MITTATEKNIIKAN KESKUS

CENTRE FOR METROLOGY AND ACCREDITATION

Publication J3/2002

UNCERTAINTY OF QUANTITATIVE DETERMINATIONS DERIVED BY CULTIVATION OF MICROORGANISMS

Seppo I. Niemelä

ADVISORY COMMISSION FOR METROLOGY

Chemistry Section
Expert Group for Microbiology

Helsinki 2002
Finland

CENTRE FOR METROLOGY AND ACCREDITATION

Publication J3/2002

UNCERTAINTY OF QUANTITATIVE DETERMINATIONS DERIVED BY CULTIVATION OF MICROORGANISMS

Seppo I. Niemelä

ADVISORY COMMISSION FOR METROLOGY
Chemistry Section
Expert Group for Microbiology

Helsinki 2002
Finland

UNCERTAINTY OF QUANTITATIVE DETERMINATIONS DERIVED BY CULTIVATION OF MICROORGANISMS

FOREWORD

The purpose of the guide is to help laboratories engaged in routine microbiological analysis to calculate uncertainties of their test results. Only test based on counting colonies or other discrete entities are considered.

In everyday speech "uncertainty" has wide connotations, even in the context of analytical measurements. In this treatise, measurement uncertainty is considered. Other uncertainties, such as the uncertainty of identifying the object of measurement (the target organism), are not considered.

With the help of this guide, it should be possible to obtain a numerical estimate of uncertainty for each individual test result. Also, advise is given on the estimation of major systematic errors.

The microbiological advisory group of the Advisory Commission for Metrology founded a special task group for measurement uncertainty with the undersigned as chairman and Maarit Niemi (Finnish Environment Institute) as secretary. The other members were Suvi Bühler (HYKS Diagnostics), Sari Hemminki (Safety Technology Authority), Seija Kalso (Helsinki Environment Centre), and Antti Nissinen (Keski-Suomen keskussairaala). The group was very helpful during the development work of the guide. An *ad hoc* uncertainty workshop with Seija Kalso, Kirsti Lahti (Finnish Environment Institute), Tuula Pirhonen (EELA) and Pirjo Rajamäki (Helsinki University, Department of Applied Chemistry and Microbiology) that met with the chairman and secretary to try out in practice the principles written out in the guide was of great help.

Seppo I. Niemelä

PREFACE TO ENGLISH EDITION

A guide on the calculation and expression of uncertainty in microbiological measurements was published (in Finnish) at the beginning of 2001 by the Centre for Metrology and Accreditation (MIKES). It was developed by the microbiology task group of the metrology council for chemistry.

The current publication is not only an English version of the Finnish original. It is at the same time a second edition. The opportunity was taken not only to correct the errors observed but also to improve the text according to some new ideas and the response received. The part dealing with confirmed counts was completely revised and expanded. Other changes are less thorough and concern mainly the order of presentation and algebraic expressions and symbols. Some new examples have been added.

This guide was mainly inspired by two documents on the expression of uncertainty in chemistry, published by the International Organisation for Standardisation (ISO) and Eurachem. These documents essentially deal with ways to construct combined uncertainty estimates for test results from a set of individual uncertainty components by a principle called the law of propagation of uncertainty.

The idea of combined uncertainty is not unknown to microbiologists. Already more than half a century ago Jennison and Wadsworth described ways to estimate the total uncertainty of the complex dilution procedure. The uncertainties associated with random scatter of particles in suspension, with volume measurements and with the reading of results have been the subject of numerous publications since the 1920's. Also systematic errors have been dealt with from time to time, the "crowding error" being one of the first to be identified. Attempts to formalise the construction of total uncertainty encompassing both systematic and random components are also known.

The long history of published work on the uncertainty of microbiological test results has had no observable impact on the daily routine of testing laboratories. The time seemed ripe, to make an attempt to show how all the manageable uncertainty components should be combined to estimate the total uncertainty of a test result when standard microbiological methods are used.

Helsinki, April 2002

CONTENTS

1 TERMS, DEFINITIONS AND SYMBOLS

In this document the term **test result** is reserved for the final reported outcome of the analysis. In microbiology as well as in chemistry the test result is usually derived from several **observed values** involving many measurements. The term **measurement** is used loosely to denote both a single observed value and the result calculated from a combination of them.

Uncertainty of measurement according to ISO (Anon 1995) is a parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand. (Measurand is a general term for any particular quantity subject to measurement.) The parameter may be a standard deviation or a given multiple of it, or a half-width of an interval having a stated level of confidence. There are two general ways for determining its value, called Type A and Type B evaluation (see Chapter 2). When the test result is derived from a combination of observed values its uncertainty usually needs to be estimated from the uncertainties of the components.

1.1 Symbols

The letter *u* is recommended as the general algebraic symbol of standard uncertainty (Anon 1995, 2000) For statistical quantities is it equal to the standard deviation. It is expressed in the same units of measurement as the quantity itself. If it is important to emphasise that the value was derived by statistical analysis of a series of replicate observations (Type A evaluation) the traditional symbol *s* for experimental standard uncertainty (standard deviation) may be used instead of *u*.

The most natural expression of uncertainty of microbiological test results is usually the **relative standard uncertainty**, i.e. the standard uncertainty divided by the mean. The acronyms RSD (relative standard deviation) and CV (coefficient of variation), frequently used for denoting the relative standard deviation, are somewhat cumbersome in mathematical formulae. The symbol *w* was chosen as a single-letter symbol for the relative standard uncertainty in mathematical expressions.

When the test result is obtained from a number of components with known or estimated uncertainties, the standard uncertainty based on this information is called the **combined standard uncertainty** and normally (Anon. 1995, 2000) denoted by u_c . In this document u_y or w_y is mostly used instead, because the test result is denoted by *y*.

Especially when the test result is used for assessing compliance with limits concerned with the public health or safety, it is pertinent to give an uncertainty value that encompasses a considerable proportion of the expected range of the observed values. The parameter is termed the **expanded uncertainty** and denoted by the symbol *U*. Its value is obtained by multiplying the combined standard uncertainty u_c with a **coverage factor** *k*. For Normal distribution about 95 % of the results is covered by the expanded uncertainty interval when the coverage factor $k = 2$ is chosen.

A complete report of all corrections, constants, and uncertainty components used in both the calculations and the uncertainty analysis may be required. A report sheet, such as is included in Annex D, is recommended for that purpose.

1.2 Quantitative instruments

There is a variety of methods and techniques for estimating the microbial content of a sample. In the cultural techniques considered in this connection, the techniques are based on estimating or counting the number of microbial particles in a test portion of the sample or in its dilution. Whichever the situation is the test suspension will be called the **final suspension**.

The word **instrument** or particle-detection instrument was chosen as a generic term for any system of tubes or plates employed for estimating the particle concentration of the final suspension. Symbol *c* is used for that concentration.

1.3 Expression of uncertainty

When uncertainty is expressed as the combined standard uncertainty u_c (that is, as single standard deviation), the following form is recommended for giving the result (Anon., 2000): "Result: x (units) with a standard uncertainty u_c (units)". In microbiology the units would almost invariably be "per gram", "per ml" or "per 100 ml". So, typically, the results might be reported as:

Total coliforms:	120 per 100 ml
Standard uncertainty:	36 per 100 ml.

As it will be evident later, the uncertainties in microbiological determinations nearly always emerge from calculations as the relative uncertainty. Even though uncertainty values for assessing compliance against limits will have to be expressed in the same units as the test result, it is quite convenient to initially report the uncertainty in relative units (in percent). This is the recommended practice in the report sheets presented in Annex D.

The use of symbol ± is not recommended in connection with standard uncertainty. It should be associated with intervals corresponding to high levels of confidence (the expanded uncertainty). If results are given in the form: "$x \pm U$", it is necessary to explain what is the level of confidence by stating, for instance, what coverage factor was used in calculating the expanded uncertainty.

1.3.1 Significant numbers

It is seldom justified to report the final test result and the uncertainty to more than two significant digits. The least significant digit in the test result and in the uncertainty should coincide. The intermediate measurements and observed values should not be rounded to the same degree. Colony numbers should always be inserted in calculations with all the digits originally observed.

1.3.2 Large measurement and uncertainty values in microbiology

The microbial content of a sample may be in the millions or hundreds of millions per gram. The test results are usually presented in the form $y = x \cdot 10^k$,where x is often a decimal number and k an integer. The result $y = 1300000\ g^{-1}$ with the uncertainty of 25 % would therefore be given as: $1.3 \cdot 10^6\ g^{-1}$ with a standard uncertainty $0.33 \cdot 10^6$.

2 PRINCIPLES OF ESTIMATING THE UNCERTAINTY

The total uncertainty of a test result typically consists of several components. In microbiology, at least three factors are always involved: the uncertainty of the inoculum volume, random scatter due to particle statistics, and the uncertainty of reading the result. Uncertainty of dilution is frequently a fourth factor.

The ISO uncertainty guide (Anon. 1995) classifies the methods of estimating the uncertainty into two types called the Type A and Type B evaluation (of uncertainty).

2.1 Type A evaluation (of uncertainty)

The Type A standard deviation (standard uncertainty) is calculated from a series of n independent parallel measurements x_1, x_2,…,x_n of the test quantity using the conventional statistical formula for experimental standard deviation:

$$s(x) = s_x = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \overline{x})^2}{n-1}} \qquad (1)$$

($\overline{x}$ is the arithmetic mean)

Type A evaluation may concern the final test result of the analytical procedure or a part of it (e.g. a volume measurement).

A fairly large number of parallels is essential for a reliable Type A estimate. For example, the uncertainty of an estimate based on 30 independent measurements is 13 %; that of a sample of two measurements is considerably higher, 76 % (Anon. 1995).

2.2 Type B evaluation (of uncertainty)

According to ISO (Anon. 1995) Type B evaluation the numerical value of the uncertainty of measurement is estimated by other means than the statistical methodes (see 2.4).

2.3 Combined uncertainty

It is generally not practicable to make series of repeated measurements in routine monitoring to get a Type A estimate of uncertainty of a final test result.

With some chemical methods it is possible to assume the general validity of method-specific repeatability and reproducibility parameters (precision estimates) determined in collaborative method performance studies. There are reasons why this approach is likely to be less successful in microbiology. One is the unpredictable colony number that varies from case to case and is usually the main cause of uncertainty. The other is the instability of samples. Uncertainty of a test result depends too much on the prevailing unrepeatable conditions under which a test is made.

The best approach in microbiology seems to be to compose an uncertainty estimate from the separate uncertainties of the unit operations of the analytical procedure, estimated by any means available. A mathematical procedure, called *the law of propagation of uncertainty* (Anon 1995), is applied.

The combined uncertainty estimate is based on recognising and listing the most important components of uncertainty of the analytical process, being careful to avoid "double-counting", i.e. including any uncertainty component more than once. Finding a value for each component by Type A or Type B processes and combining them mathematically makes it possible to compose an estimate of uncertainty for any unique measuring situation.

2.4 Sources of information

Some components of uncertainty are best estimated by calibration measurements in the own laboratory. Also the analysis of variance of earlier experiments, possibly conducted for completely other purposes, may be suitable sources of information on the variability of test results.

When necessary, other means of estimation are used. The means include assumed statistical distributions (Poisson, binomial) or assumed *a priori* distributions of possible values (rectangular and triangular distributions). Further means might include values based on experience or other information as well as equipment manufacturers' specifications, scientific literature, general experience of instrument 'errors', homogeneity of materials, calibration and certification reports, and uncertainly values quoted in handbooks. Even an educated guess might be acceptable if nothing else is available. The sources of information should be indicated in the uncertainty report.

2.4.1 Rectangular distribution

In some cases the value of an input quantity can be suspected to vary within some limits ($-a \ldots +a$) without sufficient information on the shape of the frequency distribution. Examples of such cases are the specifications given by manufacturers of volumetric equipment. In these cases a rectangular distribution (Fig. 1) might be the most suitable model.

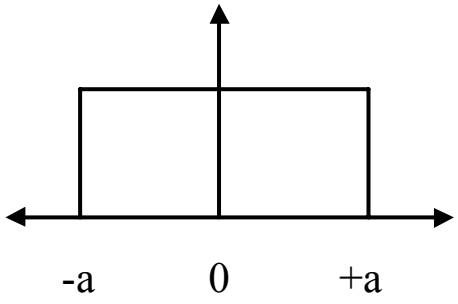

Fig. 1. *Rectangular distribution. Every value between –a…+a is considered equally possible.*

The standard uncertainty of a rectangular distribution is estimated from (Anon 1995)

$$u = \frac{a}{\sqrt{3}} \qquad (2)$$

In microbiology, a typical way of expressing the possible range is relative (e.g. *a* %). In that case the uncertainty from the formula comes out automatically as an estimate of the relative uncertainty.

2.4.2 Symmetric triangular distribution

A different kind of an *a priori* distribution follows when the information about the probability is less limited and can be described by a symmetric, triangular graph. It is considered most likely that the value of the variable is close to a fixed value but deviations within ±*a* of that value are possible. The "probability distribution" is an isosceles triangle with its apex situated at 0 or at an assumed point *g* and the lower and upper bounds at – *a* and + *a* (or at – *a* + *g* and *a* + *g*) (Fig. 2).

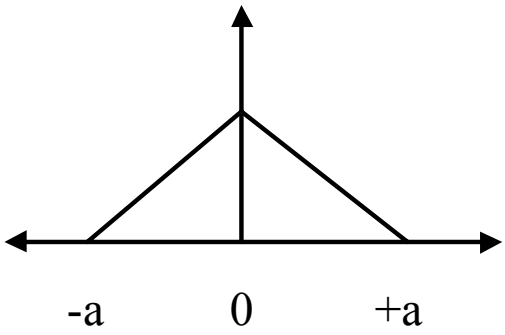

Fig. 2. *Symmetric triangular distribution. All values between –a…+a are considered possible but the values close to zero the most likely.*

The standard uncertainty of a symmetric triangular distribution is approximately (Anon. 1995)

$$u = \frac{a}{\sqrt{6}} \qquad (3)$$

The estimate is directly suitable as the value of the relative standard uncertainty when *a* is expressed in the relative scale (e.g. in percent).

2.4.3 Asymmetric triangular distribution

In some cases a triangular distribution comprising only one half of the symmetric distribution might be the most appropriate. A case in point is the change in population density of a sample during refrigerated storage. Cold storage is likely to keep the microbial density unchanged. If any change should nevertheless take place, only death (of at most *a*% of the cells) would seem logical. The probability distribution would be a right-angled triangle extending from 0 to *–a* (Fig. 3).

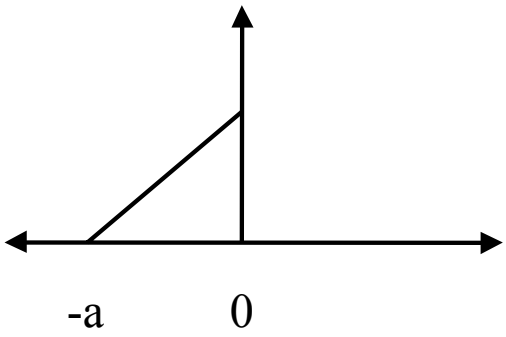

Fig. 3. *Asymmetric triangular probability distribution. No change is considered to be the most probable case. A loss of as much as a(%) of the analyte is considered possible.*

The main parameters of the distribution are

Mean: $$-\frac{a}{3} \qquad (4)$$

Variance: $$u^2 = \frac{a^2}{18} \qquad (5)$$

Standard uncertainty: $$u = \frac{a}{3\sqrt{2}} \qquad (6)$$

When *a* is expressed in the relative units, also the uncertainty estimate is relative.

