Uncertainty of measurement of microbiological counts

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Introduction

Laboratories operating under ISO/IEC 17025 accreditation and related systems are required to evaluate measurement uncertainty (MU) for the analyses they conduct, and to report it when relevant. This requirement was brought to the attention of microbiologists in the 1990s. The initiative came from the chemical profession.

A comprehensive *Guide to the expression of uncertainty in measurement* (GUM) elaborated by a wide international group of experts on metrology, published by ISO in 1993, became the basic document for MU evaluation. The principles were "interpreted" in a more practical way for analytical measurements, mainly of chemical nature, by the EURACHEM/CITAC Guide (1995, second edition in 2000). Both documents exclude measurements based on counts.

Because of the central position of counts in microbiological monitoring, microbiologists felt it necessary to make a contribution of their own to the MU discussion. The work began first in Finland (MIKES) and was soon followed by two ISO technical committees ISO/TC 34 (food and animal feeding stuffs) and ISO/TC 147 (Water).

Results by an MPN method are not regular counts. Enumeration by MPN methods, however, is increasingly important in microbiological monitoring. The MPN principle is therefore included.

Uncertainty of measurement

There is always a great deal of general uncertainty to microbiological test results. The past history of the sample may affect the viability of the target microbe, the microbial populations of the sample may interact in the detector during incubation, etc. It is not possible to know what percentage of the target population is caught by the analysis at any particular time. Different nutrient media will give different answers, and so on.

Uncertainty of **measurement** (MU), is defined by ISO (1993) as "a parameter associated with the **result of measurement**, that characterises the dispersion of the values that could **reasonably** be attributed to the measurand". The definition is quite general and leaves open the detailed content of MU and does not define what is 'reasonable' in the dispersion of the results. It leaves room for consideration what sources of variation should be included in the measurement uncertainty.

The MU value is accordingly attached to the result and not the act of measuring. The MU estimation gives a measure of the confidence that that can be put on the analytical results, not on the laboratory competency. (ISO 19036).

This is an important distinction in microbiology where a considerable parts of the variation is often generated without an identifiable cause.

Determination of MU, two approaches

Microbiological count data is generated when the microbial concentration of a **laboratory sample** (portion of material brought to the laboratory for analysis) is estimated by a microbiological counting process. In microbiology, as well as in chemistry, the test result is derived from several observed values often involving many measurements

The test result is calculated from the final counts, taking into account the dilution and the volume of the test portion. Volume measurement uncertainties are involved. Additional uncertainty is caused by incubation, operator differences and uncertainty of counting. On top of everything is the intrinsic variation, random distribution of particles in perfectly mixed suspensions.

There are two general approaches for estimating the actual or potential variability of the test result (the uncertainty of measurement). They are nowadays preferably called the **component** approach and the **global** approach.

Recent history

Development of standards or technical guides for the evaluation and expression of measurement uncertainty for microbiological counts was begun independently and almost simultaneously for food and water microbiological methods. Expert groups were available at the time in technical committees TC 34 (food and animal feeding stuffs) and TC 147 (water).

Obviously, the microbiological techniques and many methods were the same for food and water. It was natural that an attempt was made to harmonise the uncertainty estimation of water and food microbiological methods. In the end, however, it proved impossible to agree on a common standard at this stage. Food microbiologists thought the global approach more appropriate and water microbiologists wanted a guide on the component approach. There is a difference in efficiency of the approaches depending on the situation.

ISO/TC 34/SC 9 considered that the "step-by-step" (component) approach does not apply satisfactorily in the case of the microbiological analysis of food, where it is difficult to build a really comprehensive model of the measurement process. For reasons they listed for instance: "possibility of overlooking a significant source of uncertainty, the analyte is a living organism, whose physiological state can be variable, and the analytical target includes different strains, different species, and different genera". The working group concluded that, for these reasons, the microbiological analyses of food do not enable a metrologically rigorous and statistically valid estimation of MU (ISO/TS 19036:2005). This cannot be the full reason, however, because the same difficulties are present in water analysis as well.

The working group for food found that the global design is not applicable to enumeration using a most probable number (MPN) technique or the analysis of low levels of microorganisms (10 colonies per plate being the limit).

