



Transposition of the approach described in ISO/TR 13843 (2001)
to the evaluation of uncertainties associated with
water microbiological analysis results

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Context

A working group has been constituted at the AFNOR to address the issue of uncertainties evaluation in microbiology.

There have been discussions about the suitable approach that should be used to estimate this uncertainty of measurement (refer to ISO/TC 147/SC 4 N 0229 of S. Niemelä for more details of the main approaches).

AGLAE (Association Générale des Laboratoires d'Analyse de l'Environnement) has been supporting the combined uncertainty approach.

However, there have been objections from some French laboratories arguing that the workload to evaluate the effect of the different components of uncertainty with such an approach is too heavy.

Considering that point, AGLAE has developed an approach based on the combined uncertainty approach but presenting also similarities with the global approach, including notably data from internal quality control and interlaboratory studies.

This approach derives from a principle described in ISO/TR 13843 (2001) 'Guidance on validation of microbiological methods'.



1. Introduction

The aim of the present document is to set the principle of this 'mixed' approach. At this stage, only the basic concept is described and some numerical examples are given to illustrate it. Complete experimental designs will have to be implemented to evaluate the different components.

As reminded by S. Niemelä in ISO/TC 147/SC 4 N 0229 (about the global approach), the design of experiments is very important in order to represent the components correctly and to make sure the estimate is valid.

Ideological basis of this approach: once an experiment has been completed, the uncertainty estimate is assumed valid for future analyses by the same method on similar types of samples in the same laboratory.

At the end not only guidelines but a protocol will be elaborated.

2. Key calculations

Arithmetic mean \bar{x} and variance s^2 :

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n} \quad \text{and} \quad s^2 = \frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n - 1}$$

The squared coefficient of variation, CV^2 , will also be used:

$$CV^2 = \frac{s^2}{\bar{x}^2}$$

3. Model of dispersion

The uncertainty can be expressed as a combined standard uncertainty (based on the law of propagation, GUM approach):

$$CV_{tot}^2 = \sum CV^2$$

In this combined uncertainty approach, the different analytical steps are identified, the sources of uncertainties (components) are listed and then quantified. Each component is represented by a squared coefficient of variation (CV^2).



Example of enumeration of *E. coli* following ISO 9308-1 (2000).

| Different steps of the analysis (standard assay) | Representation of random error sources <i>a priori</i> of importance in the considered model (*) |
|--|---|
| 1/ Filtration of 100 ml. | $CV_{Poisson}^2$: represents the random error due to the distribution of micro-organisms in water. (without test portion volume errors) |
| 2/ Membrane transferred onto a selective culture medium. | CV_{medium}^2 : represents the random error due to disparities of culture media batches, batches of membranes, ... |
| 3/ After incubation, typical colonies are counted ('presumptive positives'). | $CV_{reading}^2$: represents the random error reading the plates. |
| 4/ A number of typical colonies is randomly picked for further biochemical testing. | $CV_{picking}^2$: represents the random error due to the random choice of colonies picked. $CV_{confirmation}^2$: represents the random error due to the setting of confirmation tests. |
| 5/ Confirmed count calculated using the proportion of confirmed colonies among the presumptive positive colonies: 30 typical colonies; 10 colonies picked for testing; confirmation of 3 <i>E. coli</i> . Gives a result of 9 <i>E. coli</i> in 100ml. | (*) : systematic errors also contribute to the total uncertainty. However, here they have not been included at all levels. Interlaboratory comparisons reveal yield differences which are not identified within a laboratory. |

3.1 Expression of the total dispersion

It can then be expressed as :

$$CV_{tot}^2 = CV_{Poisson}^2 + CV_{medium}^2 + CV_{reading}^2 + CV_{picking}^2 + CV_{confirmation}^2$$

3.2 Two comments

3.2.1 Calculation of $CV_{Poisson}^2$

The number of particles observed in the aliquot is randomly distributed with a Poisson distribution.

$$CV_{Poisson}^2 = \frac{1}{\lambda}$$

with λ average number of colonies observed.