3 CALCULATING THE COMBINED UNCERTAINTY

The *law of propagation of uncertainty* as expressed in the ISO guide (Anon. 1995) is quite complicated because of covariances. Whenever the components of uncertainty are independent (orthogonal, statistically uncorrelated) the covariances vanish and calculations become simpler. Fortunately, most of the components of uncertainty in microbiological standard methods can be assumed to be either independent or only weakly correlated. An exception is the uncertainty of the dilution factor which is obtained by division in which the same volume measurement appears in both the numerator and the denominator. This case is dealt with in 5.7.1.

The combined uncertainty of independent components is calculated as if they were vector components in Cartesian space. In other words, the combined standard uncertainty is a positive square root of the sum of the variances. (the squares of uncertainties). In some connections, a similar parameter is called the Euclidian distance or geometric sum.

To calculate the test result and its uncertainty the relation between the test result and the input quantities should be expressed in the form of a mathematical formula. The formula should include all measurements, corrections, and correction factors that may have a significant effect on the final result and its uncertainty.

The combined uncertainty of a sum or of a difference is obtained by adding the squared uncertainty components in an arithmetical way (3.1.1, 3.1.2). The combined relative uncertainty of a product or of a division is obtained by adding the squared relative uncertainty components arithmetically (3.1.3, 3.1.4).

Estimation of the combined uncertainty often involves both sums and products. In order to minimise the possible confusion caused by moving between two scales of measurement, distinct symbols (u and w) were chosen for the uncertainty in the two scales (1.1).

3.1 Basic rules for combining two independent components of uncertainty

Assume the values of two independent quantities A and B and their standard uncertainties u_A and u_B or relative standard uncertainties w_A and w_B are known.

The relation between the absolute (u) and relative (w) uncertainty is $u_X = w_X X$, where X is the measured value (or mean) of the quantity.

The combined uncertainties of the quantities derived by the basic algebraic operations ($A + B$), ($A - B$), AB and A/B from A and B are detailed below.

3.1.1 Standard uncertainty of a sum ($A + B$)

$$u_{(A+B)} = \sqrt{u_A^2 + u_B^2} \qquad (7)$$

The relative standard uncertainty of a sum is

$$w_{(A+B)} = \frac{u_{(A+B)}}{A+B} \tag{8}$$

3.1.2 Standard uncertainty of a difference (*A - B*)

The standard uncertainty of a difference is the same as that of a sum

$$u_{(A-B)} = \sqrt{u_A^2 + u_B^2} \tag{9}$$

but the relative standard uncertainty is different:

$$w_{(A-B)} = \frac{u_{(A-B)}}{A-B} \tag{10}$$

3.1.3 Standard uncertainty of a product (AB)

$$u_{AB} \approx AB\sqrt{\frac{u_A^2}{A^2} + \frac{u_B^2}{B^2}} = AB\sqrt{w_A^2 + w_B^2} \tag{11}$$

The relative standard uncertainty

$$w_{AB} = \frac{u_{AB}}{AB} \approx \sqrt{w_A^2 + w_B^2} \tag{12}$$

3.1.4 Standard uncertainty of a division

$$u_{A/B} \approx \frac{A}{B}\sqrt{\frac{u_A^2}{A^2} + \frac{u_B^2}{B^2}} = \frac{A}{B}\sqrt{w_A^2 + w_B^2} \tag{13}$$

The relative standard uncertainty of a division is the same as that of a product

$$w_{A/B} \approx \sqrt{w_A^2 + w_B^2} \tag{14}$$

3.2 Formulae for dependent variables

Whenever the two variables are correlated the value of the combined uncertainty is different from that of the independent variables. When positive, the correlation increases, when negative, it decreases the combined uncertainty according to the general formula

$$u_{(A+B)} = \sqrt{u_A^2 + u_B^2 + 2ru_A u_B} \qquad (15)$$

where u_A and u_B are the respective uncertainties of A and B and r is the correlation coefficient between A and B.

Information about the correlation (or covariance) of influence quantities in microbiological test results is seldom available.

3.3 Standard deviation, coefficient of variation and logarithms

It is a common practice in microbiology to convert test results or counts to common (base 10) logarithms before any mathematical calculations without even considering if it is necessary. A considerable part of the scientific information on the precision of microbiological test results is therefore reported in the logarithmic scale. Taking logarithms is a way to convert results to the relative scale of measurement.

3.3.1 Transformation of the scale of measurement

The key to transformations in uncertainty calculations is included in the mathematical principle expressed by Myrberg (1952) roughly as follows: *"The relative error of a quantity is approximately equal to the absolute error of its logarithm"*. The logarithms referred to are the natural (base *e*) logarithms. In terms of uncertainties this means that the relative precision and the uncertainty in natural logarithmic scale are numerically approximately the same.

Conversion from common (base 10) logarithms to natural (base *e*) logarithms is achieved by multiplying with the modulus between the two systems. The value of the modulus is 2.30259; for all practical purposes 2.303 or 2.3 are adequate approximations.

3.3.2 Relative and percent expressions

The value of the relative standard deviation is frequently expressed in percent. Because the meaning of the symbol "%" is one in a hundred, there is no need at the abstract or conceptual level to make a distinction between the two (5 % = 0.05). When computing the actual uncertainty values, one or the other must be consistently followed. In this document, one percent is mostly expressed as 0.01 in the calculations.

3.3.3 An example of transformation

The following example is no ‘proof’ of the principle in clause 3.3.1 but shows its practical value.

In a random series of parallel plates of a somewhat heterogenous sample, the following colony counts were observed: 30, 30, 31, 34, 48, 53, 97, 164, 166, 213. Various parameters were estimated from the series by standard statistics:

arithmetic mean 86.6
experimental standard deviation 69.3337
experimental standard deviation in ln scale 0.7889
experimental standard deviation in $\log_{10}$ scale 0.3426

Applying the principles described in clauses 3.3.1 and 3.3.2, the relative standard deviation can be estimated in three ways:

arithmetic (interval) scale: 69.3337 / 86.6	= 0.8006 ~ 0.80
ln scale:	= 0.7889 ~ 0.79
$\log_{10}$ scale: 2.30259 · 0.3426	= 0.7889 ~ 0.79

Even in this rather scattered series, with about 80 % relative standard deviation, the estimates did not differ markedly.

4 METROLOGICAL CHARACTERISTICS OF MICROBIOLOGICAL CULTURAL METHODS

From a wide metrological perspective all the standard microbiological methods work in the same way. The process involves careful mixing or homogenisation of the sample and suspending a measured portion of it in an aqueous solution. Further dilution of the suspension may be necessary to arrive at a concentration appropriate for the measuring instrument where the detection and counting of the particles takes place. In all that follows, the suspension (dilution) where the first reliable counts are made is called the final suspension.

With very few methods does the preparative stage involve other means than agitation to separate the analyte (target microbe) from the sample matrix and the interfering non-target species. Pasteurisation and acid treatment of the sample are two examples of such practices. For the most part, the processes that correspond to extraction, separation and fractionation in chemistry take place on or in the nutrient medium during growth.

The measuring instrument or detection system often consists of many individual detectors, of which there are two kinds: the colony count detector (petri dish) and the presence/absence detector (usually a tube or well of liquid).

4.1 The common basic equation

The basic equation for computing the test result, common to all quantitative cultural methods, is:

$$y = Fc \tag{16}$$

y is the estimated particle concentration of the sample
F is the dilution factor (e.g. 10^4), the reciprocal of dilution (10^{-4})
c is the estimated particle concentration of the final suspension
(When dilution is unnecessary $F = 1$)

4.2 Metrological types

Four instrument types are commonly used for determination of the particle concentration of the final suspension. A rare fifth type (spiral plate) is omitted.

4.2.1 The one-plate instrument

The estimate of the microbial concentration of the final suspension is based on the colony number (z) observed in one test portion (volume v) of the final suspension (Fig. 4).

$$c = \frac{z}{v} \tag{17}$$

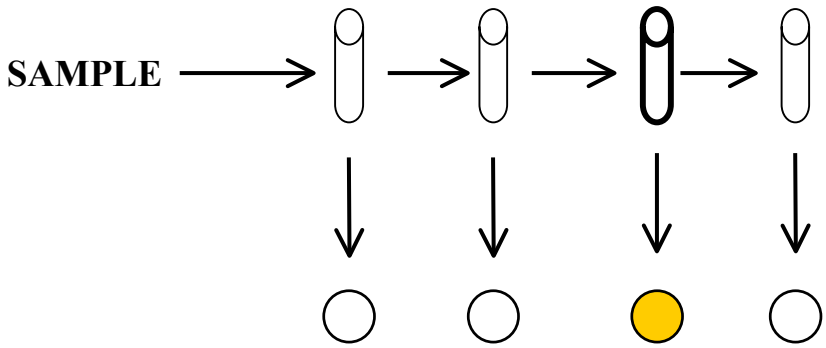

Fig. 4. *The one-plate instrument. The cylinders represent dilution flasks or tubes, the circles petri plates, arrows indicate transfer of liquid. Despite several prepared dilutions, the result is only read in one plate (shaded) from the so-called final suspension (bold). The open circles signify plates that have been discarded as uninformative or impossible, or because of a limiting rule on reliable plates.*

4.2.2 The multiple-plate instrument

The estimate of the particle concentration of the final suspension is based on the colony numbers (z_1, z_2, …) counted in parallel plates of the same suspension and/or plates from different dilutions (Fig. 5).

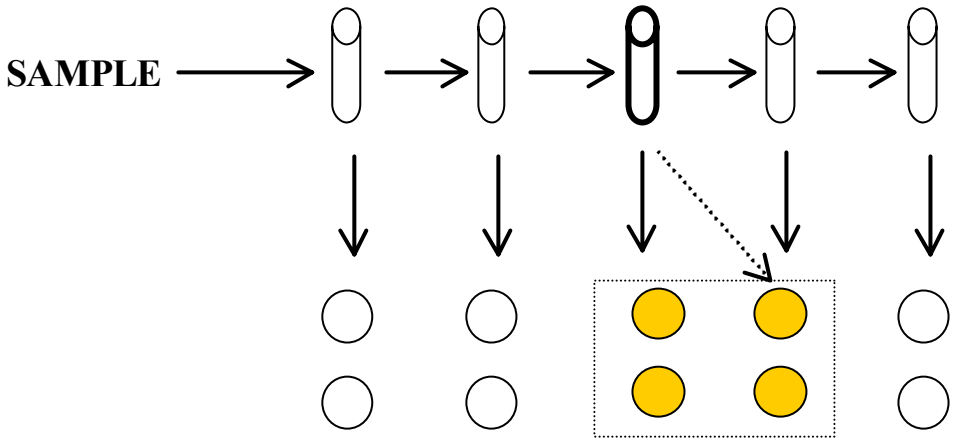

Fig. 5. *The multiple-plate instrument. From a set of plates seeded from different dilutions, a subset (shaded plates, framed) is selected for reading. They form the detection instrument. The first dilution with readable plates becomes the final suspension (bold). It is mathematically acceptable to regard inocula derived from further dilutions of the final suspension as smaller volumes of the final suspension (dotted arrow).*

The weighted average concentration of particles (Farmiloe et al. 1954) in the final suspension is:

$$c = \frac{\Sigma z_i}{\Sigma v_i} = \frac{Z}{V} \qquad (18)$$

z_i is the count on the i^{th} plate
v_i is the volume of the i^{th} test portion in terms of the final suspension

The lower case letters (z, v) denote the colony numbers and test portion volumes of individual samples. The capital letters indicate their sums.

NOTE. Often some limiting rules are given regarding the 'reliable' number of colonies per plate (10-100, 30-300, etc.). The upper and lower limits have a different reason. The upper limit is due to the expected increasing problems caused by crowding. The lower limit is defined by the statistical precision at an arbitrarily chosen limit of determination. As the statistical precision of the test result largely depends on the *sum* of colony numbers, there is no need to observe the lower reliable counts per plate in a multiple-plate system. The upper limits per plate remain in force.

4.2.3 The one-dilution MPN instrument

A series of equal test portions, volume v, from the final suspension are inoculated in n reaction vessels or reaction sites of sterile nutrient medium. When from one to n-1 of the reaction sites remain sterile following incubation, a quantitative estimate of the particle density of the final suspension is possible. It is calculated from:

$$c = \frac{1}{v} \ln\left(\frac{n}{s}\right) \qquad (19)$$

v is the test portion volume
n is the number of reaction sites inoculated
s is the number of reaction sites found sterile after incubation

From the metrological perspective the one-dilution MPN system is the counterpart of the one-plate colony count instrument. An exact count of positive (or negative) sites is involved, but the value (c) derived from the formula is often considered "only" an estimate of the hidden true concentration. The average systematic error caused by the possibility of several particles in the same sites is corrected mathematically by the use of the above formula. (No corresponding correction is normally made on colony counts.)

4.2.4 The multiple-dilution MPN instrument

The MPN estimate from a system of several dilutions and several parallel tubes (vessels, sites) per dilution has no explicit solution. It is calculated by an iterative process (e.g. Halvorson and Ziegler, 1933), which can be relatively easily computerised. Ready-made tables for several configurations are available. The test result is a function

$$c = MPN = f(n_i, z_i, k) \qquad (20)$$

where the MPN estimate is obtained from computer programs or tables as a function of the number of dilutions (k) and the numbers of parallels (n_i) and positive tubes (z_i).

The metrological counterpart among the colony-count methods is the multiple-plate instrument involving different dilutions (Fig. 5).

4.3 Confirmed test results

In many practical cases the test results are assumed to be confirmed counts. The initial presumptive counts are supposed to be corrected for false positive results by doing additional tests.

4.3.1 *In situ* confirmation

With some colony count methods, confirmation can be achieved after the initial incubation by flooding the entire plate with a special reagent or by transfer of the membrane filter onto a second reagent medium or pad. In this way a confirmation test is made on every colony, also the presumptive negative ones. The final confirmed count is based on the colonies reacting in the appropriate way.

The corresponding confirmation procedure in MPN systems would be to dispense a test reagent into every non-sterile tube irrespective of the initially observed result.

4.3.2 Total or comprehensive confirmation

Another form of confirmation is to transplant each presumptive positive colony individually to one or more confirmatory media to confirm its inclusion in the target group. This rather tedious procedure is only suited for the confirmation of moderate colony numbers. It gives a direct individually confirmed count for every plate examined but differs from *in situ* confirmation by focusing on the presumptive positive colonies only.

With MPN systems, a corresponding confirmation procedure would be to dispense the test reagent into every presumptive positive tube only.

4.3.3 Partial confirmation

When *in situ* procedures are unavailable and comprehensive confirmation is considered unpracticable, it is necessary to resort to partial confirmation. A chosen fraction or a random sample of the presumptive positive colonies is picked for confirmatory tests. This is the approach usually chosen in daily routine work.

In order to convert a presumptive count to the corresponding confirmed count the presumptive count is multiplied by the **confirmation coefficient** (true-positive rate), the proportion of confirmed cases among the sample of presumptive positives.
The confirmed count is accordingly obtained from

$$x = \hat{p}z = \frac{k}{n}z \qquad (21)$$

$\hat{p}$ is an estimate of the confirmation coefficient
z is the count of presumptive positives
n is the number of presumptive positives tested
k is the number of presumptive positives confirmed

With MPN systems, a corresponding confirmation procedure would be to test only a chosen fraction or a chosen number of the positive tubes.

This straightforward mathematical concept becomes a complicated practical matter in case of the multiple-plate instrument (5.6.1-5.6.3).

