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Water quality - The variability of test results and the uncertainty of measurement of microbiological enumeration methods

Qualité de l'eau - Variabilité des résultats d'essais et incertitude de mesure des méthodes d'énumération microbienne

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Water quality — The variability of test results and the uncertainty of measurement of microbiological enumeration methods

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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Introduction

Testing laboratories are required to apply procedures for estimating uncertainty of measurement (see ISO/IEC 17025[5]). Without such an indication, measurement results cannot be compared, either among themselves or with reference values (see ISO/IEC Guide 98-3:2008[7]).

General guidelines for the evaluation and expression of uncertainty in measurement have been elaborated by experts in physical and chemical metrology, and published by ISO and IEC in ISO/IEC Guide 98-3:2008.[7] However, ISO/IEC Guide 98-3:2008[7] does not address measurements in which the observed values are counts.

The emphasis in ISO/IEC Guide 98-3:2008[7] is on the “law of propagation of uncertainty” principle, whereby combined estimates of the uncertainty of the final result are built up from separate components evaluated by whatever means are practical. This principle is referred to as the “component approach” in this International Standard. It is also known as the “bottom-up” or “step-by-step” approach.

It has been suggested that the factors that influence the uncertainty of microbiological enumerations are not well enough understood for the application of the component approach (see ISO/TS 19036:2006[6]). It is possible that this approach underestimates the uncertainty because some significant uncertainty contributions are missed. Reference [19] shows, however, that the concepts of ISO/IEC Guide 98-3:2008[7] are adaptable and applicable to count data as well.

Another principle, a “black-box” approach known as the “top-down” or “global” approach, is based on statistical analysis of series of repeated observations of the final result (see ISO/TS 19036:2006[6]). In the global approach it is not necessary to quantify or even know exactly what the causes of uncertainty in the black box are.

According to the global philosophy, once evaluated for a given method applied in a particular laboratory, the uncertainty estimate may be reliably applied to subsequent results obtained by the method in the same laboratory, provided that this is justified by the relevant quality control data (EURACHEM/CITAC CG 4[10]). Every analytical result produced by a given method thus should have the same predictable uncertainty. This statement is understandable against its background of chemical analysis. In chemical analyses the uncertainty of the analytical procedure and the uncertainty of the final result of analysis are usually the same. The global principle dismisses the possibility that there might be something unique about the uncertainty of a particular analysis.

The uncontrollable “variation without a cause” that always accompanies counts alters the situation for microbiological enumerations. The full uncertainty of a test result can be estimated only after the final result has been secured. This applies to both the global and the component approaches.

The unpredictable variation that accompanies counts increases rapidly when counts get low. The original global design is therefore not suitable for low counts, and therefore also not applicable to most probable number (MPN) methods and other low-count applications, such as confirmed counts.

It is often necessary, and always useful, to distinguish between two precision parameters: the uncertainty of the technical measuring procedure (operational variability), which is more or less predictable, and the unpredictable variation that is due to the distribution of particles. A modification of the global principle that takes into account these two sources of uncertainty is free from the low-count restriction. This is the global model detailed in this International Standard.

In theory, the two quantitative approaches to uncertainty should give the same result. A choice of two approaches is presented in this International Standard. Offering two approaches is appropriate not only because some parties might prefer one approach to the other. Depending on circumstances one approach may be more efficient or more practical than the other.

Neither of the main strategies is, however, able to produce unequivocal estimates of uncertainty. Something always has to be taken for granted without the possibility of checking its validity in a given situation. The estimate of uncertainty is based on prior empirical results (experimental standard uncertainties) and/or reasonable general assumptions.

Water quality — The variability of test results and the uncertainty of measurement of microbiological enumeration methods

1 Scope

This International Standard gives guidelines for the evaluation of uncertainty in quantitative microbiological analyses based on enumeration of microbial particles by culture. It covers all variants of colony count methods and most probable number estimates.

Two approaches, the component (also known as bottom-up or step-by-step) and a modified global (top-down) approach are included.

The aim is to specify how values of intralaboratory operational variability and combined uncertainty for final test results can be obtained.

The procedures are not applicable to methods other than enumeration methods.

NOTE 1 Most annexes are normative. However, only the annexes relevant to each case are to be applied. If the choice is the global approach, then all normative annexes that belong to the component approach can be skipped and vice versa.

NOTE 2 Pre-analytical sampling variance at the source is outside the scope of this International Standard, but needs to be addressed in sampling designs and monitoring programmes.

NOTE 3 The doubt or uncertainty of decisions based on the use of analytical results whose uncertainty has been estimated is outside the scope of this International Standard.