3.2.2 About $CV_{picking}^2$

This component might be significant.

Example: 30 typical colonies on a plate, among them 9 are *E. coli*.

Out of the 30 presumptive positive colonies, a random subset of 10 colonies is isolated and tested.

The hypergeometric distribution gives the probability that the number of *E. coli* among the 10 presumptive positive colonies tested is 0, 1, ...10.

| Number of <i>E.coli</i> potentially present among the 10 presumptive colonies tested | Probability |
|--|-------------------|
| 0 | 0,01173958 |
| 1 | 0,08804689 |
| 2 | 0,24382214 |
| 3 | 0,32509619 |
| 4 | 0,22756734 |
| 5 | 0,08533775 |
| 6 | 0,01673289 |
| 7 | 0,00159361 |
| 8 | 6,2906E-05 |
| 9 | 6,9895E-07 |
| 10 | 0 |

Considering 95% of the cases, the number of *E. coli* out of the 10 presumptive positives randomly tested may vary between 1 and 5. The fact not to pick all the typical colonies ('partial confirmation') for confirmation tests rather than a total confirmation introduces a large uncertainty: the result '9 *E. coli* in 100ml' becomes '3 to 15 *E. coli* in 100ml'.

In terms of CV^2 :

$$CV_{picking}^2 = \frac{s^2}{\bar{x}^2} = \frac{\sum_{i=1}^n p_i \cdot (x_i - \bar{x})^2}{\bar{x}^2}$$

In this example, $CV_{picking}^2 = 0,16$ whereas $CV_{Poisson}^2 = 0,11 (= \frac{1}{9})$.

Partial confirmation of typical colonies can generate more uncertainty than the aliquot sampling, represented by the Poisson distribution.

Intra and interlaboratories data do not always give an idea of that source of error.

Indeed, it is extremely difficult to plan experiments where the materials are sufficiently representative to reflect that source of error (representative of the complexity sometimes met on natural samples).

The combined uncertainty approach is a reliable approach from a statistical point of view.

However, the main limit of that approach is the necessity to set experimental designs adapted to each source of dispersion and the cost of its implementation for laboratories might be prohibitive.

3.3 Alternative approach

A pragmatic and objective alternative might be to gather sources of dispersions related to the enumeration method. The formula:

$$CV_{tot}^2 = CV_{Poisson}^2 + CV_{medium}^2 + CV_{reading}^2 + CV_{picking}^2 + CV_{confirmation}^2$$

can be expressed as:

$$CV_{tot}^2 = CV_{Poisson}^2 + CV_{Method}^2$$

CV_{Method}^2 can be determined by an adapted design of experiment. Precision levels then have to be set:



- either repeatability (work within an analytical series) ;
- or intralaboratory reproducibility (work within a laboratory but with several analytical series) ;
- or interlaboratory reproducibility (work of several laboratories on the same material).

CV_{Method}^2 can then be expressed as :

$$CV_{Method}^2 = CV_A^2 + CV_B^2 + CV_C^2$$

with:

CV_A^2 is a component within the same analytical series;

CV_B^2 is the 'analytical series' component of the systematic error;

CV_C^2 is the 'laboratory' component of the systematic error.

Actually, CV_A^2 is the measurement error (added to Poisson distribution) within an analytical series ; CV_B^2 is the measurement error from one analytical series to another one ; CV_C^2 is the measurement error from one laboratory to another one.