4.3.4 General or universal confirmation

It has been suggested that conversion of presumptive counts to confirmed counts could also be effected by assuming a general confirmation coefficient, constant for a method and/or for all samples of a certain kind or origin. Every presumptive count belonging to the category would be multiplied by the same constant confirmation coefficient. The coefficient would be known on the basis of the cumulative information collected from occasional random checks.

It has become obvious that a general method-specific, laboratory-specific or even sample-type specific constant is not a tenable idea. Every sample is unique and its presumptive count needs to be individually confirmed by one of the other means. Use of a common confirmation coefficient cannot be recommended in any other context than for the plates within a multiple-plate instrument. Even then it needs to be considered whether the different dilutions within the multiple-plate instrument should require the use of different coefficients.

4.4 Principles of compounding the uncertainty of different metrological types

The formulae for computing microbiological test results, in their simplest forms, involve two or three factors of a product. The combined uncertainty is built from the uncertainties of the factors. Accordingly, the combined uncertainty of a total count or of a completely confirmed MPN or colony count is a function of the uncertainties of the dilution factor and of the density estimate. The combined uncertainty of partially confirmed counts is a function of the uncertainties of the dilution factor, of the density estimate, and of the confirmation coefficient.

Because there are four metrological types and different confirmation practices in each, there will be numerous types of uncertainty calculations.

4.5 Uncertainty of reading

All microbiological viable counts are based on an observation of the number of colonies or reaction sites deemed typical of the target species. In most cases this basic observation depends on the human eye and mind. Without even considering how close to or far from the true value the number of objects in the detection system is, the reading of the result itself is

always more or less uncertain because of the human factor. If the same person were to read the result a second time, the count would not invariably be the same.

In the following, the lack of repeatability of the reading is called uncertainty of reading. Generally, the sub-index T or t (t for 'tally') is used to identify this component of uncertainty. The uncertainty of reading does not refer to the possible systematic differences between persons.

A person is generally capable of repeating the reading of a colony number in easy situations within an uncertainty (standard deviation) of a couple of percent units (w_t = 0.01…0.03). With difficult sample types (mixed populations) and with the least reliable methods, the uncertainty of reading may be much greater. Values from a few percent to more than ten percent for the uncertainty of reading the total plate count of raw milk samples have been reported in the literature. Even higher uncertainty values can be expected with some selective methods. Especially in such cases it is a significant additional component to take into consideration.

The uncertainty of reading may be inadvertently included in other uncertainty components that depend on counts. Including the uncertainty of reading more than once in the combined uncertainty estimate should be avoided.

5 ESTIMATION OF THE COMPONENTS OF UNCERTAINTY

To break open the test procedure in order to compose the combined uncertainty, it is necessary to write down in complete detail what measurements the final test result consists of. As an example, consider the case illustrated by Fig. 5. The test result is based on fourteen measurements

$$y = \frac{(a_1 + b_1)}{a_1} \cdot \frac{(a_2 + b_2)}{a_2} \cdot \frac{(a_3 + b_3)}{a_3} \cdot \frac{(z_1 + z_2 + z_3 + z_4)}{(v_1 + v_2 + v_3 + v_4)} \qquad (22)$$

a_i = transfer volume of the i^{th} dilution step
b_i = dilution blank volume of the i^{th} dilution step
z_j = colony count of the j^{th} 'countable' plate
v_j = test portion volume of the j^{th} plate (in ml of the final dilution)
i = 1…3, j = 1…4

Ten of the observed values are volume measurements (a_i,b_i,v_j) and four are counts (z_j). Some uncertainty is associated with every one of them. In addition, the result is affected by the uncertainty of reading the colony numbers. In order to simplify the mathematical expression it is convenient to consider the whole dilution series as one process that results in the dilution factor F. Calculation of the uncertainty of the dilution factor F is presented in clause 5.6.

When the combined uncertainty is constructed mathematically from its separate components, the calculations always make use of the squares of the uncertainty components (variances). It is therefore appropriate in this part to deal with variances rather than standard deviations.

5.1 Uncertainty of reading

With difficult natural samples and unspecific methods, reading the result may be uncertain to the extent that it significantly affects the combined uncertainty

5.1.1 Average uncertainty of reading one plate

It is advisable to estimate the personal uncertainty by having an occasional plate read twice in the daily routine. It is best to pick the plates randomly for second counting after the initial count has been made. Not only problem cases should be chosen.

It is difficult to avoid a carry-over effect that causes underestimation of the uncertainty. The person might remember what the count was the first time and 'force' the second count close to it. Some time should be allowed between the two readings and the plates for repeated reading should be picked at random after the first count had been made.

The average relative uncertainty w_t of reading depends very little on the number of colonies. It remains about the same throughout the useful colony count range and can be estimated from repeated counts as presented in example 9.3 The uncertainty estimate becomes fairly reliable after more than 30 counts have been included.

5.1.2 Combined uncertainty of reading several plates

The relative standard deviation squared of reading the sum of counts in the case of a multiple- plate instrument (4.2.2) can be estimated from the formula

$$w_T^2 = w_t^2 \frac{\Sigma z_i^2}{(\Sigma z_i)^2} \qquad (23)$$

w_T = the combined uncertainty of reading the sum of colony counts
w_t = relative uncertainty of reading one plate (assumed constant)
z_i = the colony count of the i^{th} plate

5.1.3 Uncertainty of reading MPN test results

No uncertainty is normally believed to be associated with the reading of the number of positive or negative reaction vessels in an MPN system. The truth may not be quite that simple but no information on the uncertainty seems to be available.

5.2 The Poisson scatter of a single colony count

It is generally acceptable to assume that the colony-forming particles ('germs', 'propagules') from which the colonies of a plate potentially arise, originate in a perfectly mixed suspension. This is particularly true if the final suspension is a dilution of the original sample. The statistical distribution of the number of particles observed in a fixed volume can be predicted by the Poisson model. **Poisson scatter** is a general expression chosen to represent the variation in particle numbers observed in a series of faultlessly measured aliquots of fully mixed particle suspensions.

The relative standard deviation squared is the most appropriate expression of uncertainty in this connection:

$$w_z^2 = \frac{1}{z} \qquad (24)$$

(z is the number, or average number, of colonies observed)

5.3 The Poisson scatter of a sum of counts

In a multiple-plate instrument the estimate of the microbial concentration is calculated as the 'weighted mean', the sum of all colony counts ($Z = \Sigma z$) divided by the total test portion volume ($V = \Sigma v$). Because of the additivity of the Poisson distribution, the Poisson scatter (relative variance) of the sum of counts can be estimated from:

$$w_Z^2 = \frac{1}{\Sigma z_i} = \frac{1}{Z} \qquad (25)$$

Z is the sum of the numbers of colonies in a multiple-plate instrument.

The formula is of the same form as in the case of a single plate but the value of the estimate is usually smaller because of a greater number of colonies.

5.4 The uncertainty of the test portion volume

Even if the measured inoculum volumes were true on the average, some uncertainty is always associated with an individual measurement. It can be expressed as a standard deviation or a relative standard deviation (often in percent).

The volumetric uncertainty itself is the result of three main influences:
- repeatability of filling and emptying the measuring device
- the specification of the glassware manufacturer (stated limits of the true volume)
- temperature effect when calibration and measurement take place at different temperatures

Because of the many factors, calculation of the volumetric uncertainty from these components is fairly complicated. Estimating the uncertainty of the volume delivered by weighing repeated measurements is more practical. This practice allows simultaneous estimation of both the systematic and the random component of uncertainty. Aseptic working techniques require that a different pipette or tip should be picked for each measurement. With automatic dispensers the same instrument is filled repeatedly.

Results of selected uncertainty determinations from one laboratory are given as an example in Annex A. An extensive compilation of similar data can be found in Jarvis (1989). Another important source is Untermann (1970). The general applicability of such data is, however, never certain.

5.5 Volumetric uncertainty of a sum of test portions

It is simplest to think that the plates of a multiple-plate instrument or the tubes of an MPN series are inoculated with $V = \Sigma v$ millilitres from the final suspension although, in reality, further dilutions may be involved (see Fig. 5). The inaccuracy caused by ignoring the additional dilution steps is normally insignificant.

It is typical for the end result is that the relative uncertainty of the total volume is smaller that of the other components.

The uncertainty (variance) of the total volume (V) is the sum of the squares of the component uncertainties:

$$u_V^2 = u_{v_1}^2 + u_{v_2}^2 + \ldots \qquad (26)$$

The final uncertainty estimate is most conveniently expressed in the relative scale (percent). Calculation of the combined uncertainty may require shuttling between the log and interval scales. Examples 9.6.1 and 9.6.2 illustrate the point.

5.6 Uncertainty of confirmed counts

Confirmation coefficient p is the proportion of confirmed cases among the presumptive positive cases; the probability of success. Its value is needed whenever a confirmed count x is calculated without testing all presumptive colonies.

$$x = pz \tag{27}$$

x is the confirmed count
p is the confirmation coefficient ('true positive rate')
z is the count of presumptive positives

The true value of the confirmation coefficient p is not known. It is not advisable to assume an estimate known from previous observations, but it should be based on the topical sample itself.

In case of partial confirmation, the coefficient is estimated by isolating and testing a random subset n of the total number z of the presumptive positive colonies. Assuming that k of the n colonies are confirmed, the best estimate of the confirmation coefficient is the quotient

$$\hat{p} = \frac{k}{n} \tag{28}$$

n is the number of presumptive colonies tested
k is the number of colonies confirmed

NOTE 1. Randomness of the choice of colonies for confirmation is essential. At no stage should subjectivity, apart from recognition of presumptive positives, be allowed to direct the choice. An instruction to pick a representative of each colony type for confirmation ruins the quantitative basis of estimating the confirmed count.

NOTE 2. Because the 'germs' of colonies originally land in random places on the plate, any sector or partial area contains a random sample of the colonies. Thus, all the presumptive colonies taken from a sub-area constitutes a legitimate random sample. Should a pre-selected number be taken then a sample fulfilling the requirement of randomness can be obtained by proceeding systematically from a random starting point along a pre-selected route, picking every presumptive positive colony until the number is full. Grids of membrane filters provide a convenient route to follow.

The RSD squared of the estimate, assuming a binomial distribution, is obtained from:

$$w_{\hat{p}}^2 = \frac{n-k}{kn} \tag{29}$$

A small number of colonies picked for testing leaves a large uncertainty to the confirmed counts. Confirming 5 colonies leaves an uncertainty of 19 to 22 % and with ten colonies the uncertainty is still 10 to 16 %. It requires more than 100 colonies to estimate the confirmation coefficient to better relative precision than 5 %.

The Poisson-binomial variance of the confirmed count is

$$u_x^2 = \frac{z^2 k(n-k) + nk^2 z}{n^3} \tag{30}$$

The simplest form of the RSD squared is

$$w_x^2 = \frac{u_x^2}{x^2} = \frac{1}{z} + \frac{n-k}{kn} = \frac{1}{z} + \frac{1}{k} - \frac{1}{n} \qquad (31)$$

5.6.1 General confirmation in the multiple-plate instrument

Denoting a confirmed count with the symbol x and the presumptive count with z, the sum of confirmed counts is obtained from

$$X = \Sigma x_i = \hat{p}_1 z_1 + \hat{p}_2 z_2 + ... \qquad (32)$$

Within the multiple-plate instrument, the colonies on every plate are representatives of the same microbial population. The confirmation rate calculated from any of the plates should (in theory) be an estimate of the common confirmation rate. It should be acceptable to estimate the general confirmation rate by collecting a pre-selected number of colonies from any single plate or from different plates. (It would be most convenient to pick all colonies from the most dilute plate(s) and, if that is not enough, continue by taking the necessary additional colonies from the plates containing more colonies.)

Assuming the above to be true the common coefficient is calculated from

$$\hat{p} = \frac{\Sigma k_i}{\Sigma n_i} \qquad (33)$$

The RSD squared of the sum of confirmed counts can be obtained directly from

$$w_X^2 = \frac{1}{\Sigma z_i} + \frac{1}{\Sigma k_i} - \frac{1}{\Sigma n_i} \qquad (34)$$

It is noteworthy that in this system it is not necessary to pick colonies from every plate. (Example 9.7)

5.6.2 Dilution-specific confirmation in a multiple-plate instrument

When the dilution factor between two dilution levels is ten, as it usually is, the average numbers of colonies in plates from successive dilutions differ considerably. It is possible, even likely, that the confirmation rate seems different in the two dilutions because changes in the appearance of the colonies, due to crowding, alters the subjective evaluation of presumptive colonies. A general coefficient would not apply and a separate estimate of the confirmation rate would be required for the two dilutions. This changes to some extent the calculation of the uncertainty of the sum (5.6.2).

Assume countable parallel plates in two dilutions (Fig. 5). Assume also that it is suspected that the apparent confirmation rates differ in the dilutions. Colonies must be picked from both dilutions for confirmation.

With the following symbols

X sum of confirmed counts
Z_1 sum of presumptive counts in the first dilution
N_1 total number of presumptive colonies tested in the first dilution
K_1 total number of colonies confirmed in the first dilution
Z_2 sum of presumptive counts in the second dilution
N_2 total number of presumptive colonies tested in the second dilution
K_2 total number of colonies confirmed in the second dilution

The sum of confirmed colony counts

$$X = \frac{K_1 Z_1}{N_1} + \frac{K_2 Z_2}{N_2} \tag{35}$$

Variance of the sum

$$u_X^2 = \frac{Z_1^2 K_1 (N_1 - K_1) + N_1 K_1^2 Z_1}{N_1^3} + \frac{Z_2^2 K_2 (N_2 - K_2) + N_2 K_2^2 Z_2}{N_2^3} \tag{36}$$

The RSD squared of the sum

$$w_X^2 = \frac{u_X^2}{X^2} \tag{37}$$

(Example 9.7)

5.6.3 Plate-specific confirmation in a multiple-plate instrument

It should not be necessary to assume conditions in every plate of a multiple-plate instrument so different that a plate-specific confirmation coefficient would be required. If, however, the opposite is suspected the principles in 5.6.2 should be applied to every count. In that case colonies should be picked from every plate for confirmation.
(Example 9.7)

5.7 The dilution factor

The term ‘dilution factor’ was selected to denote the number of times the original sample was diluted to obtain the final dilution. It is the reciprocal of the dilution, and is used as a multiplier when converting counts to concentration of the analyte in the original sample.

A single dilution step is made by mixing a small volume a of a microbial suspension with volume b of sterile diluent. The dilution factor of one dilution step is therefore

$$f = \frac{a + b}{a} \tag{38}$$

Should calibration measurements indicate that the true volumes differ from the nominal ones then a and b in the formula should be replaced with the true volumes $a' = a + \Delta a$ and $b' = b + \Delta b$. The standard uncertainties in volume scale remain unchanged but the relative uncertainties change as much as a and b do.

Mostly laboratories seem to assume without controlling that the volumes a and b equal the nominal volumes (Δa and Δb equal zero).