NOTE 4 The extra-analytical variations observed in proficiency tests and intercalibration schemes are also not detailed in this International Standard, but it is necessary to take them into consideration in analytical control. The use of intercalibration data in uncertainty estimation offers the possibility for the bias between laboratories to be included (Nordtest Report TR 537[12]).

2 Key concepts

2.1 Uncertainty of measurement

Uncertainty of measurement according to ISO/IEC Guide 98-3:2008[7] is defined as a “parameter, associated with the result of measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand”. It is a measure of imprecision. The parameter is expressed as a standard uncertainty or relative standard uncertainty.

2.2 Estimation of the uncertainty of measurement

According to ISO/IEC Guide 98-3:2008,[7] the parameter can be evaluated by statistical analysis of series of observations. This is termed type A estimation of uncertainty.

Any other type of procedure is called type B estimation of uncertainty. The most common type B estimates in microbiological analysis are those based on assumed statistical distributions in the component approach.

Types A and B may refer to the uncertainty of individual components of uncertainty as well as to the combined uncertainty of the final result.

Type A evaluations of standard uncertainty are not necessarily more reliable than type B evaluations. In many practical measurement situations where the number of observations is limited, the components obtained from type B evaluations can be better known than the components obtained from type A evaluations (ISO/IEC Guide 98-3:2008[7]).

2.3 Intralaboratory reproducibility

A somewhat abstract expression of uncertainty, **intralaboratory reproducibility**, is frequently considered the most appropriate parameter of the uncertainty of measurement, see ISO/TS 19036:2006.[6] It is also known as intermediate reproducibility or intermediate precision, e.g. [time + equipment + operator]-different intermediate precision standard uncertainty as defined by ISO 5725-3.[2] The idea is to evaluate how much the analytical result might have varied if the analysis had been made by another person in the same laboratory using different equipment and batches of material and different analytical and incubation conditions than those actually employed. The value of intermediate precision estimated never belongs to any actual analytical result, but is assumed to give a general estimate of reasonable uncertainty for the application of a method in one particular laboratory.

Intralaboratory reproducibility is estimated either by combining separate components of uncertainty determined under intralaboratory reproducibility conditions (component approach) or by special experiments in which the analytical conditions are varied by design (global approach).

2.4 Combined standard uncertainty

2.4.1 General

The final test results of microbiological analyses are calculated from intermediate **observed values**. The main intermediate observation is the count. Most of the other observed values are connected with volume measurements.

Combined standard uncertainty, as defined in ISO/IEC Guide 98-3:2008,[7] is the "standard uncertainty of the result of a measurement when that result is obtained from the values of a number of other quantities, equal to the positive square root of a sum of terms, the terms being variances or covariances of these other quantities weighted according to how the measurement result varies with changes in these quantities".

NOTE 1 Observation of covariances is only necessary if significant correlations occur between components of uncertainty. Otherwise a simple root sum of variances is sufficient (see 2.4.2 and 2.5).

NOTE 2 In cases of microbiological enumeration, it can be assumed that all components of uncertainty are independent, i.e. statistically uncorrelated. In such instances, the combined standard uncertainty is the positive square root of the sum of component variances, i.e. the root sum of squares (Annex B). (ISO/IEC Guide 98-3:2008.[7])

2.4.2 Significant property of combined uncertainties

According to EURACHEM/CITAC CG 4[10], "Unless there is a large number of them, components (standard uncertainties) that are less than one-third of the largest need not be evaluated in detail". This statement implies that in borderline cases, even a single component might provide an adequate estimate of the combined uncertainty. To decide when a component is unimportant, its approximate size should be known in relation to other components. Generally at least two, usually more, components are significant and should be included.

EXAMPLE The combined uncertainty of two components, one three times the other, is calculated as $u_c(y) = \sqrt{3^2 + 1^2} = \sqrt{10} = 3{,}16$.

Without the smaller component, the estimate would be 3,00. Ignoring the smaller component underestimates the combined uncertainty in this case by about 5 %. For the sake of caution, setting a four-fold difference as the limit might be recommended.

2.5 Relative standard uncertainty

2.5.1 General

The formula for the final results of microbiological analyses involves only multiplication and division. Under such conditions, the combined standard uncertainty should be calculated from components expressed as relative standard uncertainties (ISO/IEC Guide 98-3:2008[7])(see Annex B).

With both type A and type B estimates, the symbol chosen to represent the **relative standard uncertainty** is u_{rel}.

NOTE 1 Relative standard uncertainty is often expressed as a percentage. The term commonly used for this expression is coefficient of variation (CV), C_V.

