



INTERNATIONAL INTERLABORATORY COMPARISON ON THE ACUTE TOXICITY TEST WITH THE FRESHWATER ANOSTRACAN CRUSTACEAN *THAMNOCEPHALUS PLATYURUS*

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INTRODUCTION

One of the most used acute toxicity tests with invertebrates is the 24h-48h *Daphnia magna* assay (ISO standard 6341). This assay is performed with neonates less than 24 h old collected from laboratory stock cultures and is hence – similarly to most toxicity tests - inherently dependent on the year-round culturing/maintenance of live stocks of test organisms, with all the infrastructure, work and costs which keeping of live stocks implies.

In order to bypass the latter (major) handicap, extensive research has been performed at the Laboratory for Environmental Toxicology and Aquatic Ecology at the Ghent University in Belgium, on the controlled production of “dormant eggs” of *D. magna*, 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.

The availability of *D. magna* dormant eggs subsequently triggered research in various laboratories with regard to the outcome of acute *D. magna* toxicity tests performed with neonates taken from laboratory cultures, versus neonates hatched from the dormant eggs.

All the evidence generated in these studies has been compiled in an extensive review co-authored by 9 renowned ecotoxicologists (Persoone *et al.*, 2009), which shows that the neonates hatched from dormant eggs have the same sensitivity as those taken from laboratory cultures.

In parallel to the investigations on the controlled production and use of dormant *D. magna* eggs, research has also been made in parallel at the same laboratory at the Ghent University, on the controlled production of dormant eggs of other invertebrate test species, to foster the development of additional practical and cost-effective “culture/maintenance free” microbiotests.

One of the achievements is a 24 h toxicity test with the freshwater anostracan crustacean *Thamnocephalus platyurus*.

This microbiotest appeared to have several interesting assets in comparison to the acute *D. magna* test, even the *D. magna* microbiotest which is based on the use of dormant eggs.

First of all *T. platyurus* neonates already hatch from the dormant eggs (cysts) in less than 24 h incubation, whereas the embryonic development of the dormant *D. magna* eggs takes about 72 hours till hatching.

The *T. platyurus* microbiotest is furthermore attractive because the neonates of this crustacean are much smaller than *D. magna* neonates, which allows to perform the tests in small multiwells. This results in a substantial saving of shelf space and incubator space when multiple tests need to be performed concurrently.

Extensive research performed in different laboratories revealed that in 24 h toxicity tests *D. magna* and *T. platyurus* have grossly spoken a similar sensitivity to toxicants.

Based on this fact, and taking into account the user-friendliness and the cost-effectiveness of the acute *Thamnocephalus* assay, the Environmental Agency of Flanders, Belgium, as of the turn of the century gave preference to this microbiotest in lieu of the acute *D. magna* test, for incorporation in its test battery for toxicity monitoring of river sediments.

As of to date nearly thousand sediment pore waters of rivers in the Flemish hydrographic basin have already been analysed with the *T. platyurus* microbiotest.

Scientists from 3 different countries (the Czech Republic, Poland and Hungary) also discovered that the *Thamnocephalus* microbiotest is particularly sensitive to cyanotoxins, which makes it a very interesting tool for monitoring of eutrophic water bodies in relation to the presence of cyanotoxins.

The experimental procedure of this assay will therefore be included and described in detail in the chapter “Toxicity Testing” in the upcoming and updated version of the WHO handbook “Toxic cyanobacteria in water”.

Last but not least, and in view of the need for “rapid” assays for toxicity assessment, a procedure was worked out for a “one hour” test with *T. platyurus* larvae. In this rapid microbioassay a “physiological” effect parameter (the ingestion or absence of ingestion of particles) is used to assess the “sublethal” impact of toxicants on the test organisms.

The 1 hour *Thamnocephalus* microbioassay has been evaluated in 2005 by the Environmental Protection Agency in the United States (US EPA) in the framework of the Environmental Technology Verification (ETV) Program for detection of toxicity in drinking water.

The rapid microbioassay was subjected to industrial chemicals, pesticides, rodenticides, pharmaceuticals, nerve agents and biological toxins and it detected all these chemicals (except one biotoxin) at - and in most cases even substantially below - the “human lethal dose” level.

Over the last few years correlation studies have been performed and published on the toxic response of *T. platyurus* to cyanotoxins and pharmaceutical products, with both the 24 h mortality test and the 1 h rapid sublethal microbioassay. These investigations revealed a good correlation between the 24 h LC50 and the 1 h EC50, indicating that the rapid test was a good predictor of the concentrations causing 50 % mortality after 24 h of exposure.