A dilution factor consisting of k successive dilution steps is calculated as the product:

$$F_k = \frac{a_1 + b_1}{a_1} \cdot \frac{a_2 + b_2}{a_2} \cdot \ldots \cdot \frac{a_k + b_k}{a_k} = f_1 \cdot f_2 \cdot \ldots \cdot f_k \qquad (39)$$

In most instances the dilution series is regular in the sense that volumes a and b are the same in all k steps. In that case, the total dilution factor equals

$$F_k = f^k = \left(\frac{a + b}{a} \right)^k \qquad (40)$$

5.7.1 Uncertainty of the dilution factor

The numerator and denominator in the formula for calculating the dilution factor are correlated (same a). Therefore, it is not completely correct to apply the rules of combining independent components of uncertainty (clause 3.1) when estimating the uncertainty of a dilution factor. The uncertainty variance of a dilution step is obtained from

$$u_f^2 = \frac{a^2 u_b^2 + b^2 u_a^2}{a^4} = \frac{u_b^2}{a^2} + \frac{b^2 u_a^2}{a^4} \qquad (41)$$

and the RSD squared from

$$w_f^2 = \frac{1}{(a + b)^2} \left[u_b^2 + \left(\frac{b}{a} \right)^2 u_a^2 \right] = \frac{(u_b^2 + b^2 w_a^2)}{(a + b)^2} \qquad (42)$$

a = suspension transfer volume (or a', see above)
b = dilution blank volume (or b', see above)
u_a = standard uncertainty of a
u_b = standard uncertainty of b
w_a = relative standard uncertainty of a (or a')

If the total dilution consists of k similar steps the combined RSD squared of the dilution factor is calculated from

$$w_F^2 = kw_f^2 \tag{43}$$

If the steps differ in their volume configuration, the relative uncertainty of each step should be separately estimated and the results compounded as the sum of RSD squared:

$$w_F^2 = w_{f_1}^2 + w_{f_2}^2 + \ldots + w_{f_k}^2 \tag{44}$$

6 MATHEMATICAL MODELS OF QUANTITATIVE TEST RESULTS AND THEIR UNCERTAINTIES

The mathematical uncertainty models considered in this part are reduced to the bare essentials. The only systematic 'corrections' taken into account are the dilution factor and the confirmation coefficient. More complete models are dealt with in clause 8 and in example 10.5.

Basically, the elements for computing the final test results of quantitative colony count methods are the dilution factor F, test portion volume v, and the number of colonies z and, with partially confirmed counts, the confirmation coefficient p.

The uncertainty of reading is a hidden additional component in all counts.

Time and temperature of incubation are important, sometimes critical, factors for the detection of viable microbial particles. Initial validation of a method should supply the exact permissible time and temperature limits for the incubation procedure. However, there is never absolute certainty that the laboratory actually succeeds in staying within the limits. Total failure of laboratories in inter-laboratory trials has frequently been traced back to insufficient temperature control of the incubators. This adds an element of uncertainty that has the characteristics of spurious errors and, therefore, remains beyond mathematical correction and estimation. There is no other solution but strict adherence to the time limits and rigorous quality control of incubator temperature during the incubation.

6.1 The one-plate instrument

The reasons for confining the observations to one single plate range from economy to some limiting agreement or rule, such that only plates with colony numbers between a small range (10-100, 25-250, 30-300) are acceptable. When a single colony count is available the uncertainty of the test result can only be calculated from statistical theory and from components assumed known.

The quantitative estimate is the result of the calculation

$$y = F\frac{z}{v} \tag{45}$$

F = nominal or true dilution factor
z = number of colonies observed on the plate
v = test portion volume (ml of the final dilution)

The combined relative uncertainty of the test result, without the personal uncertainty of reading, is obtained from

$$w_y = \sqrt{w_F^2 + w_z^2 + w_v^2} \tag{46}$$

w_F = relative standard uncertainty of the dilution factor (5.7)
w_z = relative Poisson scatter of the colony count (5.2)
w_v = relative standard uncertainty of the test portion volume (5.4)

The personal relative uncertainty of reading the number of colonies (w_t) adds one term to the sum of squares under the root sign:

$$w_y = \sqrt{w_F^2 + w_z^2 + w_v^2 + w_t^2} \qquad (47)$$

Should the count be confirmed, it is best to confirm every colony and substitute the confirmed count for z in the above formula (45). If only partial confirmation is practised, the test result is obtained from the calculation

$$y = pF\frac{z}{v} \qquad (48)$$

where p is an estimate of the confirmation coefficient derived by testing a sub-set of the colonies (5.6).

The relative standard uncertainty of the confirmed test result is calculated from

$$w_y = \sqrt{w_p^2 + w_F^2 + w_z^2 + w_v^2 + w_t^2} \qquad (49)$$

6.2 Multiple-plate designs

6.2.1 General

When more than one countable plate is available, the microbial density of the final suspension is obtained as the weighted mean (Farmiloe et al. 1954) by dividing the sum of colony counts by the sum of test portion volumes. In these cases, the test portions must be expressed in ml of the final suspension. The test result, with the dilution taken into account is

$$y = F\frac{\Sigma z_i}{\Sigma v_i} = F\frac{Z}{V} \qquad (50)$$

F is the dilution factor
z_i is the count in plate i
v_i is the test portion volume of plate i (ml of final suspension)

Upper case letters are used in the formula for denoting sums (Z, V) and the total dilution (F).

The similarity of the formula with that of the one-plate instrument means that the uncertainty of the test result is obtained by an analogous formula but with sums replacing individual observations:

$$w_y = \sqrt{w_F^2 + w_Z^2 + w_V^2} \qquad (51)$$

w_F relative standard uncertainty of the nominal or actual dilution factor (5.7)

w_Z relative standard uncertainty of the sum of colony counts (5.3)
w_V relative standard uncertainty of the sum of test portions (5.5)

Should the uncertainty of reading be taken into account, the value is estimated as presented in 5.1.2. If it is necessary to confirm the results, and all colonies cannot be tested, the confirmed sum (X) and its relative uncertainty w_X are estimated as presented in 5.6.1.-5.6.3.

It is possible to circumvent the often complicated calculations of the uncertainties of the sums by obtaining a direct uncertainty estimate from the set of counts available. The variation between the colony numbers observed includes not only the effects of the design (volumes and dilutions) but also the Poisson scatter and the uncertainties associated with volume measurements and reading. This special short-cut estimation procedure is explained in clause 7. See also example 10.4.

6.2.2 Confirmed counts

The confirmed test result is calculated from

$$y = F\frac{X}{V} \tag{52}$$

X is the sum of confirmed counts
F is the dilution factor
V is the total test portion volume

The relative uncertainty is estimated from

$$w_y = \sqrt{w_F^2 + w_X^2 + w_V^2} \tag{53}$$

The most peculiar feature of the confirmed sum and its uncertainty in the case of partial confirmation is the dependence of the numerical values on inexact impressions. The values are calculated in one of three ways, depending on what is *assumed to happen* at the bench level when presumptive target colonies are picked for confirmation (5.6.1-5.6.3). See example 9.7.

6.3 The single-dilution MPN instrument

The test result is calculated from

$$y = F\frac{1}{v}\ln\left(\frac{n}{s}\right) \tag{54}$$

y = the estimated (number) concentration of the organism per unit volume of original sample
F = nominal or actual dilution factor of the final suspension
v = volume of one test portion (the average)
n = total number of reaction vessels (tubes)
s = number of reaction vessels remaining sterile after incubation

There are tables for the test result for some selected series of tubes (n = 5, 10, 15, 20, 25). Some sources are Niemelä (1983) and ISO CD 8199. The tables give the MPN estimate and its 95 % confidence interval. In addition, the most versatile computer programs (e.g. Hurley and Roscoe, 1983) provide an estimate of the standard uncertainty in $\log_{10}$ units.

The dilution factor (F) and the volume measurements (v) are subject to normal measurement uncertainty, n is a chosen constant with no uncertainty, and s is a random variable believed to follow the binomial probability law. Note that in this case v denotes the average volume. Its uncertainty should be estimated accordingly (6.3.1).

6.3.1 Uncertainty of the average test portion

The random relative uncertainty of the total volume $V = nv$ measured into the series of tubes is smaller than the relative uncertainty of an individual volume (v) because it is obtained from

$$w_V^2 = \frac{nv^2 w_v^2}{V^2} = \frac{nv^2 w_v^2}{(nv)^2} = \frac{w_v^2}{n} \qquad (55)$$

w_V = the relative standard uncertainty of the total (and mean) test portion volume
n = number of growth cells (tubes) in the MPN series
v = volume of one test portion (the mean)
w_v = relative standard uncertainty of one volume measurement
V = sum of all test portions = nv

The relative standard uncertainty of the sum and the mean are the same. The value from this calculation is usually so small that it can be ignored when computing the combined uncertainty.

6.3.2 Relative standard uncertainty of the MPN estimate

When the information sources, such as tables, give the 95 % confidence interval, then the relative standard uncertainty of the MPN estimate or of the final test result can be obtained from the given limits with the help of the logarithmic transformation. Tables and computer programs may also provide the standard deviation in $\log_{10}$ units (S.E. of $\log_{10}$ MPN). An estimate of the relative standard deviation can be derived from such information in one of three ways:

i) Given the upper x_U and lower x_L limits of the 95 % confidence interval the relative uncertainty is estimated as one half of the half-width of the confidence interval (in ln scale) according to:

$$w_{MPN} = \frac{\ln(x_U) - \ln(x_L)}{4} \qquad (56)$$

ii) A 'standard error' of $\log_{10}$MPN from a computer program (e.g. Hurley and Roscoe, 1983) can be converted into the relative uncertainty (w_{MPN}) simply by transforming to the natural logarithmic scale (multiplying with 2.303).

iii) Lacking other sources of information, the relative standard uncertainty can be obtained by assuming the number s of sterile tubes to be a random quantity that varies according to the binomial distribution. It should, accordingly, have a variance of $s(n-s)/n$.

The simple (approximately 68 %) upper and lower boundaries of the MPN estimate are first computed from

$$x = \frac{1}{v} \ln \left[\frac{n}{s \pm \sqrt{\frac{s(n-s)}{n}}} \right] \tag{57}$$

(The plus sign in the denominator gives the lower boundary x_A and the minus sign the upper boundary x_H.) The relative uncertainty of the MPN estimate is calculated by dividing the difference of the natural logarithmic values by two:

$$w_{MPN} = \frac{\ln(x_H) - \ln(x_A)}{2} \tag{58}$$

6.3.3 Combined uncertainty of the test result

Considering all the elements of uncertainty, the combined relative uncertainty of the single-dilution MPN estimate can be estimated as:

$$w_y = \sqrt{w_{MPN}^2 + w_F^2 + w_V^2} \tag{59}$$

Frequently, the MPN component is so dominant that it hardly matters if the other two, especially the volume component, are ignored.

It is always assumed that there is no uncertainty to the reading of the number of positive or negative tubes in an MPN series.

6.4 The multiple-dilution MPN instrument

The test result is calculated as

$$y = F \cdot MPN \tag{60}$$

where F is the actual or nominal dilution factor and MPN is the most probable number corresponding to the number and distribution of positive reaction vessels (tubes, wells, etc.) observed in the parallel series of tubes in two or more dilutions. When tables are used, three dilutions are usually presupposed. The most versatile computer programs have no

restrictions regarding the numbers of dilutions, numbers of parallel tubes per dilution, and the dilution steps.

The underlying assumptions in the MPN systems are that all dilutions and volume measurements within the detection instrument are made without any systematic or random uncertainty, and that suspensions at all stages are so well mixed that the Poisson distribution prevails throughout.

If Poisson distribution is not true, the most important basic assumption of the MPN statistics is not valid. The estimate should not be accepted. Generally, however, the Poisson assumption is not questioned when using an MPN system. In some of the modern MPN tables, the problem is taken into account by excluding such combinations of tubes that are highly unlikely when Poisson distribution is valid. Computer programs give a result for every combination of positive tubes but may report about the plausibility of the observed test result. In that case, it is up to the user to decide whether the result should be accepted or not.

The model of the combined relative uncertainty is simply

$$w_y = \sqrt{w_{MPN}^2 + w_F^2} \tag{61}$$

There is no simple pocket-calculator solution either to the MPN estimate or its exact uncertainty. The user is dependent on ready-made tables or computer algorithms. Both sources provide the 95 % confidence interval in the form of the lower and upper limits. The relative uncertainty of MPN (the Poisson scatter) can be obtained from this information as described in 6.3.2 (alternative i). Different methods of estimating the confidence interval have been employed in the tables and computer programs of different authors. For that reason the limits given may differ to some extent.

Some computer programs (e.g. Hurley and Roscoe, 1983) provide not only the confidence limits but also an estimate of the standard uncertainty in $\log_{10}$ scale. This can be converted to the relative standard uncertainty as described in 6.3.2 (alternative ii).

Finally, the uncertainty can be estimated by first calculating the standard uncertainty of $\log_{10}$MPN by the use of Cochran's (1950) approximation

$$s_{LogMPN} \approx 0.58\sqrt{\frac{\log_{10} f}{n}} \tag{62}$$

f = dilution factor between two successive dilutions
n = number of parallel tubes per dilution

The standard uncertainty becomes an estimate of the relative standard uncertainty of MPN when converted to ln scale by multiplying with 2.303.

The above uncertainty model does not include any uncertainties associated with dilution and volume measurements within the multiple-dilution instrument. They could be estimated, but it has become evident that the effect on the combined uncertainty is insignificant. The basic assumptions regarding faultless volume measurements are normally sufficiently true.

7 A SHORT-CUT TO THE UNCERTAINTY OF MULTIPLE- PLATE INSTRUMENTS

The differences between the colony numbers in the multiple-plate system are partly due to design (volumes and dilutions) and partly due to random variation. The random component consists of the Poisson scatter, effects of volume uncertainties, and the uncertainty of reading the counts. It is possible to remove the variation due to design by the use of the log-likelihood ratio statistic G^2. By doing so the uncertainty of the microbial concentration in the final suspension can be estimated directly without the need to compose it from separate uncertainty components. The concentration in the final suspension is calculated as the weighted mean

$$c = \frac{\Sigma z_i}{\Sigma v_i} \tag{63}$$

z_i the observed colony count of the i^{th} plate
v_i the volume of final suspension inoculated into the i^{th} plate

The combined relative uncertainty of the test result is calculated as

$$w_y = \sqrt{w_c^2 + w_F^2} \tag{64}$$

where w_F is the relative uncertainty of the dilution factor (5.7).

To obtain the relative uncertainty estimate that includes all the random components affecting the counts within the instrument, the log-likelihood ratio statistic is first computed

$$G_{n-1}^2 = 2\left[\sum_{i=1}^{n} z_i \ln\left(\frac{z_i}{v_i}\right) - \left(\sum_{i=1}^{n} z_i\right) \ln\left(\frac{\Sigma z_i}{\Sigma v_i}\right)\right] \tag{65}$$

The formula may look forbidding but is quite simple. Considering that the sums in the second term of the equation are the total number of colonies and the total volume it can also be written

$$G_{n-1}^2 = 2\left[\sum_{i=1}^{n} z_i \ln\left(\frac{z_i}{v_i}\right) - Z \cdot \ln\left(\frac{Z}{V}\right)\right] \tag{66}$$

z_i colony count of the i^{th} plate
v_i test portion volume (of final suspension) of the i^{th} plate
n number of plates
Z sum of all colony counts
V sum of all test portion volumes

To facilitate the calculation of G^2 an algorithm written in BASIC computer language (Anon. 1994) has been presented in Annex C, with a worked example.

The relative uncertainty variance of the density estimate c of the final suspension, needed in the uncertainty formula (64), is calculated from

$$w_c^2 = \frac{G_{n-1}^2}{n-1} \cdot \frac{1}{Z} \qquad (67)$$

n number of plates
Z sum of colony counts
$G^2_{(n-1)}$ likelihood-ratio index with (n-1) degrees of freedom

NOTE. The reasoning behind the short-cut procedure is rather indirect. Knowing that the G^2 statistic is asymptotically distributed as χ^2 with the same number of degrees of freedom, and utilising the connection between χ^2 and variance, a connection is established between G^2 and variance.