NOTE 2 When it is important to stress that the standard uncertainty has been calculated by the type A process, the symbol used is s.

NOTE 3 Any systematic or random variation that takes place in the process before the final suspension, such as subsampling, matrix, and dilution effects, influence the target concentration in the final suspension proportionally. Relative variances of these components are therefore additive. Such effects after inoculation as incubation, and reading, can be more complicated statistically and are not well enough known. Proportionality can still be the best simple approximation. Systematic errors in these influences are usually treated as if they were random effects.

2.5.2 Logarithms and relative standard uncertainty

"Global" estimates of experimental standard uncertainty are traditionally made by calculation with common logarithms. When using such estimates in further calculations together with other estimates, it is necessary to express all components of uncertainty on the same scale of measurement, either by converting relative standard uncertainties into logarithms or logarithms into relative standard uncertainties.

In most cases, absolute standard uncertainty calculated in natural logarithmic scale and the relative standard uncertainty in interval scale can be assumed to be numerically equal. Values calculated in common logarithms can be converted to natural logarithms and vice versa by use of appropriate coefficients. The mathematical relationships between relative standard uncertainty and standard uncertainty on different logarithmic scales are shown in B.9.

2.6 Relative variance

The square of the relative standard uncertainty is called the relative variance (ISO/IEC Guide 98-3:2008).[7]

2.7 Expanded uncertainty and expanded relative uncertainty

Especially when the test result is used for assessing limits concerned with public health or safety, it is pertinent to give an uncertainty value that encompasses a large fraction of the expected range of the observed values. The parameter is termed the **expanded uncertainty**, for which the symbol is U.

The value of U is obtained by multiplying the combined uncertainty with a **coverage factor** k:

$$U = ku_{\text{c}}(y)$$

The value of k is typically in the range 2 to 3. On the relative scale

$$U_{\text{rel}} = ku_{\text{c,rel}}(y)$$

For normal distributions, about 95 % of the results are covered by the expanded uncertainty interval $\mu \pm U$, where μ is the mean, when the coverage factor k = 2 is chosen. When k = 3, coverage corresponds to about 99 %.

Microbiological test results almost never fit a normal distribution perfectly. Distributions are often markedly asymmetrical (skewed). When there are sufficient reasons for assuming distributions to be other than normal (e.g. Poisson or negative binomial or log–normal distributions) and plausible estimates of the relevant parameters are available, upper and lower 95 % boundaries can be based on these distributions. Annex N gives more details about estimation and use of expanded uncertainty.

3 Microbiological methods

3.1 Common basis

Microbiological enumeration methods based on culture are technical variants of the same basic principle. The analysis often begins with the mixing of a measured portion of the laboratory sample into a suitable liquid medium to produce a homogenate called the **initial suspension**. It may have to be diluted further to produce a **final suspension** of appropriate density for detection and enumeration of the target microorganism. In water analysis, the water sample is the initial suspension and, when dilution is unnecessary, also directly serves as the final suspension.

3.2 Quantitative instruments

Measured portions of the final suspension are transferred into a detection instrument for quantitative evaluation.

The detection instruments in microbiological analyses vary from a single Petri dish to systems of many parallel plates in different dilutions and to most probable number (MPN) systems of diverse complexity.

3.3 Uncertainty structure

A complete microbiological analytical procedure consists of five or six successive steps:

a) subsampling and mixing;

b) dilution;

c) delivery of test portions(s) into the detection system of nutrient media;

d) development during incubation;

e) counting and possibly confirming the (presumptive) target organisms.

The operational variability consists of the effects of these technical steps. They are individually estimated for use in the component approach. When estimating the uncertainty of the final result, the uncertainty due to random distribution of particles in suspension is additionally taken into account (5.2). In the traditional global approach all operational components and the random distribution of particles are estimated together.

3.4 Expression of combined uncertainty

3.4.1 Two-component model

For many practical and illustrative purposes it is sufficient to consider the uncertainty of microbiological test results to consist of two groups of components.

Combined uncertainty of measurement is obtained by combining the operational variability and the intrinsic variability (distribution uncertainty).

In microbiological contexts both variances are to be expressed as relative (or logarithmic) variances. The symbols used in this connection in this International Standard are:

$$u_{\mathrm{c,rel}}(y) = \sqrt{u_{\mathrm{o,rel}}^2 + u_{\mathrm{d,rel}}^2} \tag{1}$$

where

$u_{c,rel}(y)$ is the combined relative standard uncertainty;

$u_{o,rel}$ is the relative operational variability (experimental relative standard uncertainty);

$u_{d,rel}$ is the relative intrinsic variability (relative distribution uncertainty).