Therefore, under repeatability conditions (within an analytical series):

$$CV_{tot}^2 = CV_{Poisson}^2 + CV_A^2$$

Under conditions of intralaboratory reproducibility (within a laboratory but in different analytical series):

$$CV_{tot}^2 = CV_{Poisson}^2 + CV_A^2 + CV_B^2$$

Under conditions of interlaboratory reproducibility (from one laboratory to another):

$$CV_{tot}^2 = CV_{Poisson}^2 + CV_A^2 + CV_B^2 + CV_C^2$$

4. Quantifying CV^2 of the model

To quantify the different CV^2 of the model (CV_A^2 , CV_B^2 , CV_C^2), one might proceed as follows:

4.1 First term : CV_A^2

The estimate of CV_A^2 can be realised on the basis of internal quality control results or external quality control (provided that there are duplicates). More generally, any series of repeated analyses under repeatability conditions, preferably on naturally contaminated samples, might quantify CV_A^2 .



Example to illustrate the concept: determination of CV_A^2 from 15 measurements repeated on the same material within an analytical series:

| x_i | $(x_i - \bar{x})^2$ |
|--------------------------|--|
| 38 | 29,16 |
| 33 | 0,16 |
| 42 | 88,36 |
| 37 | 19,36 |
| 36 | 11,56 |
| 26 | 43,56 |
| 34 | 1,96 |
| 35 | 5,76 |
| 40 | 54,76 |
| 29 | 12,96 |
| 27 | 31,36 |
| 22 | 112,36 |
| 29 | 12,96 |
| 28 | 21,16 |
| 33 | 0,16 |
| $\sum_{i=1}^n x_i = 489$ | $\sum_{i=1}^n (x_i - \bar{x})^2 = 445,6$ |
| $\bar{x} = 32,6$ | $s^2 = 31,8285714$ |

Estimate of λ : $\bar{x} = 32,6$.

Estimate of observed CV_{tot}^2 : $\frac{s^2}{\bar{x}^2} = \frac{31,83}{(32,6)^2} = 0,0299$.

Calculation of $CV_{Poisson}^2$: $CV_{Poisson}^2 = \frac{1}{\bar{x}} = \frac{1}{32,6} = 0,0307$.

In these conditions of repeatability: $CV_{tot}^2 = CV_{Poisson}^2 + CV_A^2$

$CV_A^2 = CV_{tot}^2 - CV_{Poisson}^2 = 0,0299 - 0,0307 = 0,0000$.

This negative result means that the dispersion of observed counts follows Poisson distribution. The error due to the enumeration method does not seem significant (so $CV_A^2 = 0$).

4.2 Second term : CV_B^2

As for CV_A^2 , CV_B^2 can be evaluated from data of internal quality control. However, provided that the materials used for that control are representative of routine samples: the global level of contamination (of the whole flora) and the physiological state of the enumerated germs should be considered.

The use of stable materials (reference or even better certified) is necessary.



Example to illustrate the concept: determination of CV_B^2 using data from internal quality control; one measurement per day on a reference material, during 30 days.

| x_i | $(x_i - \bar{x})^2$ |
|--------------------------|---|
| 34 | 6,93444444 |
| 24 | 54,2677778 |
| 16 | 236,134444 |
| 35 | 13,2011111 |
| 41 | 92,8011111 |
| 32 | 0,40111111 |
| 35 | 13,2011111 |
| 38 | 44,0011111 |
| 41 | 92,8011111 |
| 30 | 1,86777778 |
| 25 | 40,5344444 |
| 33 | 2,66777778 |
| 28 | 11,3344444 |
| 46 | 214,134444 |
| 35 | 13,2011111 |
| 15 | 267,867778 |
| 34 | 6,93444444 |
| 30 | 1,86777778 |
| 26 | 28,8011111 |
| 30 | 1,86777778 |
| 25 | 40,5344444 |
| 23 | 70,0011111 |
| 42 | 113,067778 |
| 39 | 58,2677778 |
| 18 | 178,667778 |
| 39 | 58,2677778 |
| 32 | 0,40111111 |
| 26 | 28,8011111 |
| 28 | 11,3344444 |
| 41 | 92,8011111 |
| $\sum_{i=1}^n x_i = 941$ | $\sum_{i=1}^n (x_i - \bar{x})^2 = 1796,96667$ |
| $\bar{x} = 31,3666667$ | $s^2 = 61,9643678$ |

Estimate of observed CV_{tot}^2 : $\frac{s^2}{\bar{x}^2} = \frac{61,96}{(31,4)^2} = 0,0630$.