To date both the 24 h and the 1 h *T. platyurus* microbioassays are applied by many laboratories in different countries, and more than 150 papers have already been published on the use of these two assays in various domains.

PROPOSAL TO THE INTERNATIONAL STANDARDISATION ORGANISATION (ISO) FOR A NEW WORK ITEM ON THE *THAMNOCEPHALUS PLATYURUS* MICROBIOTEST

The *T. platyurus* assay has already been incorporated in some countries in regional or national regulations for toxicity testing but requests have also been formulated from various sides to propose this microbioassay to “international” organisations for endorsement as a “standard toxicity test”, for specific applications in a regulatory framework.

An initiative was therefore taken in 2009 by R. Baudo, from the National Research Council in Italy, on behalf of the Italian Organization for Standardization (UNI) to propose the *T. platyurus* microbioassay to the International Standardisation Organisation (ISO) for 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 the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca)” 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 acute *T. platyurus* assay was subsequently sent out in February 2010 by the ISO to the ISO member countries for comments and voting.

Following the approval by a majority of the votes, a DIS version of the *T. platyurus* acute toxicity test is now being prepared taking into account suggested improvements, and will be circulated by ISO for voting prior to publication as a new ISO standard.

ORGANISATION OF AN INTERNATIONAL INTERLABORATORY COMPARISON ON THE ACUTE TOXICITY TEST WITH THE FRESHWATER CRUSTACEAN *THAMNOCEPHALUS PLATYURUS*

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

It was agreed that since Italy had proposed the new assay to the ISO, this country would organise the comparison, under the coordination and supervision of the “Consiglio Nazionale delle Ricerche (CNR) – Istituto per lo Studio degli Ecosistemi (I.S.E.)” in Pallanza.

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 acute *T. platyurus* microbioassay for toxicity research or for toxicity monitoring, and an invitation was sent out in February 2010 by the CNR - I.S.E. for their possible 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 acute 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 intercomparison are that the participants should strictly follow the “Operational Procedure” of the intercomparison assay (which is included in the testkit) and that the detailed results of their toxicity test should be sent to the organiser I.S.E. “within the stipulated deadline” (i.e. within two months after reception of the testkit).

A request was made by the I.S.E. 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 I.S.E.

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

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

PARTICIPANTS IN THE INTERNATIONAL INTERLABORATORY COMPARISON ON THE ACUTE TOXICITY TEST WITH THE FRESHWATER CRUSTACEAN *THAMNOCEPAHLUS PLATYURUS*

About 30 laboratories, organisations, institutes and companies in countries worldwide were contacted for participation in the *T. platyurus* ringtest.

Twenty three laboratories, institutes, organisations and companies from 14 countries eventually indicated their interest to participate in this interlaboratory comparison and have sent their Participation Form to the 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
 - MicroBioTests Inc. – Mariakerke-Gent
- The Netherlands
 - AquaSense – Grontmij - Amsterdam
- Estonia
 - National Institute of Chemical Physics and Biophysics - Laboratory of Molecular Genetics - Tallinn
- Hungary
 - National Institute of Environmental Health – Dept of Water Biology and Ecotoxicology - Budapest
- Poland
 - Medical University of Gdansk – Interdepartmental Institute of Maritime and Tropical Medicine – 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
- Environmental Biotechnology Department – Silesian University of Technology - Gliwice

- Lithuania - Laboratory of Hydrobotany – Institute of Botany in the Centre of Nature Research - Vilnius

- Portugal - Laboratorio de Referencia do Ambiente – Zambujal Amadore

- Czech Republic - Institute of Chemistry and Technology of Environmental Protection – Faculty of Chemistry – Brno University - Brno
- Institute of Botany – Czech Academy of Sciences - Brno

- Slovak Republic - Public Health Authority of the Slovak Republic – National Reference Center for Ecotoxicology - Bratislava

- Italy - Acquedotto Pugliese S.p.A. – Unita Vigilanza Igienica – Bari
- EcoBioqual srl – Torino
- ARPA Sicilia – Laboratorio di Metrologia – Palermo
- Dipartimento di Scienze della Vita – Facoltà di Scienze, Seconda Università di Napoli – Caserta

- Greece - University of Thessaly – Department of Planning and Regional Development - Volos

- AMERICA
- Canada - Environment Canada, Science and Technology Branch – Quebec Laboratory for Environmental Testing – Quebec

- Guatemala - Servicios y Productos Ambientales, SEPRA – Guatemala City

- ASIA
- South Korea - Department of Biology – University of Incheon - Incheon

REFERENCE CHEMICAL

Similarly to several other acute toxicity tests, potassium dichromate ($K_2Cr_2O_7$) was selected as the reference chemical for the International Interlaboratory Comparison on the acute toxicity test with *Thamnocephalus platyurus*.