The important water microbiological methods of health-related indicator species and pathogenic organisms must function at very low counts, at the border of the limit of detection. Also methods based on the MPN technique are almost necessary in water analysis. The component approach, which has no limitations concerning low counts and the MPN principle, therefore, became the chosen approach for water.

Component approach

The component approach is also referred to as the "step-by-step" or "bottom-up" procedure. It is based on identifying the components of uncertainty of the analytical process. Each of them is separately evaluated in whatever way is feasible. The individual components are mathematically combined to correspond with the design of the test procedure. This is the procedure introduced by the original uncertainty document GUM (ISO 1993).

In practice it means forming a mental picture of the process, which is almost the same for all microbiological methods (Fig. 1). It is also helpful to write down the entire formula for calculating the final result. Often it appears that the result is based on ten or more elemental measurements

(mostly volumes). The uncertainties of the elemental operations are combined by what is called the law of propagation of uncertainty to give an estimate of the combined uncertainty.

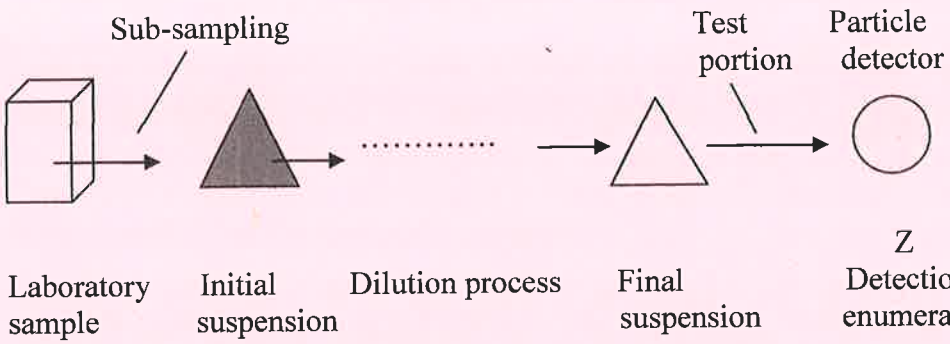

Fig. 1. Graph of a typical microbiological counting procedure

A simple procedure without duplication is normal in daily routine analysis of samples. Formerly, it was common to duplicate the last step.

The law of propagation of uncertainty

The law is based on the idea of the additivity of variance. The combined standard uncertainty (u_{comb}) is obtained as the 'quadratic sum' of n independent components of uncertainty ($u_1, u_2, ..., u_n$):

$$u_{comb} = \sqrt{u_1^2 + u_2^2 + ... + u_n^2}$$

The uncertainty components are expressed in a scale where variances are known or believed to be additive (usually logarithmic or relative scale).

It is sometimes instructive to group the components of uncertainty. Division into three groups: sub-sampling (or matrix), analytical procedure, and detector might help decide which of the approaches, component or global, would be best.

An important property of the quadratic sum is namely the powerful influence of large values. Unless there is a large number of small values, contributions that are less than one third of the largest need not be quantified in detail. If matrix variability is dominant, like it probably is in difficult-to-mix solid foods, the choice is the global approach. If the matrix uncertainty is negligible, like it is in water and liquid foods, then the best choice is the component approach.

Component estimation

What is being measured should be clearly defined. The equation used to calculate the value of the measurand at the end of the method process is a good starting point.

On the whole, general quantitative microbiological analyses are very straightforward, most being based on the same general principles, i.e. subsampling, dilution, plating, incubation, and counting (with, on occasion, confirmation of the identity of organisms) Forster (2003).

Not all identified contributions to uncertainty will make a significant contribution to the total uncertainty.

There are two general ways of determining the values of variance components, called Type A and Type B evaluation.

Type A evaluation consists of calculating the standard deviation (standard uncertainty) from a series of independent parallel measurements $x_1, x_2, \ldots, x_n$ of the test quantity using the conventional statistical formula for experimental standard deviation. It can concern the entire analytical process (the final result) or parts of it (for instance repeatability of pipetting or uncertainty of counting, etc.).

In Type B evaluation, the numerical value of a component of uncertainty is obtained by other means than statistical methods, most importantly from assumed statistical distributions. Some may be accounted for by data already available in the laboratory or in published tables.

The global approach

In the global approach, special experiments are necessary. The entire analytical process is repeated, at least in duplicate, in order to obtain an empirical estimate of the variability of the test result (Fig. 2).