Calculation of $CV_{Poisson}^2$: $CV_{Poisson}^2 = \frac{1}{\bar{x}} = \frac{1}{31,4} = 0,0319$.

Under these conditions of intralaboratory reproducibility: $CV_{tot}^2 = CV_{Poisson}^2 + CV_A^2 + CV_B^2$

$CV_B^2 = CV_{tot}^2 - CV_{Poisson}^2 - CV_A^2 = 0,0630 - 0,0319 - 0,0000 = 0,0311$.



(CV_A^2 previously evaluated)

4.3 Last term : CV_C^2

The only way to establish it is to proceed to interlaboratory comparisons (proficiency testing schemes), organised with representative materials, as varied as possible.

Example to illustrate the concept: determination of CV_C^2 from interlaboratory comparisons; one measure by 73 laboratories.

$$\sum_{i=1}^n x_i = 2003$$

$$\bar{x} = 27,4383562$$

$$\sum_{i=1}^n (x_i - \bar{x})^2 = 21603,9726$$

$$s^2 = 300,055175$$

Estimate of observed CV_{tot}^2 : $\frac{s^2}{\bar{x}^2} = \frac{300,06}{(27,4)^2} = 0,3986$.

Calculation of $CV_{Poisson}^2$: $CV_{Poisson}^2 = \frac{1}{\bar{x}} = \frac{1}{27,4} = 0,0364$.

Under these conditions of interlaboratory reproducibility:

$$CV_{tot}^2 = CV_{Poisson}^2 + CV_A^2 + CV_B^2 + CV_C^2$$

$$CV_C^2 = CV_{tot}^2 - CV_{Poisson}^2 - CV_A^2 - CV_B^2$$

$$CV_C^2 = 0,3986 - 0,0364 - 0,0000 - 0,0311 = 0,3310$$

(CV_A^2 and CV_B^2 previously evaluated)

At the end :

The model $CV_{tot}^2 = CV_{Poisson}^2 + CV_A^2 + CV_B^2 + CV_C^2$ is estimated.

$$CV_{tot}^2 = CV_{Poisson}^2 + 0,0000 + 0,0311 + 0,3310$$

$$CV_{tot}^2 = CV_{Poisson}^2 + 0,3621$$

The intralaboratory error is low (within an analytical series, it is even negligible). However, with the material considered during the interlaboratory comparison, the measurement error (laboratory component of the systematic error, CV_C^2) is significant.

4.4 Comments

4.4.1 When data come from a nested design, calculations have to be adapted.

4.4.2 Before calculating any CV^2 , it is relevant to try to know whether data follow a Poisson distribution by testing for over-dispersion. In such cases, there is no need to calculate CV^2 , it then equals zero.

5. Expression of the uncertainty of measurement

In this global approach, several levels of estimate can be distinguished. One should be careful so that there are no ambiguities in the identification and interpretation of these levels.

- 1- Thus, if the contamination level of several sites has to be compared, it is recommended to ask one single laboratory to analyse the samples within the same analytical series (all the analyses on the same day). The uncertainty established under repeatability conditions will then have to be considered.
- 2- If the contamination level of one site is investigated over time, the analyses should then also be performed by one single laboratory and the uncertainty established under reproducibility conditions will be considered.
- 3- However, when test results from several laboratories have to be compared or when one test result has to be compared to a regulation value, the uncertainty established under interlaboratory reproducibility conditions will have to be considered.

