



Guidelines PT 2025

Battery of ecotoxicological tests

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1. INTRODUCTION

The purpose of the Proficiency Tests (PT) is to verify the participants' performance in accordance with the official methods used.

ARPAE LM RA has initiated the accreditation process as a Proficiency Test Provider (PTP), which will finish in October 2025 with the scheduled inspection visit. The proposed schemes will be subject to accreditation and will be carried out in compliance with the standard UNI CEI EN ISO/IEC 17043:2024.

This guideline and all the documentation sent to the participants, are an integral part of the Quality Management System (QMS) documents, drawn up in compliance with the UNI CEI EN ISO/IEC 17043:2024 standard.

The proficiency tests are organized by PTP RA for the determination of the ecotoxicity of chemical substances in aqueous solution.

The organization of the proficiency tests aims to improve the quality and accuracy of ecotoxicological tests data, as well as to evaluate the ability of each laboratory to obtain comparable results at national and European level.

All analytical determinations concerning the test samples are carried out by an Arpae laboratory accredited according to the UNI CEI EN ISO/IEC 17025:2018 standard.

2. CONFIDENTIALITY

The laboratories are identified only and exclusively through a numerical code (Lab.id); the identification code is assigned randomly and sent to each participant via email upon registration for the tests. The identification code for each laboratory is changed at least once a year.

The communication of a participant's results to third parties occurs only with the prior knowledge and written authorization of the participant.

This authorization is not foreseen if the request is required by law, however the Provider must give written notice to the participant.

3. SAMPLES FOR PT BATTERY OF ECOTOXICOLOGICAL TESTS

The preparation for the proficiency test (PT) includes:

❖ *the selection of the matrix:*

- Milli-Q water, grade 1 water with conductivity <10 $\mu\text{S}/\text{cm}^2$, for the EC50 determination with *Daphnia magna*, *Pseudokirchneriella subcapitata*, and *Aliivibrio fischeri*.

- ISO test water (ISO 6341 - 6.3 Dilution and culturing water; LG OECD 202 – Annex 3 ISO test water) for the % of immobilisation with *Daphnia magna* and for the OECD 236 - Fish Embryo Test (FET)

❖ *the selection of the active substance*: the substance to be used as a toxicant in the test is selected based on the following characteristics:

- availability of literature data
- low toxicity to the operator
- easy availability and accessibility
- low cost
- easy disposal at the end of the test

A defined quantity of active substance is homogenized with an appropriate volume of matrix; subsequently, it is divided into containers.

Given the nature of the samples, salts in aqueous solution, and the inherent chemical stability and miscibility of such substances, the assessment of homogeneity and stability prior to distribution is generally not deemed necessary, as the associated risk is considered negligible.

Nonetheless, a homogeneity and stability verification study is conducted at least once for each reference toxicant in compliance with the requirements of UNI CEI EN ISO/IEC 17043:2024 and ISO 13528:2022 standards. The statistical assessment of homogeneity is performed as specified in clause B.2.4 of ISO 13528:2022 by applying a one-way ANOVA F-test at a significance level of $\alpha = 0.05$.

Stability is evaluated by means of a one-sided t-test, in accordance with clause B.5.4 of ISO 13528:2022, employing a 95% confidence interval.

In the event that the study reveals evidence of insufficient homogeneity or compromised stability, the ongoing proficiency testing (PT) round is immediately suspended, and a revised operational plan (Annex 1-P73001/PT) is developed and implemented.

If the scheduled activities communicated to participants are not adhered to, prompt notification is issued through the communication channels established in the operational plan.

The samples are kept at a refrigerated temperature before shipping and they are shipped at room temperature.

The final report will highlight the approach for assessing homogeneity and stability. The results of the homogeneity and stability tests are processed using appropriate statistical techniques, which will be described in the final report.

4. COMMUNICATIONS

Communications between PTP RA and PT participants take place using the following Annexes:

- *Annex 1: Annual PT program* - sent by email with the presentation letter.
- *Annex 2: Participation and sample transport costs* - sent by email with the presentation letter.
- *Annex 3: PT registration form* - sent by email with the presentation letter; each participant must give it back by the specified deadline.
- *Annex 4: List of reference toxic substances*.
- *Annex 6: Data return sheet*: sent for the insertion of results and other useful information

If, during the year, changes should arise concerning company names, shipping addresses, billing data, email addresses and reference personnel, it is the responsibility of the participating laboratory to notify the coordinator by completing a new *Annex 3 - PT registration form*.