The short-cut procedure is no more reliable than a Type A estimation of uncertainty with a small number (n) of independent observations. G^2 is a random variable with an uncertainty of its own.

It may happen that the observed value of $G^2/(n\text{-}1)$ is smaller than 1.0 (its expected value in a perfect Poisson distribution). In such instances the value $G^2 = (n\text{-}1)$ should be substituted for the observed value. A high value (more than five) of $G^2/(n\text{-}1)$ is a warning sign of technical or other data quality problems in the series of plates. The basic data should be critically examined.

7.1 Confirmation in the short-cut procedure

In the short-cut procedure, every presumptive count must be individually converted to a corresponding confirmed count. When only a part of the colonies is confirmed, conversion to confirmed counts should apply general, dilution-specific, or plate specific confirmation coefficients, depending on the assessment of the situation. Also a mixed procedure is permissible. All colonies when numbers are small and a sample when numbers are large, may be tested. The confirmed counts should be substituted for z in the relevant formulae (63, 65, 66, 67). The added uncertainty due to partial confirmation will probably have an effect on the estimate of G^2.
(Example 10.4)

8 "COMPLETE" MODELS, SYSTEMATIC CORRECTIONS AND THEIR UNCERTAINTIES

According to the basic principles of metrology, any reported test result should be corrected for all known systematic errors. The uncertainties of the systematic corrections should be taken into account as additional components in the combined uncertainty of the measurement.

Apart from determination of the 'efficiency of plating', (EOP) in bacteriophage assays, microbiologists are not used to the idea of systematic corrections. The general formula for computing the test result is used without questioning its accuracy. There are, however, well known biotic and abiotic factors, many of them systematic, why the observed number of colonies z might differ from the true number of 'colony-forming particles' in a plate.

8.1 Nature of systematic corrections in microbiology

Suppose a true but unknown number of colonies x in a particle detector. The number z observed may differ from x for many reasons.

Spurious errors, such as contamination and antibiotic or competitive interactions between colonies, are frequent in microbiological determinations. Test results are also extremely sensitive to slight inaccuracies in temperature with methods that employ high incubation temperature for selective purposes. Spurious errors are unpredictable and cannot be corrected mathematically. The only protection against them is a functioning quality assurance system and high expertise at the bench level.

In addition to the random and spurious errors, a number of systematic effects may affect the result. Among them are the occasional inability of a viable particle to express itself as a recognisable colony, geometrical overlap of neighbouring colonies, systematically deviant 'style' of counting, decreased yield of a batch of medium, etc. The test result might also differ from the true concentration of the analyte in its original source because of changes between sampling and analysis.

Characteristically, the systematic effects on microbiological test results are multiplicative. Instead of calculating the test result y from the obvious basic observations (z, v, F, p) a value y_{corr} corrected for m systematic effects is obtained from

$$y_{corr} = K_1 \cdot K_2 \cdot \ldots \cdot K_m \cdot y \qquad (68)$$

In principle, the correction factors are constant, but their values are usually the result of empirical observations. Each of the values is therefore more or less uncertain. The uncertainty of the correction factors should be included in the combined estimate of the uncertainty of the ultimate test result. Because the formula for the test result is a product, the components of uncertainty should be combined in the relative scale of measurement.

A microbiological test result might be considered "completely corrected" when the following systematic correction factors have been applied (if relevant):

actual dilution factor F"
confirmation coefficient p

personal yield coefficient K_H
sample stability coefficient K_S
yield of the medium coefficient K_A
material/environment coefficient K_M
overlap correction factor K_L

The "completely corrected" test result is calculated as

$$y = K_L \cdot K_M \cdot K_A \cdot K_S \cdot K_H \cdot p \cdot F' \cdot x \qquad (69)$$

Its relative uncertainty due to the mathematical relation of the components, with the uncertainty components of counting (w_T) and final suspension density (w_c) added, is

$$w_y = \sqrt{w_{F'}^2 + w_p^2 + w_{K_H}^2 + w_{K_S}^2 + w_{K_A}^2 + w_{K_M}^2 + w_{K_L}^2 + w_c^2 + w_T^2} \qquad (70)$$

A systematic component has no effect when the coefficient is 1.0 and its uncertainty is zero.

8.1.1 A question of 'metrological ethics'

The values of correction factors are mostly derived from calibration experiments. It is possible that the value observed does not differ significantly (statistically) from 1.0 because of a high uncertainty of the determination. It should be permissible to assume 1.0 to be the true value of the correction factor in such instances.

It has not been discussed by the scientific community what to do about the uncertainty in such cases. It does not seem right to ignore the uncertainty of the statistically non-significant correction factor. The reason for the statistical non-significance may be that not enough effort was spent on the experiment (too few replicate measurements) so that the uncertainty of the coefficient remains high.

Whenever the value of a correction factor is found to be statistically no different from 1.0, it seems recommendable to substitute the value 1.0 for the empirical value. The uncertainty of the empirical value should, however, be adopted as the estimate of uncertainty.

8.2 The actual dilution factor *F'* and its uncertainty

At an early stage in the development of quantitative microbiology it was recognised that the volume measurements involved in a dilution series may be inaccurate. It has been the subject of many publications over the past half century. An extensive treatment of the subject can be found in Jarvis (1989).

As a rule, laboratories seem to assume the nominal pipette and dilution blank volumes to be true, and base the calculation of the dilution factor on the nominal volumes. Should calibration measurements indicate otherwise this should be taken into account by inserting the actual volumes $a' = a + \Delta a$ and $b' = b + \Delta b$ in the formulae for computing the dilution factor (5.7) and its uncertainty (5.7.1). The actual dilution factor F' may differ markedly from the nominal one (F) when a high degree of dilution is necessary.

8.3 The confirmation rate (true-positive rate) *p*

Although not a practice to be recommended, a confirmation coefficient determined from a small sample of presumptive positive colonies is occasionally the only available means of converting presumptive counts to confirmed counts. When this practice is used, the confirmation coefficient is a systematic correction factor with a high degree of uncertainty (5.6).

8.4 Personal yield coefficient K_H

Every person reading colony numbers has a personal 'style' of enumeration. It often leads into detectable systematic differences between the results of different persons. The differences are not great in simple situations, such as counting colonies of pure cultures. They may be considerable in crowded plates and in natural samples with complex populations of microbes, and especially with methods where recognition of the target colonies is based on colour and shape. This fact is easily demonstrated by presenting the same plates to two or more persons for reading. Such experiments provide data for estimating personal yield coefficients in comparison with a common reference. The uncertainty of the coefficients can be estimated in the same experiment. There is a wealth of literature on this subject.

The uncertainty of the personal yield coefficient includes the personal uncertainty of reading, which should not be double-counted by being separately taken into account in the combined uncertainty.

The most significant problem in this connection is that the true result is not known. There are no absolute reference points and an arbitrary one must be chosen.

8.4.1 An 'infallible' expert as reference

One solution to the reference is to choose one person as an expert whose result is agreed to be always correct. The count by the expert (E) and that by the test person (X) are recorded on a number of normal routine plates. The correction coefficient to multiply the test person's results with is the ratio of the expert's sum of counts to that of the test person.

$$K_H = \frac{\Sigma z_E}{\Sigma z_X} \tag{71}$$

Coefficients smaller than 1.0 mean that the counts by the test person in question should be reduced, because the laboratory has decided to standardise its results according to the counting convention of one chosen expert.

To estimate the uncertainty of K_H the relative difference of the two counts should be computed for each pair (z_E, z_X) of counts

$$d = \frac{z_E - z_X}{z_E} \tag{72}$$

z_E number of colonies observed by the expert
z_X number of colonies observed by the test person

The standard uncertainty (standard deviation) of d (s_d) is calculated according to the standard statistical practice (Type A estimation). The standard deviation of the mean d (standard error) is an approximate estimate for the relative uncertainty w_{KH} of K_H.

$$w_{K_H} = \frac{s_d}{\sqrt{n}} \tag{73}$$

Choosing one person as the expert means that his/her personal yield coefficient has the value 1.0 and an uncertainty of 0.
(Example 9.3)

8.4.2 The average count as reference

Another possible reference point for personal yield coefficients is the average count of all participants. Coefficient K_H and its relative uncertainty are calculated as presented in clause 8.4.1, with the exception that the expert's counts (z_E) are replaced with the average counts of all participants.

In this solution the results reported by the laboratory will be corrected to correspond to the average counting convention of all technicians involved in reading plates. None of the persons may be exactly 'average'. The uncertainty of every personal yield coefficient differs from zero.
(Example 9.3)

8.5 The common reading uncertainty of a laboratory

A laboratory might not wish to pay attention to the possibly different counting styles of individuals. In that case the value of the yield coefficient is independent of the person and has the value $K_H = 1$.

The uncertainty of the general counting practice of the laboratory as a whole can be estimated with experiments where all technicians read the numbers of colonies of the same plates. The standard uncertainty or standard uncertainty in ln scale is computed separately for the counts of each plate and their quadratic average is determined. The simplest procedure for that is to perform a one-way analysis of variance after ln transformation of the counts.
(Example 9.4)

8.6 Concentration change during storage. Stability coefficient K_S and its uncertainty.

The microbial concentration may change between the sampling and analysis of the sample, but it is not always possible to guess the direction of the change or if there has been any change at all. Empirical observations are rare, and their results can hardly be generalised. It is unlikely that laboratories have any useful empirical data of their own. Any corrections

attempted and uncertainties estimated may have to be based on rectangular or triangular *a priori* distributions chosen more or less 'by the feel' (2.4).

If there is a possibility of a significant change it should be estimated and its uncertainty should be taken into account. If death and multiplication are considered equally possible the coefficient has the value $K_S = 1.0$. If only one direction of change is considered possible, the coefficient will differ from 1.0.
(Example 9.5.2)

8.7 Yield coefficient of the nutrient medium, K_A

Every batch of medium should be tested for recovery as part of the quality assurance system. A quantitative yield coefficient, a measure of efficiency, is determined by studying reference samples. The samples are of necessity artificial in nature and there is no perfect traceability to a true value. The efficiency can only be related to the conventional true value empirically determined for the batch of reference samples.

The uncertainty of the yield coefficient depends on the relative uncertainty of the conventional reference value and that of the test result
(Example 10.5).

8.7.1 External reference samples

When commercial certified reference samples are available the certificate will provide the experimentally determined conventional true average value. Its uncertainty or expanded uncertainty will most probably be given as the standard deviation in $\log_{10}$ units or as the confidence interval. The relative uncertainty can be derived from this information by conversion to ln units or by applying the principles illustrated in clause 6.3.2.

Assuming a conventional true reference value in the interval scale x_R and a test value x_A with the batch of medium being tested, the ratio $K_A = x_R / x_A$ gives the correction factor. In principle, every test result should be multiplied by this factor as long as the same batch of medium is being used.

The relative uncertainty (variance) of the correction factor can be derived from the relative uncertainties of the reference value and that of the test value according to principles presented earlier (3.1).

$$w_{K_A}^2 = w_{x_R}^2 + \frac{1}{Z_A} \qquad (74)$$

Z_A total number of colonies on which the test result x_A is based
w_{KA} relative standard uncertainty of the reference value

8.7.2 Non-selective/selective ratio coefficient

A correction factor of a different kind stems from the observation that the recovery of microbial colonies from pure culture suspensions is almost invariably markedly lower on a

selective nutrient medium than on a non-selective one. The recovery is frequently only 70 - 80 %. Should the result on the non-selective medium be considered the true value, the results of the selective medium are to be multiplied by a factor larger than 1 to correct for the count.

The practical significance of the viable but non-recoverable cells, missed by selective cultivation, is not clear. An assumption that the same ratio could be projected to natural samples is obviously not valid. The ratio estimate might, however, have some special uses.

Membrane filters may be agents that lower the count of colonies. The recovery ratio on the same medium with and without the use of a membrane filter might be considered a relevant systematic correction factor.

The coefficient is calculated as given in clause 8.7.1, but its uncertainty is estimated differently. Provided that the test portion volumes are the same, the relative variance of the yield coefficient is obtained by the rule of combining independent uncertainties of a division (3.1.4)

$$w^2_{x_R/x_A} = \frac{1}{Z_R} + \frac{1}{Z_A} \qquad (75)$$

Z_R and Z_A are the total numbers of colonies observed on the non-selective and the selective medium.

8.8 Matrix effect. Correction factor K_M.

It is entirely possible that the solids of a sample are directly or indirectly the cause of lowered recovery of target micro-organisms from the test portion. The reasons might be anything from adsorption to particles to death or destruction of viable cells during maceration of the sample.

The quantitative effect is probably the loss of viable cells. The correction factor should consequently have a value larger than 1. It is, however, difficult to estimate the value because the true concentration of the analyte in the sample cannot be known. Spiking with pure cultures and testing for recovery seems to be the only approach.

The non-homogeneity of solid samples and the patchy distribution of microbes increase the variation considerably. The variance becomes greater than the Poisson scatter of perfect suspensions. The added variance can be interpreted as the uncertainty of the matrix coefficient.

Determination of the uncertainty of the matrix effect would require a complex Analysis of Variance design. Pure matrix variance values do not seem to be available in the literature.

Uncertainty estimates that include four or five components, including the matrix, are available to some extent. Such data are created in studies where parallel samples are tested. The ‘within-samples sum of squares’ in the Analysis of Variance includes the effects of the matrix, the dilution series, the test portion volume, Poisson scatter, and the uncertainty of reading. Sometimes useful information is recoverable from the results of collaborative

method performance tests, provided that the primary observations are made available. Unfortunately, the primary counts are not often reported.

8.9 Overlap correction factor K_L

The number of colonies observed in or on a plate is, at the most, equal to the number of so-called colony forming units or particles (CFU, CFP) present in the test portion. Resulting from purely geometrical overlap incidents, the observed number of colonies (z) is generally smaller than the original number of CFU. Some colonies 'disappear' by merging indistinguishably with other similar colonies. This phenomenon is different from the loss of recovery caused by excessive background colonies. (Background growth sometimes not only masks but may also alter the appearance of the target colonies, changing some of them into false negatives.)

The loss due to the geometrical overlap of target colonies is proportional to the space or area occupied by the colonies, the 'coverage'. The table below has been constructed from previously published data (Niemelä 1965). It applies to the membrane filtration and spread plate methods. With the pour plate technique the overlap phenomenon is only marginally observable and can be ignored.

The value of the correction factor is $K_L = CFU/z$. As the true number (CFU) is not known, only an average expected value for K_L can be quoted on the basis of the observed coverage (Table 8.1).

Table 8.1 *Expected values of the overlap correction factor K_L as a function of the coverage (proportion of growth area covered by target colonies) adapted from Niemelä (1965).*

Coverage %	K_L
5	1.02
10	1.04
15	1.06
20	1.08
25	1.11
30	1.14
35	1.18
40	1.24

At 30 % and higher coverage most technicians seem to feel the counting task overwhelming.

The uncertainty of the overlap correction factor undoubtedly varies with the coverage but is not known well enough. The average value of the relative uncertainty can be estimated to be of the order $w_{KL} = 0.05$

8.10 Correction of the reading error

When considering the uncertainty of reading when there is no reference, such as an expert or a group of other persons, the correction factor can only have the value $K_T = 1$. Its relative uncertainty w_T is estimated from data on repeated counting of plates. If the personal coefficient for correcting the yield K_H and its uncertainty w_{KH} is employed, the personal uncertainty of reading w_T should not be applied at the same time.