In order, however, to avoid custom problems with shipment of “hazardous chemicals” the organiser decided not to include the reference chemical in the testkit and each participating laboratory therefore was asked to use potassium dichromate (of analytical grade) obtained from their own supplier for the intercomparison assay.

The following dilution series of potassium dichromate had to be prepared with test medium for the test: 0,32 - 0,18 - 0,10 - 0,056 - 0,032 mg/l.

DATA TREATMENT

It was stipulated by the organiser that the statistical analysis of the data and the calculation of the 24 h LC50 would be made by the I.S.E.

Participants could, if they wished, calculate themselves the 24 h LC50 of their test, but they had anyhow to send to the organiser their Result Sheet (an Excel Sheet sent by email to all the participants), with all the mortality data.

The participants were requested to type in the mortality scores on the Excel Result Sheet (which calculates automatically the mortality percentages) and to send this Sheet 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 discussion of the results in this report.

RESULTS OF THE INTERNATIONAL INTERLABORATORY COMPARISON ON THE ACUTE TOXICITY TEST WITH THE FRESHWATER CRUSTACEAN *THAMNOCEPAHLUS PLATYURUS*

No complaint was received by the organiser from the participants, and in fact all were able to perform satisfactorily the test within the agreed timeline.

All laboratories respected the validity criteria that the percentage mortality in the controls should not be higher than 10 %. Actually, on the whole, only 5 dead organisms (out of 690 exposed organisms) were observed in the negative controls (accounting for an overall percentage mortality of 0,72 %).

All data have been analyzed with the US EPA Benchmark Dose Software (BMDS), Version 2.1. For each laboratory, the LC50 and its 95 % confidence limits have been calculated using the log-probit model.

The results are summarized in figure 1, that shows the results of the laboratories (numbered from 1 to 23), along with the overall LC50 (horizontal broken line).