The results of uncertainties could be presented in such a table :

| Observation level of uncertainty | Source of data | | |
|----------------------------------|----------------|---------------------------------------|---|
| | Laboratory | 'Laboratory community' ('profession') | Laboratory seen through Proficiency Testing |
| $U_{\text{Repro inter}}$ | | | |
| $U_{\text{Repro intra}}$ | | | |
| U_{repea} | | | |
| U_{Poisson} | | | |

For some parameters (example : *E. coli*), the regulation value is the absence in 100ml. It is not relevant to express the uncertainty as '30 to 50 *E. coli* in 100ml'. The use of the limit of detection is then recommended, it should be mentioned in the above table.

For other parameters (*Legionella*, culturable micro-organisms), the regulation value is superior to zero. Another expression of the uncertainty might be the confidence interval around the regulation value.

5.1 Limit of detection

- When Poisson distribution prevails, the probability of a positive result $p(+)$ can be calculated with the following formula (ISO/TR 13843) :

$$p(+)=1-e^{-\lambda}$$

where e is the base of natural logarithm;
 λ is the mean number of particles in the test portion volume.

One of the definitions currently used for the limit of detection is the concentration to which the probability of detection of the presence of analyte equals 95% [$p(+)=0,95$].



Then $e^{-\lambda}=1-0,95=0,05$. This equation can be solved to give $\lambda=-\ln(0,05)=3,0$.

Consequently, for a mean microbial load of 3 per test portion volume, the chances of detection of one particle in a test portion volume are 95% (provided Poisson distribution applies).

- In our example, at the interlaboratory level: $CV_{tot}^2 = CV_{Poisson}^2 + 0,3621$.

The dispersion is not limited to Poisson distribution.

When over-dispersion is observed, the negative binomial model is applicable (ISO/TR 13843).

The limit of detection, if defined in terms of probability of a positive result, can be calculated from the probability of negative results. The probability of a negative result (null) has been established as (but using the symbols of the present document):

$$p_0 = (1 + LD \cdot CV_{Method}^2)^{-\frac{1}{CV_{Method}^2}}$$

This equation can be solved to give the limit of detection (LD) when the probability of negative results and the over-dispersion (CV_{Method}^2) have been indicated:

$$LD = \frac{p_0^{-CV_{Method}^2} - 1}{CV_{Method}^2}$$

At 95% ($p_0 = 0,05$), in our example:

$$LD = \frac{0,05^{-0,3621} - 1}{0,3621} = 5,4$$

At the interlaboratory level (level of ‘laboratory community’), the uncertainty should be expressed by the limit of detection of 5,4 *E. coli* per 100ml in average.

This means that only mean microbial load superior to 5,4 per 100ml can reasonably be considered as detected. Inversely, any negative result (presence not detected) has to be considered as a mean microbial load inferior to 5,4 *E. coli* per 100ml

5.2 Confidence intervals

The value of the total uncertainty (CV_{tot}^2) will allow to calculate the upper and lower confidence limits around the regulation value (using the negative binomial model).

5.3 Introduction of a limit of quantification

The idea of a limit of quantification could also be introduced: a tolerable uncertainty is set, for example the uncertainty observed with a mean count of 25 germs per test portion volume.

Then :

$$CV_{LQ}^2 = \frac{1}{25} = 0,04$$

In our example $CV_{tot}^2 = CV_{Poisson}^2 + 0,3621$, $CV_{tot}^2 = 0,04$ cannot be reached.

In that case, the method should be considered as a presence/absence method.



If CV_{Method}^2 (0,3621 in our example) had been lower (0,01 for example), a limit of quantification could have been established as follows:

$$CV_{\text{tot}}^2 = CV_{\text{Poisson}}^2 + 0,01$$

At the limit of quantification: $CV_{LQ}^2 = 0,04 = CV_{\text{Poisson}}^2 + 0,01$

$$CV_{\text{Poisson}}^2 = 0,04 - 0,01 = 0,03 = \frac{1}{LQ}$$

The limit of quantification is 33,3 germs in average per 100 ml.