9 WORKED EXAMPLES I. ESTIMATES OF INDIVIDUAL COMPONENTS OF UNCERTAINTY.

The individual uncertainty components are always squared when used in the construction of combined uncertainty estimates. It is therefore appropriate to present the uncertainty estimates in this part in their squared form, as variances.

9.1 Poisson scatter of a single colony count

Assume $z = 36$ colonies were observed on a single plate.
According to formula (24) the RSD squared is

$$w_z^2 = \frac{1}{36} = 0.0278 \qquad (76)$$

9.2 Poisson scatter of a set of counts

The following counts have been observed in four plates that form a multiple-plate detection instrument involving two parallel plates in two successive dilutions.

Diln.	Counts		Sum.
10^{-4}	185	156	341
10^{-5}	17	22	39
		Total:	$Z = 380$

The relative variance $w_Z^2 = \frac{1}{380} = 0.0026$

9.3 Personal uncertainty of counting

A technician has noted down results of her/his own repeated reading (z_1, z_2) of several plates within a time span insufficient to cause real changes in the colony counts. The plates were chosen at random during the daily routine work. A small part of the results is shown in the table below:

Plate #.	z_1	z_2	**ln** z_1	**ln** z_2	$(\ln z_1 - \ln z_2)^2$
1	343	337	5.84	5.82	0.0004
2	40	39	3.69	3.66	0.0009
3	57	62	4.04	4.13	0.0081
4	399	397	5.99	5.98	0.0001
5	112	130	4.72	4.87	0.0225
6	349	325	5.86	5.78	0.0064
7	85	84	4.44	4.43	0.0001
8	129	122	4.86	4.80	0.0036
9	16	17	2.77	2.83	0.0036
10	27	27	3.30	3.30	0.0000
				Sum	0.0457
				Mean:	0.00457

The mean of the squared differences divided by two is perhaps the simplest way to obtain the RSD squared of the average repeatability of reading.

$$w_t^2 = \frac{0.0457}{2\ x\ 10} = 0.0023 \qquad (77)$$

The same result emerges as the within-plates sum of squares in a one-way Analysis of Variance after ln transformation of the counts.

A third possibility of estimating the variance does not involve logarithms. It might be considered a more natural approach. The estimate of the personal uncertainty of reading is calculated by the formula (78)

$$w_Z^2 = \frac{2}{n}\sum_{1}^{n}\left(\frac{z_1 - z_2}{z_1 + z_2}\right)^2 \qquad (78)$$

Applied to the same results, the calculations proceed as presented in the table below

Plate	z_1	z_2	$(z_1 - z_2)$	$(z_1 + z_2)$	$[(z_1 - z_2)/(z_1 + z_2)]^2$
1	343	337	-6	680	0.000078
2	40	39	1	79	0.000160
3	57	62	-5	119	0.001765
4	399	397	2	796	0.000006
5	112	130	-18	242	0.005532
6	349	325	24	674	0.001268
7	85	84	1	169	0.000035
8	129	122	7	251	0.000778
9	16	17	-1	33	0.000918
10	27	27	0	54	0.000000
				Sum.	0.010540

The sum of the last column multiplied by $2/n = 0.2$ gives the squared relative uncertainty of reading as: $w^2_t = 0.2 \times 0.010540 = 0.002108$. The value in this case was slightly smaller than the estimate using logarithms.

9.4 Laboratory-specific uncertainty of counting

Suppose that four technicians involved in daily routine microbiological analyses (A, B, C, D) read the same eight randomly selected plates independently. The results were the following:

Plate	A	B	C	D	Mean	s	w_t
1	21	23	24	26	23.5	2.8	0.089
2	38	38	42	40	39.5	1.91	0.048
3	27	29	34	30	30.0	2.94	0.098
4	16	22	19	21	19.5	2.65	0.136
5	33	25	33	38	32.25	5.38	0.167
6	67	65	74	66	68.0	4.08	0.060
7	160	166	176	174	169.0	7.39	0.044
8	89	81	94	92	89.0	5.72	0.064

s = standard deviation
w_t = relative standard deviation (s/Mean)

The sum of squared w_t values equals 0.0758, and the mean 0.0095. It is the sought estimate of the average relative variance of reading in the laboratory as a whole, without regard to any person in particular.

The estimate applies, of course, only to similar situations as in the experiment, which was determination of the Standard Plate Count (SPC) of unpasteurised raw milk samples.

9.5 Personal yield coefficient and its uncertainty

9.5.1 An infallible expert as reference

Let person A (from the table of results presented in Example 9.4) be the chosen expert and person C be the test person. Calculate for each plate in turn the relative difference $d = 2(A-C)/A$

Plate	A	C	A-C	*d*
1	21	24	-3	-0.143
2	38	42	-4	-0.105
3	27	34	-7	-0.259
4	16	19	-3	-0.188
5	33	33	0	0.000
6	67	74	-7	-0.104
7	160	176	-16	-0.100
8	89	94	-5	-0.056
Sum	451	496	Mean	-0.119

$s_d = 0.079$ Variance: $(0.079)^2 = 0.0062$

On the basis of the sums of counts the personal yield coefficient of person C is $K_H = 451 / 496 = 0.91$. Her/his result should be multiplied by 0.91 to correct them to the average level that corresponds to the style of counting of the expert.

To calculate the RSD squared of the correction factor, the standard deviation of the mean is first computed by dividing the standard deviation by the square root of the number of plates: $0.079 / \sqrt{8} = 0.0279$. Dividing it by the correction factor: $w_{KH} = 0.0279 / 0.91 = 0.037$ gives the relative standard deviation of the mean, and the square of this value $(0.037)^2 = 0.00137$ is the RDS squared that is needed in further calculations.

9.5.2 The mean value as reference

Results of person C and the mean (m) of all participants were extracted from the table of results in Example 9.4. The relative differences are obtained from $d = (m - C)/m$

Plate	*C*	*m*	*d*	
1	24	23.5	-0.021	
2	42	39.5	-0.063	
3	34	30.0	-0.133	
4	19	19.5	0.026	
5	33	32.25	-0.023	
6	74	68.0	-0.088	
7	176	169.0	-0.041	
8	94	89.0	-0.056	
Mean	62.0	58.8	-0.050	$s_d = 0.0478$

The value of the correction factor $K_H = 58.8 / 62.0 = 0.95$
Standard deviation of mean d $0.0478 / \sqrt{8} = 0.0169$
The relative uncertainty of the correction factor $w_{KH} = 0.0169 / 0.95 = 0.018$
RSD squared: $(0.018)^2 = 0.000324$

9.6 Uncertainty of the combined volume of test portions

9.6.1 Without additional dilution

Assume a detection instrument consisting of four countable plates (Fig. 5). Two plates have been inoculated with 1 ml and the other two with 0.1 ml of the final suspension.

Assume it known from calibration experiments that the relative standard uncertainty of the 1 ml inocula is $w = 2$ % and that of the 0.1 ml measurements equal to $w = 8$ %

In order to calculate the uncertainty of the test result (the weighted average) we need to estimate the relative uncertainty of the total volume $V = 2$ x 1 ml + 2 x 0.1 ml = 2.2 ml

Because the total volume is a sum the given relative uncertainty estimates must first be converted to millilitres. 2 % of 1 ml is $u = 0.02$ ml and 8 % 0.1 ml is $u = 0.008$ ml. The standard uncertainty of the sum of volumes $V = 1 + 1 + 0.1 + 0.1 = 2.2$ ml is obtained from

$$u_v = \sqrt{0.02^2 + 0.02^2 + 0.008^2 + 0.008} = \sqrt{0.000928} = 0.0305\ ml \qquad (79)$$

The relative uncertainty of the total volume is accordingly $w_V = 0.0305$ ml/2.2 ml = 0.014 (1.4 %). It is smaller than the relative standard deviation of any of the component volumes. The square, i.e. the RSD squared $0.014^2 = 0.000196$, is the value needed in further calculations.

9.6.2 With additional dilution

Assume a detection instrument consisting of four plates such that two plates were inoculated with 1 ml of the final suspension and two plates with 1 ml of its further dilution 1:10 (1 ml + 9 ml). See Fig. 5.

Assume it known from calibration experiments that the 1 ml measurements have a relative uncertainty of 2 % and the 9 ml dilution blank volumes the relative uncertainty of 1 %.

The estimate of the relative variance of the dilution factor $f = (1 + 9) / 1 = 10$ is obtained directly from formula (42). For that calculation we need

a = 1 ml, relative standard uncertainty w_a = 0.02 (2 %)
b = 9 ml, standard uncertainty u_b = 0.09 ml (1 %)

$$w_f^2 = \frac{\left(u_b^2 + b^2 w_a^2\right)}{\left(a + b\right)^2} = \frac{\left(0.09^2 + 9^2 \cdot 0.02^2\right)}{\left(1 + 9\right)^2} = \frac{0.0081 + 81 \cdot 0.0004}{100} = \frac{0.0402}{100} = 0.0004 \qquad (80)$$

It is worth noticing that the relative uncertainty of the dilution factor, the square root of 0.0004, in this case is the same as the relative uncertainty of the 1 ml measurement (0.02). This is a typical result with dilution steps 1:10 and higher. The uncertainty of the dilution blank contributes almost nothing to the combined uncertainty of the dilution factor.

The total volume of test portions, in terms of the final suspension, is V = 2 x 1 ml + 2 x 1 ml / f = 2 ml + 2 ml / 10 = 2.2 ml. Note that it involves the dilution factor. Its variance is
$u_v^2 = 2 \cdot u_{1ml}^2 + 0.1^2\left(2 \cdot w_{1ml}^2 + w_f^2\right) = 2 \cdot 0.02^2 + 0.1^2\left(2 \cdot 0.02^2 + 0.0004\right) = 0.000812$

Its square root is 0.0285 ml. Related to the total volume, this means a combined relative uncertainty of w_V = 0.0285 ml / 2.2 ml = 0.013 (1.3 %).

It is a rather complicated calculation and requires great care. In practice, the part of uncertainty that stems from the second dilution is insignificant, unless the dilution factor f is considerably less than 10. It would not have made a significant difference if the combined uncertainty of the total volume had been calculated as in example 9.6.1, ignoring the dilution coefficient entirely.

The combined uncertainty of volume V could have been calculated as in 9.6.1 with the difference that all test portions would have had the same relative uncertainty 2 %. This would mean the standard uncertainty 0.02 of 1 ml and 0.002 of 0.1 ml. That would lead to the standard uncertainty of the total volume $u_v = \sqrt{2 \cdot 0.02^2 + 2 \cdot 0.002^2} = 0.0284\ ml$. The relative uncertainty is the same as above w_V = 0.0284 ml / 2.2 ml = 0.013 (1.3 %).

NOTE 1. Ignoring the dilution uncertainty only makes a difference to the fourth decimal.

NOTE 2. The inaccuracy would not have been marked even if only the test portions of the first dilution had been considered. The total relative uncertainty would have been based on the two 1 ml test portions measured directly from the final suspension.

$$u_V = \sqrt{2 \cdot 0.02^2} = 0.0283 \text{ ml} \quad (81)$$

The relative uncertainty is w_V = 0.0283 ml / 2.2 ml = 0.013 (1.3 %).

9.7 Several plates. Sum of confirmed counts and its uncertainty.

Presumptive colony counts from two dilutions were

Dilution	Counts
10^{-3}	66 and 80 colonies
10^{-4}	7 and 4 colonies

Results of confirming selected colonies, and the confirmed counts for each plate, calculated from the observed values, were as shown in the table below

Plate number	Presumptive count, z	Colonies tested, n	Colonies confirmed, k	Confirmed count, x
1	66	8	6	49.5
2	80	9	6	53.3
3	7	5	4	5.6
4	4	4	4	4.0

There are three ways of calculating the sum and uncertainty of the sum of confirmed counts. The choice depends on an intuitive impression of the behavior of the microbiological population on the plates.

i) The most cautious approach is to consider each plate a unique confirmation situation, like in the table above. The confirmed sum is X = 49.5 + 53.3 + 5.6 + 4.0 = 112.4

The uncertainty (variance) of the sum is the sum of the variances of the individual confirmed counts.

Variance of x_1 is (formula 30)

$$u_{x_1}^2 = \frac{z_1^2 k_1 (n_1 - k_1) + n_1 k_1^2 z_1}{n_1^3} = \frac{66^2 \cdot 6 \cdot (8-6) + 8 \cdot 6^2 \cdot 66}{8^3} = 139.2188 \quad (82)$$

Similarly for x_2, x_3, and x_4 the variances are:

$$u_{x_2}^2 = \frac{80^2 \cdot 6 \cdot (9-6) + 9 \cdot 6^2 \cdot 80}{9^3} = 193.5802 \quad (83)$$

$$u_{x_3}^2 = \frac{7^2 \cdot 4 \cdot (5-4) + 5 \cdot 4^2 \cdot 7}{5^3} = 6.0480 \quad (84)$$

$$u_{x_4}^2 = \frac{4^2 \cdot 4 \cdot (4-4) + 4 \cdot 4^2 \cdot 4}{4^3} = 4.0000 \qquad (85)$$

Their sum is the variance of X: $u_X^2 = 342.8470$

The standard uncertainty $u_X = 18.5161$ and the relative standard uncertainty $w_X = 18.5161 / 112.4 = 0.165$ (16.5 %).

ii) In the second approach it is assumed that each dilution may have a different confirmation rate but the confirmation rates of the parallel plates are the same.

The confirmed counts are based on the sums of each dilution:

Dilution	Presumptive Count	Colonies Tested	Colonies Confirmed	Confirmed Count
10^{-3}	146	17	12	103.1
10^{-4}	11	9	8	9.8

The confirmed sum X = 103.1 + 9.8 = 112.9

The variance of the sum of confirmed counts consists of the variances of the sums of counts of the two dilutions.

Dilution 10^{-3}: $u_1^2 = \frac{146^2 \cdot 12 \cdot (17-12) + 17 \cdot 12^2 \cdot 146}{17^3} = 333.0690$

Dilution 10^{-4}: $u_2^2 = \frac{11^2 \cdot 8 \cdot (9-8) + 9 \cdot 8^2 \cdot 11}{9^3} = 10.0192$

The variance of X: $u_X^2 = 333.0690 + 10.0192 = 343.0882$

The standard uncertainty $u_X = 18.5226$ and the relative standard uncertainty $w_X = 18.5226 / 112.9 = 0.164$ (16.4 %).

iii) When the confirmation rate is considered sample-specific, i.e. the same in all the plates of a sample, the calculations are based on the grand totals of counts.

Total presumptive count $Z = 66 + 80 + 7 + 4 = 157$
Total number of colonies tested N = 8 + 9 + 5 + 4 = 26
Total number of colonies confirmed K = 6 + 6 + 4 + 4 = 20

The confirmed sum of counts $X = 157(20/26) = 120.8$

The variance of X: $u_X^2 = \frac{157^2 \cdot 20 \cdot (26-20) + 26 \cdot 20^2 \cdot 157}{26^3} = 261.1903$

The standard uncertainty $u_X = 16.1614$ and the relative standard uncertainty $w_X = 16.1614 / 120.8 = 0.1338$ (13.8 %).

The relative standard uncertainty could have been also obtained directly from

$$w_X = \sqrt{\frac{1}{Z} + \frac{1}{K} - \frac{1}{N}} = \sqrt{\frac{1}{157} + \frac{1}{20} - \frac{1}{26}} = 0.1338 \qquad (86)$$

Comparison of the three alternatives:

Type of confirmation	**Sum of confirmed counts**	**Variance of the sum**	**Relative standard uncertainty**
Plate-specific	112.4	342.09	0.165
Dilution-specific	112.9	343.09	0.164
Sample-specific	120.8	261.19	0.134

10 WORKED EXAMPLES II. COMPUTING THE COMBINED UNCERTAINTY OF TEST RESULTS.

10.1 Single plate, undiluted sample

Assume that results on the microbial content of liquid samples (water, urine, milk, etc.) are determined by seeding a single plate using a calibrated loop with a volume of 1 µl.