5. ASSIGNED VALUE, ROBUST RELATIVE STANDARD DEVIATION AND PERFORMANCE ASSESSMENT

Before calculating the assigned value and the intercomparison standard deviation, participants' results are assessed for gross errors. In this context, we define gross errors (also called *blunders*) as results that deviate by an order of magnitude or more from the median. These results will be excluded from subsequent calculations of the assigned value and the intercomparison standard deviation but will be highlighted in the final report/data statistics tables, indicating the participant(s) who returned such data.

The distribution of participant results is described using graphical tools such as "Kernel density plots" and histograms to highlight any bimodal and asymmetric trends.

Additionally, a box plot is employed to evaluate the presence of outliers.

Outliers are not excluded from the calculation, as the influence of anomalous results on statistical evaluation is minimized by applying robust statistics analysis through Algorithm A, as described in Annex C of ISO 13528:2022. As stated in section 6.5.2 of ISO 13528:2022, Algorithm A can compensate for the presence of anomalous data up to a maximum of 20% of the entire dataset. In the case of a percentage greater than 20%, the outliers are removed and the mean and standard deviation were calculated.

The assigned value is calculated either as robust mean using the Algorithm A as described in Annex C of ISO 13528:2022 or as mean when Algorithm A is not applicable. The uncertainty of the assigned value is always calculated and included in the report.

The PT standard deviation (σ_{pt}) is set as:

- 30% of the assigned value for the "Determination of EC50" scheme
- 40% of the assigned value for the "Determination of % of immobilization with D. magna" scheme

For the "Determination of the percentage of immobilization with Daphnia magna" scheme, the coordinator, based on his experience and the distribution of the data, may decide to use a σ_{pt} other than that provided for in section 8.2 of the ISO 13528:2022 standard. This choice will be justified in the final report.

To evaluate the performance of each participant, the z-score parameter is calculated using the following mathematical calculation:

$$z = \frac{(xi - x_{pt})}{\sigma_{pt}}$$

where

xi: represents the value detected by the laboratory

x_{pt} : represents the value detected by the robust statistical analysis

σ_{pt} : represents the PT standard deviation

For tests where the uncertainty of the assigned value, $u(x_{pt})$, is not negligible compared to the PT standard deviation, σ_{pt} , i.e., $u(x_{pt}) > 0.3\sigma_{pt}$, the z'-score is calculated as described in ISO 13528, paragraph 9.5.1:

$$z' = \frac{(x - x_{pt})}{\sqrt{u^2(x_{pt}) + \sigma_{pt}^2}}$$

The meaning of 'z-score' and 'z'-score' is presented in the following table:

z-score value	assessment
$ z \leq 2$	satisfying
$2 < z < 3$	questionable
$ z \geq 3$	unsatisfying

Since the assigned value of the PT is calculated as the consensus value among participants, in the case of a bimodal distribution, the z-score will not be calculated, highlighting this in the final report and still providing participants with as much information as possible, such as descriptive statistics and graphical analysis.

As reported in point D.1.3.1 of ISO 13528:2022, the assigned value can be estimated as a robust mean using algorithm A only with $p > 12$. This is because, if the number of participants was less than 13, the uncertainty of the assigned value could never satisfy the condition:

$$u(x_{pt}) < 0.3pt$$

If the number of participants is less than 13 ($p < 13$), the PTP may decide not to calculate the quantitative performance of the participants or to calculate it as a z-score (or z'-score), taking care to clarify the meaning of these values in the final report. The uncertainty of the assigned value must always be calculated and reported in the final report, along with the approach chosen for managing this data.

Analyzing the results of determining the percentage of *Daphnia magna* immobilization can be complex in some cases due to a strong asymmetry in the data distribution. In this case, the PTP may decide not to provide quantitative performance through the calculation of the z-score, but to provide a qualitative assessment in which the result is verified using the acceptability limit of a discharge as a reference (ref. Italian Legislative Decree 152/06).

For example, if the majority of participants (percentage greater than 70%) return an immobilization percentage lower than 50%, a "*positive rating*" will be given to all laboratories with an

immobilization percentage lower than 50% and a "negative rating" to the remaining laboratories. The opposite is true if the prevalent immobilization percentage is higher than 50%.

