,5 from the initial length of the test organisms”;
- the percentage mortality in the highest test concentration was for all the laboratories except one, above 50 %, allowing to calculate LC50’s;
- the statistical analysis of the mortality data singled out 1 outlier (Mandel’s statistics), and 21 out of 22 results were therefore accepted;
- the estimated mean 6 d LC50 is 5,79 mg/l $CuSO_4.5H_2O$ with an associated mean repeatability standard deviation s_r (within-laboratory variability) of 11,9 % CV and a mean reproducibility standard deviation s_R (between-laboratory variability) of 30,88 % CV;
- since the percentage mortality in the highest test concentration was > 30 %), calculation of the sublethal “growth inhibition” was in principle not necessary. Data have, however, also been submitted by the participants on which statistical analysis has been performed to the ostracod length data;
- for 8 laboratories the growth inhibition percentage in the highest test concentration was below 50 %, so the EC50 could only be calculated for 15 data sets;
- after exclusion of 2 outliers (Mandel’s statistics) the estimated 6 d EC50 was 6,58 mg/l $CuSO_4.5H_2O$ with a mean repeatability standard deviation s_r of 3,1 % CV and a mean reproducibility standard deviation s_R of 18,4 % CV;
- the estimated 6 d LC50 and 6 d EC50 are quite near to each other; correlation analysis between the fifteen LC50 and EC50 data pairs revealed a highly significant correlation ($P < 0,005$) between the assessed mortality and growth inhibition effects, explaining 50 % of the variability.

From the results of this extensive International Interlaboratory Comparison, it can safely be concluded that the determination of the subchronic toxicity to *Heterocypris incongruens*, following the method outlined by the ISO/CD 14371 (2010), fulfils the requirements for a reliable and precise ecotoxicological test.

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