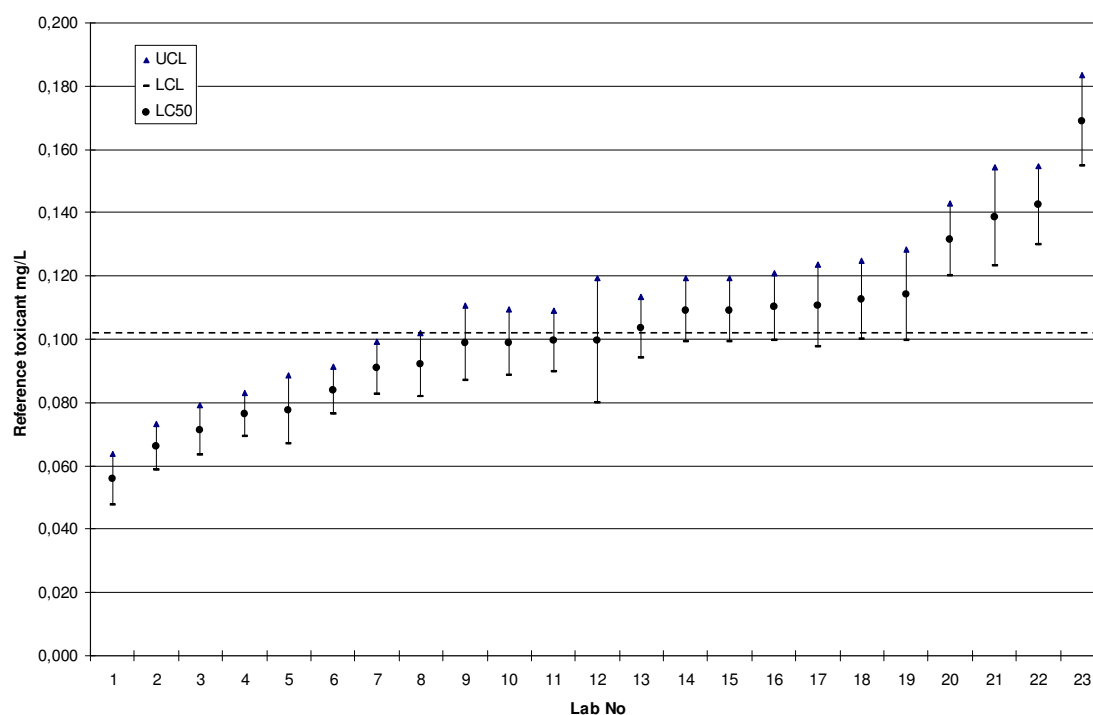


Fig. 1 – Results of the interlaboratory comparison (LC50 plus upper 95 % confidence limit UCL, and lower 95 % confidence limit LCL for each participant laboratory) on the acute toxicity test with the freshwater crustacean *Thamnocephalus platyurus*. The horizontal broken line indicates the overall LC50.

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

Figures 2 and 3 show the results of the Grubb's test applied to the h (1 outlier, no stragglers) and k (1 straggler, no outliers) statistics.

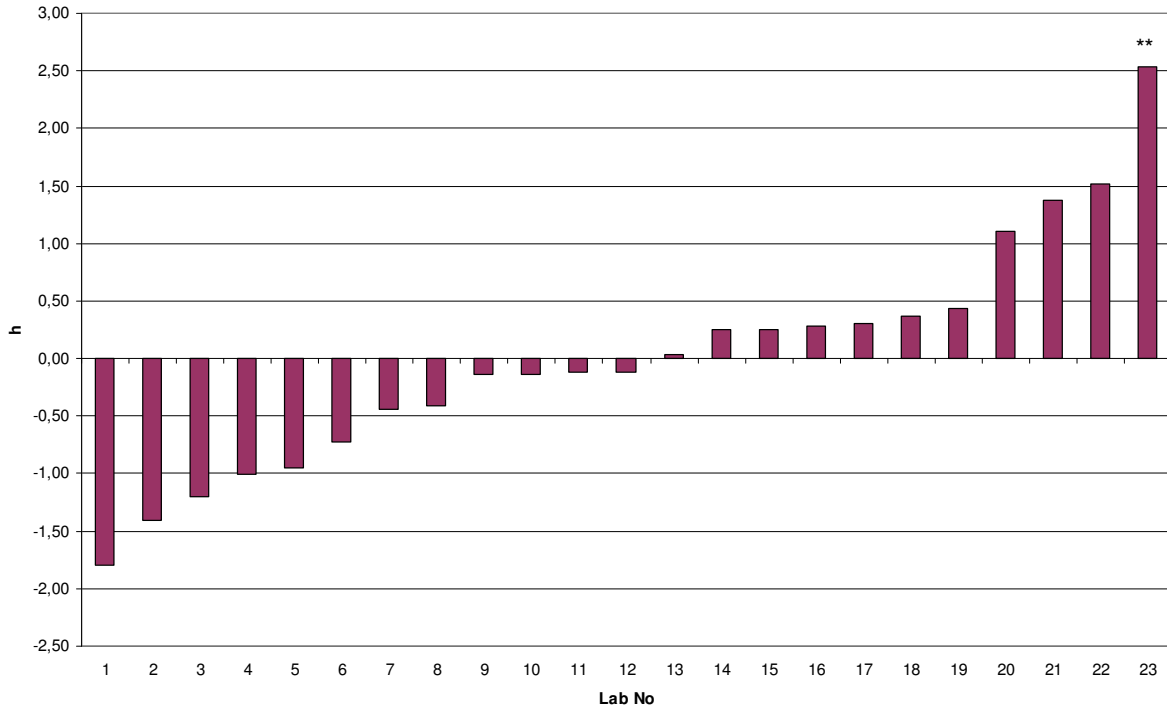


Fig. 2 - Mandel's between-laboratory consistency statistic, h, grouped by laboratories.