Equation (1) is applied in both the modified global and the component approaches to construct the combined relative uncertainty of measurement of the final result.

NOTE Subscripts can be used to indicate the experimental conditions or level of uncertainty (r for repeatability, R' for intermediate or intralaboratory repeatability and R for interlaboratory repeatability).

3.4.2 Operational variability (technical uncertainty)

Operational variability is the combination of all the uncertainties associated with the technical steps of the analytical procedure. It includes the variability of the subsampling, mixing, and dilution of the laboratory sample to prepare the final test suspension. It also includes the possible effects of incubation and the uncertainty of reading the result. Bias components are involved but form parts of random variation.

3.4.3 Intrinsic variability (distributional uncertainty)

Intrinsic variability is the unavoidable variation without a cause that is associated with the distribution of particles in the final suspension and in the detection instrument. In microbiological suspensions it is usually believed to follow the Poisson distribution. When partial confirmation is practised or the MPN principle is used, the intrinsic variation increases considerably and no longer follows the Poisson distribution (Annexes D and E).

NOTE The intrinsic variability can be decreased by using replicate plates and for MPN estimates by increasing the number of parallel tubes.

4 Choices of approach

4.1 General

The tradition of evaluation, presentation and use of measurement uncertainty is short in microbiology. Different parties still have different interpretations and understanding of the meaning and use of measurement uncertainty. Because of this fluid state, there is no unique right way of determining, expressing and using the uncertainty of measurement.

This International Standard is primarily intended to provide guidelines for laboratories on how to get started with establishing the practices of evaluating the uncertainty of measurement. Basic global and component approaches are described. While the recommendations presented do give valid approaches to the evaluation of measurement uncertainty for many purposes, there exist other uncertainty evaluation systems, both wider and narrower in scope than the present protocol. They can provide solutions to specific demands or different quality control situations. Some of them are briefly characterized in the remainder of this subclause.

In addition to the two basic approaches in this International Standard, there exist other approaches to the analysis and expression of the uncertainty of measurement. They have gained favour particularly in those parts of the world where they have been developed. Five examples are given below. Their common feature is that they are mainly or completely based on data generated in connection with internal and external quality assurance activity. They address the technical aspects of method validation and analytical competence of laboratories, and the associated uncertainties, rather than the measurement uncertainty of test results. Also the statistics may differ. For instance, robust statistics instead of standard statistics may be employed.

The methods in the Nordtest Report TR 537[12] and NMKL Procedure No. 8[11] are based on stable reference samples which permit some control of the bias components within and between laboratories. The connection and applicability to microbial populations of real natural samples necessarily remain somewhat obscure.

NMKL Procedure No. 8[11] for the uncertainty in quantitative microbiological examinations is widely accepted among food analysts in the Nordic countries. It uses internal control data as well as results of validation

data from collaborative studies for estimating the measurement uncertainty at the participating laboratories. Nordtest Report TR 537[12] deals for example with intralaboratory bias.

The most comprehensive of the systems is, at the time of publication, under preparation by AFNOR (see Reference [9]). It is reported to address the evaluation of different levels of uncertainty (repeatability, intermediate and interlaboratory reproducibility) from internal and external quality control data and to employ Bayesian statistics in confidence interval (CI) estimation.

BS 8496[8] is designed to detect the presence of overdispersion (termed “uncertainty of measurement”) between duplicate counts of natural drinking water samples. A value for the uncertainty of measurement is not determined.

A system in use in New Zealand (Reference [16]) is based on special experiments with natural water samples. The design is an extension of the basic global design. Data by three technicians analysing several water samples in quintuplicate are used to estimate general measurement uncertainty values. Operational and intrinsic components are not separated. As a consequence, low and “normal” counts (limit set at 20 colonies) require separate assessment.

4.2 Choices of evaluation approach

The uncertainty of measurement established under intralaboratory reproducibility conditions (the intermediate precision) is the focus of this International Standard. Under these conditions, the components of uncertainty can be identified and both the global and component approach basically apply. Experiments based on natural samples are considered important.

Both main approaches to uncertainty of measurement described in this International Standard should, in principle, give the same results. There are few objective reasons for choosing one approach rather than the other. Subjective preferences or requests by a customer or an accreditation authority may be equally valid reasons. Neither of these approaches might be the one to choose, if one of the approaches outlined in 4.1 is more fitting to the quality control system and quality control data possessed by the laboratory.