By its own measurements the laboratory has ascertained that the average volume delivered does not differ from the nominal value (v = 1 µl). Its volumetric relative uncertainty (relative standard deviation) has been found to be about 12 % (w_v = 0.12).

Assume the number of colonies was z = 75.

The formula for computing the test result is $y = z/v$. To express the result per the standard volume 1 ml, the test portion volume should be expressed in millilitres: v = 0.001 ml. The test result y = 75 / 0.001 ml = 75000 ml^{-1}.

The combined relative uncertainty according to clause 3.1.4 is:

$$w_y = \sqrt{w_v^2 + w_z^2} = \sqrt{0.12^2 + \frac{1}{75}} = \sqrt{0.0144 + 0.0277} = 0.17 \qquad (87)$$

Expressed in the units of the test result u_y = 75000·0.17 = 12750 ml^{-1}

Reporting the test result together with uncertainty:
y = 75000 ml^{-1} with combined standard uncertainty u_y = 13000 ml^{-1} estimated on the basis of the Poisson scatter attached to a mean value of 75 colonies and the volumetric uncertainty (12 %).

10.2 Single plate, diluted sample

Assume a sample diluted to 10^{-4} , with the only plate found to contain z = 125 colonies after incubation.

Assume further that the dilution series was prepared in four steps of 1:10 with every step consisting of the volumes 0.5 ml + 4.5 ml. The plate was inoculated with a 1 ml test portion of the final suspension (10^{-4}). The laboratory did not have experimental data of its own on the average accuracy and precision of the volume measurements.

The 1 ml and 0.5 ml volumes were measured using 1 ml graduated glass pipettes and the 4.5 ml dilution blanks were measured with an automatic dispenser after sterilising the dilution solution in bulk.

With accurate data missing it was assumed that there were no systematic errors in the average volume measurements v = 1 ml, a = 0.5 ml and b = 4.5 ml. Accordingly, the nominal dilution factor $F = 10^4$ was also assumed true.

Table A1 in Annex A provides the following information:
4.5 ml dispenser volume (b) standard uncertainty (u_b = 0.024 ml).
1 ml graduated glass pipette (v) standard uncertainty u_v = 0.025 ml.

No direct information on the uncertainty of the 0.5 ml volume (a) is available, but 0.5 ml was measured using the graduated 1 ml pipettes. It can be assumed that the precision of filling and emptying the pipette would be the same in both cases. The uncertainty (in millilitres) would be the same for 0.5 ml as for 1 ml.

A summary of the relevant volumetric information
a = 0.5 ml, Δa = 0.0 ml, u_a = 0.025 ml, w_a = 0.05 (5 %)
b = 4.5 ml, Δb = 0.0 ml, u_b = 0.024 ml, w_b = 0.005 (0.5 %)
v = 1.0 ml, Δv = 0.0 ml, u_v = 0.025 ml, w_v = 0.025 (2.5 %)

The RSD squared of one dilution step f calculated according to formula (42)

$$w_f^2 = \frac{u_b^2 + b^2 w_a^2}{(a+b)^2} = \frac{0.024^2 + 4.5^2 \cdot 0.05^2}{(0.5+4.5)^2} = \frac{0.0512}{25} = 0.002048 \qquad (88)$$

The RSD squared of the total dilution factor $F = f^4$ is, according to 5.7.1
$w_F^2 = k \cdot w_f^2 = 4 \cdot 0.002048 = 0.0082$

The combined relative uncertainty of the test result according to clauses 5.7.1, 5.4 and 5.2 is calculated as

$$w_y = \sqrt{w_F^2 + w_v^2 + \frac{1}{z}} = \sqrt{0.0082 + 0.025^2 + \frac{1}{125}} = \sqrt{0.0168} = 0.1297 \quad (13\%) \qquad (89)$$

The test result $y = 125 \cdot 10^4 = 1.25 \cdot 10^6$ ml^{-1}. Its uncertainty in the same units of measurement is $u_y = (0.1297 \cdot 1.25)10^6 = 0.16 \cdot 10^6$ ml^{-1}
The uncertainty could equally well be given as 13 %.

10.3 Diluted sample, several plates.

A series of counts was obtained from dilutions 10^{-5} and 10^{-6} with three parallel plates in each. The results are shown below. The dilution series was prepared as successive 1:10 steps (1 ml + 9 ml). Because the first countable plates were found in dilution 10^{-5} it becomes the "final suspension".

Dilution	v_i a)	z_i b)	Sum
10^{-5}	1	122	
	1	74	
	1	92	288
10^{-6}	0.1	12	
	0.1	15	
	0.1	10	37
Total	3.3		325

a) test portion volume expressed in ml of final suspension (10^{-5})
b) colony count

The nominal dilution factor of the final suspension was $F = 10^5$, the total test portion volume $V = 3.3$ ml and the total number of colonies observed $Z = 325$. The test result according to the principle of weighted mean is $y = 10^5 \times (325 / 3.3) = 98 \times 10^5$ ml^{-1}

In order to compute the combined relative uncertainty of the test result, at least three components must be estimated:

1) the Poisson scatter w_Z of the total count Z
2) the uncertainty w_V of the total volume V
3) the uncertainty w_F of the dilution factor F

In order to compute the combined uncertainty it is most convenient to express the components of uncertainty as RSD squared

According to clause 5.3 $w_Z^2 = \frac{1}{Z} = \frac{1}{325} = 0.003077 \qquad (w_Z = 0.0555)$

When the laboratory has no calibration results of its own the uncertainties quoted in Annex A may be used. The standard uncertainty of 1 ml graduated glass pipettes is given as $u_a = 0.025$ ml.
Thus, the variance of the sum of volumes $V = 1 + 1 + 1 + 0.1 + 0.1 + 0{,}1 = 3.3$ ml

$$u_V^2 = 3 \cdot 0.025^2 + 3 \cdot 0.0025^2 = 0.001894 \qquad (90)$$

The RSD squared is $w_V^2 = 0.001894 / 3.3^2 = 0.000174$ ($w_V = 0.0132$)

Even though a dispensable intermediate result, the squared relative uncertainty of the final suspension density ($c = Z / V$) can be calculated as $w_c^2 = 0.003077 + 0.001894 = 0.004971$

To estimate the uncertainty of the final dilution factor the relative uncertainty (variance) of one dilution step is calculated first and then multiplied by the number of steps (five). The uncertainty estimate of 9 ml dilution blanks can be obtained from Annex A as 0.024 ml.

Estimating the uncertainty of one dilution step according to clause 5.7.1 yields

$$w_f^2 = \frac{u_b^2 + b^2 w_a^2}{(a+b)^2} = \frac{0.024^2 + 9^2 \cdot 0.025^2}{(1+9)^2} = 0.000512 \qquad (91)$$

The RSD squred of the total dilution factor is five times the above

$$w_F^2 = 5 \cdot 0.000512 = 0.002560 \qquad (92)$$

Finally, the total uncertainty of reading was added to the estimated components. Each of the six colony counts was assumed to have been read with the relative uncertainty determined in example 9.3, i.e. with the relative variance $w_t^2 = 0.0023$. The relative variance of the sum, according to 5.1.2 is

$$w_T^2 = 0.0023 \cdot \frac{122^2 + 74^2 + 92^2 + 12^2 + 15^2 + 10^2}{325^2} = 0.000638 \qquad (93)$$

The relative uncertainty of the test result is the vector sum of the relative uncertainty components

$$w_y = \sqrt{w_Z^2 + w_V^2 + w_F^2 + w_T^2} =$$
$$\sqrt{0.003077 + 0.000172 + 0.00256 + 0.000638} = 0.0803 \qquad (94)$$

The test result is $y = 10^5(325 / 3.3) = 9.8 \cdot 10^6$ with 8.0 % relative uncertainty.

10.4 Several plates. The short-cut solution.

The same data as in example 10.3. The uncertainty of the test result estimated according to the short-cut procedure (clause 7).

Dilution	v_i [a)]	z_i [b)]	**Sum**	$z_i \ln(z_i/v_i)$
10^{-5}	1	122		586.0906
	1	74 *)		318.5008
	1	92	288	416.0045
	0.1	12		57.4499
	0.1	15		75.1595
	0.1	10	37	46.0517
Total	3.3		325	1499.2570

a) test portion volume in ml of final dilution (10^{-5})
b) colony count
*) The person reading the counts considered this result possibly suspicious due to excessive overgrowth

It would be permissible to exclude the suspicious count (74) on technical grounds. It is, however, of some interest to explore what effect, if any, retaining or deleting this one number has on the final outcome.

To estimate the combined uncertainty, the first step is to calculate the log-likelihood ratio estimate for the detection instrument (i.e. the set of plates):

$$G^2_5 = 2[586.0906 + ... + 46.0517 - 325\ln(325 / 3.3)] =$$
$$= 2(1499.2570 - 1491.7184) = 2 \cdot 7.5386 = \mathbf{15.0772} \qquad (95)$$

Dividing G^2 with its degrees of freedom yields $G^2 / (n\text{-}1) = 15.0772 / 5 = 3.0154$
The value is considerably higher than one. A significant amount of other variation than Poisson-type scatter of particles seems to be involved.

The relative standard uncertainty (squared) of the microbial content of the final suspension is, according to Clause 7

$$w_c^2 = \frac{G^2_{(n-1)}}{(n-1)} \cdot \frac{1}{\sum z_i} = 3.0154 \cdot \frac{1}{325} = 0.009278 \qquad (96)$$

The only missing component is the uncertainty of the final dilution factor F. It was already computed in example 10.3. The RSD squared was estimated as $w_F^2 = 0.002560$.

The relative standard uncertainty of the test result according to the short-cut calculations is

$$w_y = \sqrt{w_c^2 + w_F^2} = \sqrt{0.009278 + 0.002560} = 0.109 \ (10.9\ \%) \qquad (97)$$

The value is distinctly higher than the estimate (8.0 %) found in example 10.3. There are at least two reasons. In example 10.3 the value of the uncertainty of reading may have been too low. The true but unknown uncertainty of reading is included in the G^2. Secondly, the particle distribution was naively believed to follow the Poisson distribution. In the real data, at least the technically suspicious result, which may have been an 'accident', increases the scatter.

10.4.1 Calculation with the suspicious observation excluded

Excluding the suspicious colony count (74) has an effect on the test result and its uncertainty estimate.
The sum of counts is decreased to $V = 325 - 74 = 251$
The total test portion volume is decreased to 3.3 ml – 1 ml = 2.3 ml
Degrees of freedom (n-1) are decreased to the value 4
318.5008 is subtracted from the sum of $z_i \ln(z_i / v_i)$. The new sum becomes:
1499.2570 - 318.5008 = 1180.7562

The new likelihood ratio index has the value $G_4^2 = 2[1180.7562 - 251\ln(251 / 2.3)] = 2(1180.7562 - 1177.8285) = 2 \cdot 2.9277 =$ **5.8554**

The index divided by the degrees of freedom becomes considerably lower: 5.8554 / 4 = 1.4639. Despite the lower total count the uncertainty of the microbial density estimate of the final suspension is decreased: $w_c^2 = \dfrac{1.4369}{251} = 0.005832$ ($w_c^2 = 0.009278$ in 10.4 above)

The weighted mean is slightly increased $x = 251 / 2.3 = 109$ so that the test result becomes $y = 10.9 \times 10^6$ ml^{-1}.

The effect on the combined uncertainty is quite marked

$$w_y = \sqrt{w_c^2 + w_F^2} = \sqrt{0.005832 + 0.002560} = 0.0916 \left(9.2\ \%\right) \qquad (98)$$

The new test result is $y = 1.1 \times 10^7$ ml^{-1} with the combined standard uncertainty $u_y = 0.1 \times 10^7$ ml^{-1} (9.2 %)

10.5 Several plates. "Fully corrected" test result and its uncertainty.

In example 10.3 the given information lead into the results:
$F = 10^5$, $x = 98$ ml^{-1} test result $y = 9.8 \cdot 10^6$ ml^{-1}, relative uncertainty 8.0 %

Supposing an attempt were made to include corrections for all systematic effects and their uncertainties. The following correction factors will be taken into account:

the actual dilution factor F'
personal yield coefficient K_H
stability of the sample K_S
yield coefficient of the nutrient medium K_A
matrix effect K_M

The actual dilution factor F'

The sample was diluted in five steps, 1 + 9 ml each. According to Table A1 the real volume of the dilution blanks should have been $(b + \Delta b) = 9.0 - 0.3 = 8.7$ ml and the real 1 ml volume $(a + \Delta a) = 1 - 0.01 = 0.99$ ml.
The actual dilution factor $F' = ((8.7 + 0.99) / 0.99)^5 = 9.7879^5 = 89835 = 0.898 \cdot 10^5$; its uncertainty is the same as with the nominal dilution factor but the relative uncertainty is different. According to example 10.3 $w_F^2 = 0.002560$. With the actual dilution factor taken into account the relative uncertainty becomes

$$w_{F'}^2 = \left(\frac{F}{F'}\right)^2 \cdot 0.002560 = \left(\frac{1.0}{0.898}\right)^2 \cdot 0.002560 = 0.003175 \quad (w_{F'} = 0.0563) \qquad (99)$$

Personal yield coefficient K_H

Assume that the person in question has been found to count 5 % fewer colonies than the rest of the technical personnel on the average. Assume that the uncertainty of this relative difference had been estimated as 7 percent units. Thus:
$K_H = 1 + 0.05 = 1.05$, $w_{KH} = 0.07$

Sample stability coefficient K_S

Assume that the microbial content of the sample might have changed during storage by 20 % at the most, but not even the direction of the change is predictable. Any change between –20 %...+20 % is considered equally plausible. The *a priori* 'probability distribution' is a rectangular distribution. Because of the symmetrical situation the average value of the stability coefficient can only be guessed as $K_S = 1$. Its uncertainty is estimated according to (2.4.1) and is in relative units because a was given in percent $w_{KS} = 0.20 / \sqrt{3} = 0.12$

Yield coefficient of the nutrient medium K_A

The batch of medium was tested after preparation by culturing six certified reference samples. The expected count of the reference samples, according to the certificate, had a mean value 56 with 95 % of the counts expected to fall within the range 48...66. The relative standard uncertainty estimated according to (6.3.2): (ln66-ln48) / 4 = 0.0796. According to the certificate, estimation of the mean and uncertainty was based on the study of 356

samples. The relative uncertainty of the mean can therefore be estimated as $w = 0.0796 / \sqrt{356} = 0.0042$

The test of the medium with six reference samples gave a mean of 51. Its relative uncertainty according to (6.3.2) is $(\ln 66 - \ln 48) / 4\sqrt{6} = 0.033$

The relative variance of the yield coefficient is ($w^2_{KA} = 0.0042^2 + 0.033^2$) = 0.0011 ; w_{KA} = 0.033.

The factor that should be used for correcting the yield is $K_A = 56 / 52 = 1.10$ (8.7.1). There is no conflict with 'metrological ethics' because the coefficient evidently is statistically significant.

Matrix correction factor K_M

Assume that the sample material had been found to have an intrinsic matrix heterogeneity that can be characterized by the added relative uncertainty $w_M = 0.17$.