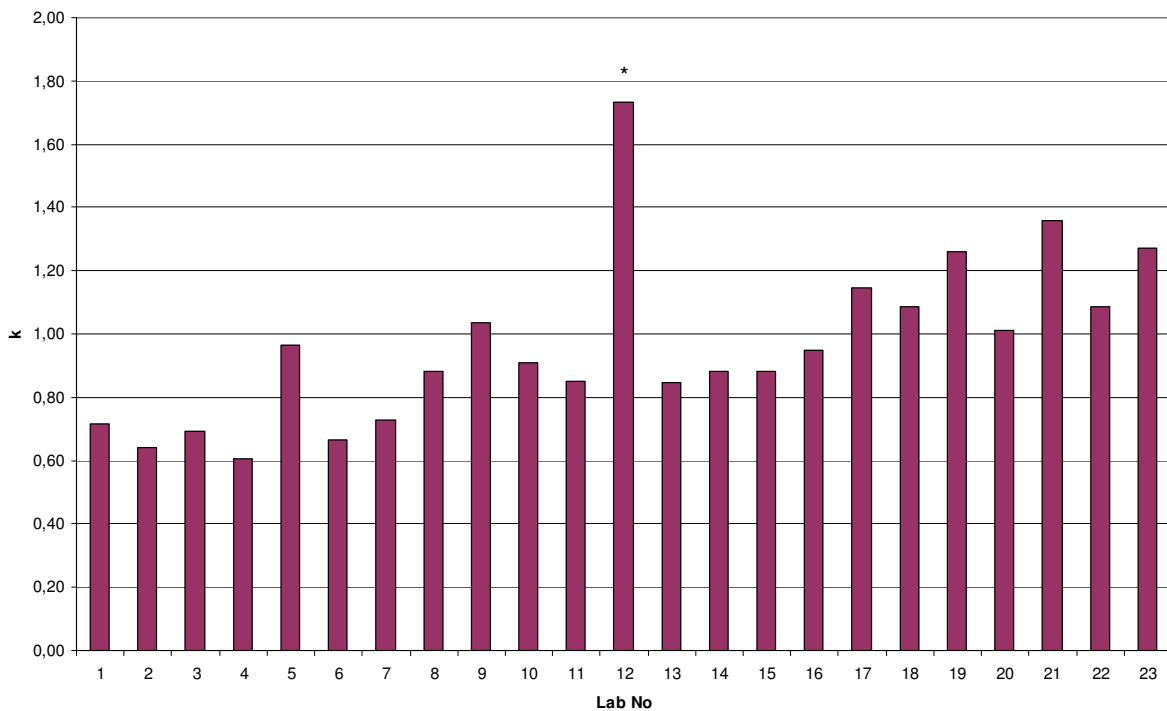


Fig. 3 - Mandel's within-laboratory consistency statistic, k, grouped by laboratories.

The final results are summarized in Table 1.

Therefore, after having excluded 1 outlier (Mandel's h statistic), the mean LC50 value is 0,100 mg $K_2Cr_2O_7/L$ (95 % confidence limits: 0,086 – 0,113), with a repeatability standard deviation s_r (within-laboratory variability) of 0,010 (9,74 as CV%), and a reproducibility standard deviation s_R (between-laboratory variability) of 0,024 (23,74 CV%).

Tab. 1 – Computed values for the acute toxicity test with the freshwater crustacean *Thamnocephalus platyurus*.

	All data	Without h and k outliers	Without h and k stragglers
N. labs	23	22	15
Mean	0,103	0,100	0,099
s_r	0,010	0,010	0,009
CV%	9,59	9,74	9,22
s_R	0,03	0,024	0,015
CV%	26,63	23,74	14,71
N. h straggler	0	2	0
N. h outlier	1	0	0
N. k straggler	1	1	0
N. k outlier	0	0	0
95 % UCL	0,118	0,113	0,109
95 % LCL	0,088	0,086	0,089

CONCLUSIONS

The International Interlaboratory Comparison on the determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca), following the method outlined by the ISO/CD 14380 (2010), clearly demonstrated that:

- the hatching of the *Thamnocephalus platyurus* cysts from the vial contained in the testkit in all cases provided enough living organisms to perform the test;
- all 23 participating laboratories, following the provided detailed instructions, were able to satisfactorily perform the test;
- the validity criterion that the percentage mortality in the controls should not be higher than 10 % was achieved by all the participating laboratories;
- the statistical analysis of the mortality data singled out only 1 outlier (Mandel's h statistic), and 22 out of 23 results were therefore accepted;
- the mean repeatability standard deviation s_r (within-laboratory variability) is lower than 10 % CV;
- the estimated LC_{50} 24 h has an associated reproducibility standard deviation s_R (between-laboratory variability) of 23,74 CV%.

Therefore, it can be safely concluded that the determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca), following the method outlined by the ISO/CD 14380 (2010), fulfils the requirements for a reliable and precise ecotoxicological test.

ACKNOWLEDGEMENTS

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