If a laboratory already has a good quality control system for monitoring details of the analytical procedure, it probably has most of the necessary data available to calculate a component uncertainty estimate. If not, then the global approach would seem to provide the fastest way to get started with estimation of the uncertainty of measurement.

According to recent observations, two components of operational uncertainty are expected to be larger than others. They are the subsampling variance (matrix effect) with solid materials, and the incubation effect with many methods. Subsampling variance often exceeds the particle distribution effect in solid samples. With difficult microbial populations and poor selective methods, the incubation effect can become as important as the particle distribution effect, whereas with good selective methods, simple microbial populations, and easily interpreted colony morphology, the incubation effect is insignificant.

The incubation effect is evaluated by observing the possible overdispersion of parallel counts of final suspensions. Such tests belong to the quality control arsenal of all laboratories irrespective of which evaluation approach they prefer. Evaluation of the incubation-effects component is therefore usually possible without any special arrangements.

With water samples, the subsampling variance is not expected to exceed the Poisson distribution variance significantly. Other liquid samples and finely powdered materials might be in the same category. In laboratories where the quality control is based on details of procedure, the estimate of measurement uncertainty can be constructed from the normally available quality control data. A global approach in such cases would be superfluous.

With solid samples the situation is different. Significant overdispersion between subsamples is the rule. In these cases, either the global approach should be chosen or the subsampling variance component should be evaluated by a dedicated experiment. The dedicated experiment for subsampling variance (Annex H) is a statistically somewhat more complex design than the entire global experiment (Annex F). The question is which is considered a more useful parameter to evaluate, the global operational uncertainty or the subsampling variance. The choice depends on subjective preference.

Evaluation of the operational variance by the global approach is based on subtraction. The smaller the operational component is in comparison with the distribution uncertainty, the greater its relative imprecision. The global approach is less efficient with low counts than the component approach. This is a typical situation

with water samples. With increasing heterogeneity of samples, the efficiency of the global approach improves progressively. As soon as the operational variance is expected to become larger than the distribution variance, the global approach is a reasonable choice. This is a likely situation with solid samples.

When the estimate of uncertainty is to include interlaboratory biases, the evaluation is based on intercalibration data using the same reference samples for all laboratories. In such cases, the analysis can only be based on the global approach. The components of uncertainty cannot even be identified. Such evaluations are not within the scope of this International Standard. Those interested in the approach are advised to consult relevant protocols (e.g. Nordtest Report TR 537[12]).

4.3 Choices of expression and use of measurement uncertainty

Customers, accreditors and the laboratory may have different expectations and uses of the measurement uncertainty information. Observation of these requirements determines whether the uncertainty should be given as operational uncertainty, combined uncertainty, expanded uncertainty or an interval based on expanded uncertainty of measurement, and in which specific form or scale of measurement. Both the use and the expressions relevant to various uses are presented in Annex N.

5 The component approach to the evaluation of operational uncertainty

5.1 General

In the component estimation, individual contributions to the uncertainty of measurement (subsampling, dilution, inoculation, incubation, and reading) evaluated separately are mathematically combined using the law of propagation of uncertainty (ISO/IEC Guide 98-3:2008[7]). Computationally, it means forming the root sum of squares of the component uncertainties. The combined estimate produced can be called the intralaboratory reproducibility when the components are determined under reproducibility conditions within one laboratory.

5.2 Identification of the components of uncertainty

Statistically thinking the uncertainty structure in microbiological enumerations consists of three layers: a) *before*; 2) *within*; and 3) *after* the final suspension. For a more detailed list, see 3.3.

Uncertainty *before* the final suspension consists of the subsampling and matrix variation, as well as dilution. Influence quantities before the final suspension affect the combined uncertainty proportionally to the mean concentration. Whatever additional variation occurs in subsampling or during dilution is transported to the mean of the final suspension proportionally.

Uncertainty *within* the final suspension consists of the random distribution of particles in suspension. Together with the distribution of colonies on the plate, and the possible contribution of the uncertainty of partial confirmation, they constitute the intrinsic variation. Intrinsic variation does not contribute to the operational uncertainty.

Variation *after* the final suspension includes the uncertainties connected with the reading of the results and influences of the incubation environment and time on the apparent observed result. The uncertainties may include both additive (e.g. contamination) and proportional elements (e.g. uncertainty of counting).

Experiments and examples for the quantitative estimation of the components of uncertainty are detailed in the annexes. The variance components for subsampling and incubation effects require special experiments. The other three operational components are available from quality control procedures.

5.3 Evaluation

When components are independent (statistically uncorrelated) and the influence quantities are multiplicative, the combined relative operational uncertainty is calculated as the positive square root of the sum of relative variances.