The process has also been called the "top-down" approach. The term "global" was invented to convey the idea that even all unknown sources of uncertainty are believed to be included.

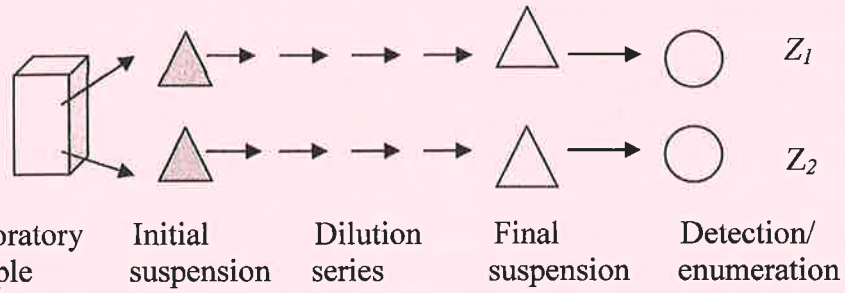

Fig. 2. Minimum design for a basic experiment for the global uncertainty of measurement.

The standard statistical formula for standard deviation is applied to calculate an estimate of the uncertainty of measurement.

Microbiological testing laboratories generally have a program whereby a certain number of samples are analysed at least in duplicate. Duplicate data for a particular test and for particular types of samples collected over a period of time can be analysed to determine the standard deviation. If all normal sources of variation in the method are taken into account it is called the intermediate (or intra-laboratory) precision of the test method. The sources of variation include storage effects, laboratory environmental effects, operator affects, effects of using different items of equipment, different batches of media, etc. This is the preferred extent of uncertainty likely to be reported as a global estimate of MU..

Subsampling procedures are normally included in it whereas external sampling is not.

The MU estimate does not characterise the analytical method itself independently from the laboratory which implements it. The critical factors associated with the method or the laboratory should be identified and demonstrated to be under control. A re-assessment of the MU is required following changes to any of the critical factors.

In food microbiology, the effect of the matrix on MU cannot be avoided whereas the influence of the contamination level can be eliminated by log transformation. In water microbiology the matrix effect is generally negligible and the contamination level is the main cause of differences in MU.

Problems of the global approach

In the global method, the sources or causes of variation are not identified and assessed individually. This may be seen as an advantage because the unknown sources of uncertainty are assumed to be included even though it is not known why and how.

Standard deviation based on two values is very imprecise. The experimental unit depicted in Fig. 2 should be repeated many times. ISO/TS 19036 proposes a minimum of ten repetitions

The aim of the global approach is to generate MU estimates that need not be reassessed unless some important factor in the analysis chain is changed significantly. A major problem here is that it is not possible to know what factors are important because they are not evaluated separately in the global approach.

Chemists sometimes speak of the MU of methods. In microbiology it is not possible to think of the uncertainty of methods independent of the matrix, the target microbe, and the concentration of the analyte.

In food microbiology, the effect of the matrix on MU cannot be avoided. The influence of the contamination level can be eliminated by log transformation.

The standard deviation of reproducibility shall be estimated for each type of target microorganism and for each matrix, for a given method that the laboratory uses for producing its routine results. This may lead to very extensive trials when the laboratory analyses a large variety of matrices.

Many microbiological laboratories have procedures available for monitoring variability in results. The analysis of a natural sample is *duplicated* under intermediate reproducibility conditions. (Same sample, same day, same laboratory, but different operators and equipment.) It is relatively easy to incorporate such duplication within daily routine analyses. It only takes some planning and a little extra work.

For global MU evaluation, the estimate should not depend on the concentration of the analyte. This is only possible if the variability of sub-sampling and the analytical procedure (dilution, incubation, counting, etc.) overwhelm the intrinsic variability of the final count that follows the Poisson distribution. The relative Poisson variation (distribution uncertainty) increases dramatically when the counts per plate become small. That is the main reason why MU standard for food microbiology (ISO/TS 19036:2006) had to exclude the estimation of uncertainty of low numbers (less than 10 colonies counted). MPN methods that always operate with low density suspensions are also not included in the global standard.

Problems of the component approach

Many separate calibration experiments are needed to establish reliable estimates of a great number of individual operational steps in the component approach. The components are derived by various means, such as from assumed statistical distributions, empirical calibration data or QA data, and literature. Some of them are quite easy, for instance checking the uncertainty of volumes by weighing.