6. STEPS TO FOLLOW

For the year 2025, participation in the proficiency test involves 4 essential steps:

- 1 Each laboratory must complete and return to PTP RA the *Annex 3: PT registration form*, by the specified deadline; the tests of interest must be clearly indicated. The email address provided through the registration form is the only one that will be used for communications. Any changes must be notified.
- 2 Participating laboratories must respect the deadlines indicated for sending the results, using exclusively the *Data Return Sheets*.
- 3 Once all the results have been submitted, PTP RA evaluates the results and sends to the participants the Final Report in pdf format. This report will include information on the test preparation, statistical evaluation and graphical representation of the results, and any other relevant information.
- 4 Participating laboratories will receive by 31/12/2025 the invoice for the PT participation that includes the costs for samples and shipping. Participating laboratories must pay the amount reported in the invoice. The costs are those reported in *Annex 2: Participation and sample transport costs*.

7. SAMPLE QUANTITY

Participating laboratories, depending on the chosen tests, may receive:

1. For round 1S-E25 PT FET:

- A PET bottle containing 1000 ml of sample for performing the Fish Embryo Test (FET) for the determination of the LC50

2. For round 2S-E25 PT ECOTOX:

- A PET bottle containing 500 ml of sample for the determination of the % of immobilization with *Daphnia magna*.

- A PET bottle containing 1000 ml of sample for the EC50 determination tests with *Daphnia magna*, *Pseudokirchneriella subcapitata* and *Aliivibrio fischeri*.

8. SAMPLE SHIPMENT

All test samples are stored refrigerated until shipment.

The expected shipping dates are those indicated in *Annex 1: Annual PT Program*.

In the event of damage or loss of samples, the participating laboratory may request a second shipment free of charge.

9. SAMPLE HANDLING

Once received, the test samples must be kept under refrigeration until analysis to prevent any deterioration. All the participants may use their own standard analytical procedures and reference materials for testing. The sample should be handled as a routine sample.

10. RESULTS

The results are returned via an Excel spreadsheet (*Annex 6: Data return sheet*) which must be returned in signed PDF format within the timeframe established by the provider.

Until the deadline, an unlimited number of changes and/or additions can be made by submitting a new Excel spreadsheet (*Annex 6: Data return sheet*) to replace the one previously sent. After the deadline, it is no longer possible to modify the results sent.

The samples received are considered to be at a concentration of 100%, and any dilution must be referred to this concentration.

The results must be expressed as a percentage of the initial sample, using a period as decimal separator; no separator is needed to identify thousands.

For each test, only one data is used for each participating laboratory; if more than one result is submitted, the participating laboratory must nominate one result from those submitted, which will be used for performance evaluation. Any other results submitted are evaluated based on the statistical data obtained in the PT and communicated via email to the participating laboratory. The results provided by participants are never rounded and are used in the calculations as submitted by the participants; descriptive and performance statistics are calculated and reported in the report with four significant figures. The z-score is reported with two decimal places.

11. APPEALS AND COMPLAINTS

In accordance with ARPAE's Quality Management System (QMS), participants to the PT have the opportunity to submit appeals or complaints to the PT coordinator by sending a written communication via email to the following address:

interconfronto-ra@arpae.it

The nature of the complaint is related to the service provided: delays in the scheduled timeline, damaged samples.

An appeal can be submitted after the final report has been issued. The participant may contest the evaluation of their performance by presenting objective documentation of their reasons. Once an appeal or complaint has been received, PTP RA has 30 calendar days to respond via email and, if necessary, amend the final report.

12. REMAINING SAMPLES

At the conclusion of each PT scheme, remaining samples are stored at a controlled freezing temperature, for a period of 30 calendar days from the sending date of the Final Report revision 0.

The samples will remain available to the participants who may request an additional aliquot to use the sample for their own purposes.

Please note that the stability of the material is guaranteed until the results transmission deadline, that is communicated by email and specified in *Annex 1: Annual PT program*. The participating laboratory is responsible for the shipping logistics and costs of the samples.

13. ARCHIVING

All the records related to PT schemes (e.g., results sent by participants, statistical analysis, reports, documents and any relevant communication) are stored on a computer medium by the PTP, for at least 5 years. The electronic storage of these files is managed in accordance with I71101/LM.