A special problem are the causes of uncertainty that do not appear in the calculation of the final result. Effects of incubator environment (time, temperature, and humidity variations, positional effects), personal differences in recognition and counting of colonies and equipment influences should be estimated. They might require special trials.

Nevertheless laboratories shall at least attempt to identify all the components of uncertainty and make a reasonable estimation. In the evaluation of the measurement uncertainty of a method, the EURACHEM guide requires the analyst to look closely at all possible sources of uncertainty within a method and states that "in practice, a preliminary study will quickly identify the most significant sources of uncertainty" which will be the dominating influences in the total uncertainty of the method. "Method" in this case meaning the medium, matrix, microbial population, incubation environment complex.

MPN

MPN methods are mostly connected to simple designs. The practical procedure might involve only direct measuring of a sub-sample volume and the counting of positive tubes. It is safe to assume that the water sample can be mixed perfectly. The uncertainty of the MPN test result therefore consists of three components: uncertainty of volume measurement, distribution uncertainty, and the uncertainty of counting.

In a perfectly mixed sample there is no other sub-sampling variability than that connected with the inherent distribution variation (Poisson and binomial in the case of MPN). It is great at the low particle density where MPN methods operate and it can be modelled mathematically,

Volume uncertainty is negligible compared with the distribution uncertainty. It is only the uncertainty of counting that might add significantly to the combined uncertainty.

Methods and bias in microbiology

An important principle in general metrology is to correct the result for systematic error (bias). Physicists and chemists are familiar with it and apply correction factors routinely. Microbiologists are very reluctant to apply corrections even when it is apparent that the count may be seriously affected (too low or too much under influence of personal interpretation, for instance).

Microbiological methods can be considered what the EURACHEM guide calls 'empirical methods' (Forster, 2003). They are methods where the analytical results are dependent on the procedures used in the analysis. The method accordingly defines the measurand or, in other words, the "right" answer is not only a property of the sample or the target organism, but also of the method. (Microbiologists know well that different media give different answers in the same sample.) Possibly the majority of quantitative microbiological methods can be considered to be empirical methods, where results generated are dependent on the nutrient medium in use, time and temperatures of incubation, and inclusion or exclusion of resuscitative steps in the methods. The bias associated with the results cannot be accurately defined and is conveniently ignored or considered zero. Microbiologists are comfortable with that. In fact, they have always been reluctant to apply any bias corrections in their results for the following obvious reasons (Forster 2003):

1) "It is virtually impossible to know the exact microbial concentration of any sample, natural or artificial."
2) "Certified reference materials for running as controls alongside tests are not generally available and where these are available, it will be unlikely that they will be matrix matched."

In other words, the true value is not known.

Uncertainty in microbiology. Some historical notes.

Long before the introduction of the GUM and EURACHEM documents there already was a long tradition of estimating the variability of microbiological count data.

Almost a century microbiologists have been assured by statisticians that the variability of parallel counts can be modelled by the Poisson distribution, which means that the standard deviation (standard uncertainty) is the square root of the number of colonies counted. Dutifully, this concept has been repeated over and over again in standards, method protocols, and guides on counting. 95% CIs have been available in MPN tables almost from the beginning.

It also has been known since 1920s that this estimate might underestimate the uncertainty because of so called over-dispersion that may exist for many reasons.

Traditionally, however, results from microbiological analyses are presented unaccompanied by any form of uncertainty estimation. This may change although it does not seem that customers often request that information.

Expression of uncertainty

The final stage is to multiply the global or component (combined) estimate of standard uncertainty by a chosen coverage factor *k*, in order to obtain an expanded uncertainty.

The expanded uncertainty U is required to provide an interval which may be expected to encompass a large fraction the distribution of values which could reasonably be attributed to the measurand, i.e, an interval within which the value of the measurand is believed to lie, with a high level of confidence. For most purposes, a coverage factor of 2 is chosen (confidence level of approximately 95%).

If requested, the uncertainty information can be reported as a confidence interval or as confidence limits. For example: Result: *x* (units) with a confidence interval of *y* to *z*, or, *x* (units) with confidence limits of *y* and *z*.

What is preferably determined is the experimental intra-laboratory standard deviation of reproducibility on the final result of the measurement.

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