Whether the matrix has an effect on the estimate of the microbial density is assumed unknown. There is no alternative but to select $K_M = 1$ as the value of the correction factor.

Summary

Density of the final suspension $x = 98$ ml^{-1} (example 10.3)
Uncertainty of the total number $w_Z = 0.0555$ (example 10.3)
Uncertainty of the total test portion volume $w_V = 0.013$ (example 10.3)
Uncertainty of reading $w_T = 0.0253$ (example 10.3)

$F' = 0.898 \cdot 10^5$, $w_{F'} = 0.0563$
$K_H = 1.05$, $w_{KH} = 0.07$
$K_S = 1.0$, $w_{KS} = 0.12$
$K_A = 1.10$, $w_{KA} = 0.033$
$K_M = 1.0$, $w_{KM} = 0.17$

The "fully corrected" test result: $y = 1.05 \cdot 1 \cdot 1.1 \cdot 1 \cdot 0.898 \cdot 10^5 \cdot 98 = 1.02 \cdot 10^7$

The relative uncertainty of the corrected result

$$w_y = \sqrt{0.0563^2 + 0.07^2 + 0.12^2 + 0.033^2 + 0.17^2 + 0.555^2 + 0.013^2 + 0.0253^2} = 0.237 \quad (100)$$

The uncertainty is quite high compared to the uncorrected results presented before: 0.080 (example 10.3) and 0.109 (example 10.4).

10.6 MPN, single dilution

Assume a single-dilution MPN design with n = 15 tubes with a test portion volume v = 5 ml of undiluted water sample in each tube.

Assume that after incubation ten positive tubes were observed (s = 5 tubes remained sterile). The task is to estimate the microbial content of the water sample, and the uncertainty of the test result.

Formula (5.3.1) for the bacterial content of the final suspension, which in this case is the same as the sample,

$$x = \frac{1}{5}\ln\left(\frac{15}{5}\right) = 0.2\ x\ 1.10 = 0.22 ml^{-1} \qquad (101)$$

The same result is found by the computer program (Hurley and Roscoe 1983) or by consulting appropriate tables (Niemelä 1983, ISO CD 8199).

There are three alternative starting points for estimating the uncertainty: the $\log_{10}$ standard deviation given by a computer program, the 95 % confidence interval from computer programs or tables, or independent calculation based on the basic observed and design values (s and n).

i) The computer programs give the value 0.14435 for the $\log_{10}$ standard deviation. Multiplying it with the modulus between the base e and base 10 logarithms yields the first estimate of the relative standard uncertainty $w_x = 2.3 \cdot 0.14435 = 0.332$

ii) The 95 % confidence interval of the MPN estimate from two different sources:

Upper x_Y	Lower x_A	Source
0.426	0.096	Niemelä 1983
0.422	0.114	Hurley and Roscoe 1983

Applying formula (5.3.3) gives, due to the different confidence limits, the estimates of relative uncertainty w_x = 0.373 in the first case and w_x = 0.327 in the second. The two estimates based on the computer program, 0.332 and 0.327, are essentially the same.

iii) Starting with the basic observations the 'one-sigma' (68 %) confidence limits are first obtained (formula 57):

$$x_U = \frac{1}{5}\ln\left(\frac{15}{5 - \sqrt{\frac{5 \cdot 10}{15}}}\right) = 0.3106 \qquad (102)$$

Similarly, at the lower limit x_L = 0.1575

The estimate of relative uncertainty according to (6.3.2) is (ln 0.3106-ln 0.1575) / 2 = 0.340

Thus, the test result is y = 0.22 ml^{-1} and there is a choice between three estimates of its relative standard uncertainty: w = 0.33, 0.37 or 0.34.

10.7 MPN, several dilutions

Assume a sample first diluted to 10^{-6} . Five 1 ml aliquots each from the final suspension and from its further 1:10 and 1:100 dilutions were cultured.

Assume the numbers of positive tubes in the series of three dilutions was found to be 5-2-0. The task is to estimate the bacterial content of the original sample and the relative uncertainty of this test result.

Different sources yield different estimates for the MPN of the bacterial density (x) and the 95 % confidence limits (x_U, x_L) of the final suspension

x	x_U	x_L	Source
4.9	12.6	1.7	Swaroop (1951)
4.9	15.2	1.6	Hurley ja Roscoe (1983)
5	17	2	deMan (1975)

The confidence limits as such constitute an appropriate expression of the expanded uncertainty of the MPN of the final suspension.

If the uncertainty of the MPN value should be combined with other components, for instance the uncertainty of the dilution factor, the relative uncertainty is also needed. Estimating the relative uncertainty as given in clause 6.3.2 the different confidence limits quoted in the table give the values: w_x = 0.50, 0.56, 0.54.

Computing independently from the basic design parameters (n, f) the approximate solution by Cochran (1950) would yield an estimate of $\log_{10}$ standard deviation 0.259 (6.4). Conversion to ln scale gives the relative standard deviation $w_c = 2.303 \cdot 0.259 = 0.60$.

The additional relative uncertainty due to the dilution factor $F = 10^6$ might at the most be of the order of w_F = 0.10 (see Annex A, Table A2). It adds barely noticeably to the combined uncertainty:

$$w_y = \sqrt{w_{MPN}^2 + w_F^2} = \sqrt{0.60^2 + 0.10^2} = 0.61 \qquad (103)$$

11 BIBLIOGRAPHY

Anon. 1994. IDF Provisional International Standard 169:1994. *Quality Control in the microbiological Laboratory. Analyst performance assessment for colony count.* International Dairy Federation, Brussels.

Anon. 1995. *Guide to the expression of uncertainty in measurement.*
First Edition, corrected and reprinted. International Organization for Standardization, Geneve.

Anon. 2000. EURACHEM/CITAC Guide. *Quantifying Uncertainty in Analytical Measurement*, second ed. Editors: Ellison, S.L.R., M. Mosslein and A.Williams, London.

Cochran, W.G. 1950. Estimation of bacterial densities by means of the "Most Probable Number". Biometrics, **6:**105-116.

Farmiloe, F.J., S.J. Cornford, J.P.M. Coppock and M. Ingram. 1954. The survival of *Bacillus subtilis* spores in the baking of bread. J.Sci.Food Agric. **5:** 292-304.

Halvorson, H.O. and N.R. Ziegler, 1933. Application of statistics to problems in bacteriology I. A means of determining bacterial population by the dilution method. J. Bact., **25:**101-121.

Hurley, M.A. and M.E. Roscoe, 1983. Automated statistical analysis of microbial enumeration by dilution series. J.Appl.Bacteriol., **55:**159-164.

ISO/CD 8199:2002 (revised draft) – Water quality- General guide to the enumeration of micro-organisms by culture.

Jarvis, B. 1989. *Statistical Aspects of the Microbiological Analysis of Foods*. Progress in Industrial Microbiology, vol. 21. 179 pp. Elsevier, Amsterdam.

de Man, J.C. 1975. The probability of most probable number. European J.Appl.Microbiol. **1:**67-78.

Myrberg, P.J. 1952. *Differentiaali- ja integraalilaskennan oppikirja*. Tiedekirjasto N:o 23, Kustannus Oy Otava, Helsinki

Niemelä, S. 1965. The quantitative estimation of bacterial colonies on membrane filters. Ann.Acad.Sci.Fenn., Ser.A. IV. Biologica. No. 90.

Niemelä, S. 1983. Statistical evaluation of results from quantitative microbiological examinations. NMKL Report no. 1, second edition. Nordic Committee on Food Analysis. Ord & Form AB, Uppsala.

Niemelä, S. 1998. *Mikrobiologian biometria.* Luentomoniste, Helsingin yliopiston soveltavan kemian ja mikrobiologian laitos. Yliopistopaino, Helsinki.

Untermann, F. 1970. Varianzanalytische Untersuchungen über die Fehlergrösse der „drop-plating“ –Technik bei kulturellen Keimzahlbestimmungen an Lebensmitteln. Zbl. Bakteriol. 1. Abt., Orig., **215:** 563-571.

Swaroop, S. 1951. The range of variation of the most probable number of organisms estimated by the dilution method. Indian J. med. Res. **39:**107-134.

ANNEX A. Observed and computed values of components of uncertainty

Table A1. *Examples of empirical volumetric uncertainties based on Type A estimation (Niemelä 1998). v = notional volume.*

Device	***v***	**Δ*v*(ml)**	***u*(ml)**	***w****
Semi-automatic pipette	0.1		0.0051	0.05
Semi-automatic pipette	1.0		0.0087	0.009
Graduated 1 ml (glass)	0.1		0.0081	0.08
Graduated 1ml (glass)	1.0	-0.01	0.025	0.025
Graduated 5 ml (glass)	5.0		0.027	0.005
Dispensing pump*	4.5	-0.02	0.024	0.005
Dispensing pump*	9.0	-0.3	0.024	0.003
Dispensing pump**	9.0	-0.6	0.089	0.01
Dispensing pump**	99.0	-3.5	0.249	0.0025

* sterilised in bulk before dispensing
** sterilised after dispensing
*** relative standard uncertainty

Table A2. *The relative uncertainty of the dilution factor (F) under different assumptions of the uncertainty of the pipetted (1 ml) volume (1 %, 2 %, 3 %, 4 %). The uncertainty of the diluent volume is assumed the same (1 %) in all cases. Estimated according to clause 5.7.*1

Dilution	**Relative uncertainty of 1 ml pipettes**			
factor *F*	**1 %**	**2 %**	**3 %**	**4 %**
10^1	0.014	0.022	0.032	0.041
10^2	0.020	0.032	0.045	0.058
10^3	0.024	0.039	0.055	0.071
10^4	0.028	0.045	0.063	0.082
10^5	0.032	0.050	0.071	0.092
10^6	0.035	0.055	0.077	0.100

ANNEX B. Examples of estimated relative standard uncertainty of the bacterial content of materials and natural sites.

The values given include three uncertainty components: non-homogeneity of the matrix, uncertainty of reading and uncertainty of the inoculum volume (w_M, w_t, w_v)

Site or material	*RSU**	Reference
River Kymi at Myllykoski, within 15 min		1
Endo- ja SB-colony count	0.10	
River Vantaa, 5.0 km lower reach, Working hours, week-days		2
mFC colony count	0.24**	
KF colony count	0.25**	
River Keravanjoki, 4.4 km middle reach Working hours, week-days		3
mFC colony count	0.26**	
KF colony count	0.27**	
Sterilised milk spiked with a mixture of pure cultures		3
Standard Plate Count, dilution 1:10	0.06	
Petrifilm Total Count, dilution	0.00	

* *RSU* relative standard uncertainty

** Estimated using the regression procedure described in example C7 of ISO/TR 13843: Water quality – Guidance on validation of microbiological methods.

References:

1. Niemelä, S.I. & E.K. Tirronen. 1968. Suolistobakteerimääritysten luotettavuus. Vesi **1:**5 - 16.

2. Niemi, R.M. & J.S. Niemi. 1990. Monitoring of fecal indicators in rivers on the basis of random sampling and percentiles. Water, Air, and Soil Pollution **50:**331 - 342.

3. Ginn, R.E., V.S. Packard & T.L. Fox. 1986. Enumeration of total bacteria and coliforms in milk by dry rehydratable film methods: Collaborative study. J. Assoc. Off. Anal. Chem. **69:**527 - 531.

ANNEX C. A BASIC computer program for computing the value of the log-likelihood ratio index G^2 (Anon. 1994).

```
10   PRINT "LIKELIHOOD RATIO INDEX G^2"
20   INPUT "NUMBER OF TERMS, n=";N
30   C=0: V=0: S=0: T=0: D=0
40   FOR I=1 TO N
50   PRINT "I=";I
60   INPUT "COLONY COUNT=";C
70   INPUT "VOLUME=";V
80   IF (C=0) THEN W=0: GOTO 100
90   W=C*LOG(C/V)
91   REM IN THIS BASIC LANGUAGE VERSION LOG=NATURAL LOGARITHM
100  S=S+W
110  T=T+V
120  D=D+C
130  NEXT I
140  Y=2*(S-D*LOG(D/T))
141  REM IN THIS BASIC LANGUAGE VERSION LOG=NATURAL LOGARITHM
150  Y=(INT(1000*Y+0.5))/1000
160  PRINT
170  PRINT "INDEX G^2=";Y
180  PRINT
190  PRINT
200  INPUT "ANOTHER SET? (Y/N)";H$
210  IF H$="Y" OR H$ ="y" GOTO 20 ELSE 220
220  END
```

Assume that the above commands have been saved on a diskette (A:) in a file named "A:G2-INDEX".

After loading or activating the BASIC interpreter, the programme is started by inserting the diskette into Drive A and typing
LOAD "A:G2-INDEX" (press Enter).

A worked example:

Assume colony counts (*z*) on two parallel plates from two successive dilutions (coefficient 10):

Diln.	z	Relative volume
10^{-4}	268	10
	314	10
10^{-5}	31	1
	15	1

The value of the likelihood-ratio estimate does not depend on the unit of measurement of the test portions. It is simplest to express the volumes relative to the smallest test portion, in this case 10^{-6} ml. The smallest volume is chosen as the unit (1) and the other volumes are its multiples.

Running the program

Activate the BASIC interpreter and start the calculations by entering the text and numbers printed in **boldface**

Ok
LOAD"A:G2-INDEX"
Ok
RUN
LIKELIHOOD RATIO INDEX G^2
NUMBER OF TERMS, N=? **4** (enter the number of plates here)
I=1
COLONY COUNT=? **268**
RELATIVE VOLUME=? **10**
I=2
COLONY COUNT=? **314**
RELATIVE VOLUME=? **10**
I=3
COLONY COUNT=? **31**
RELATIVE VOLUME=? **1**
I=4
COLONY COUNT=? **15**
RELATIVE VOLUME=? **1**

INDEX G^2= 11.847

ANOTHER SET (Y/N)? (Answer Y or N depending whether you are finished or wish to continue with another set of numbers)

Exit the programme by entering
Ok
SYSTEM

NOTE. A typing error entered cannot be edited afterwards. The whole process must be started again. If the typing error is noticed immediately after entering it, the program is aborted by pressing CtrlC. The text appears on the screen:
Break in 70
Ok

Restart by entering
RUN
LIKELIHOOD RATIO INDEX G^2
NUMBER OF TERMS, N=?
Etc…...

ANNEX D. D1. Report sheet. One-plate instrument.

Sample: _____ Date: _____

Basics:

Dilution factor *F* of final suspension: _____
Test portion volume: _____
Number of colonies: _____

Systematic correction coefficients applied:
(Figures in parentheses refer to the clause of the main text)

		Coefficient	**w**	**w²**
confirmation *p*	(5.6)			
personal yield KH	(8.4)			
sample satability KS	(8.6)			
medium yield KA	(8.7)			
overlap KL	(8.9)			
dilution factor F	(8.2, 5.7)			

(When a coefficient is unknown or unused its value is taken as 1 and the uncertainty as 0.)

Test result: $y = p \cdot K_H \cdot K_S \cdot K_A \cdot K_L \cdot F \cdot \frac{Z}{V}$

Uncertainty components of the "particle detection instrument":

		w	**w²**
reading (t)	(4.5, 5.1.1)		
primary count (z)	(5.2)		
test portion (v)	(5.4)		

Relative uncertainty of the test result:

$$w_y = \sqrt{w_p^2 + w_{K_H}^2 + w_{K_S}^2 + w_{K_A}^2 + w_{K_L}^2 + w_F^2 + w_T^2 + w_Z^2 + w_V^2}$$

Final report:

Microbial content of the sample *y*: _____

Relative uncertainty, $100w_y$: _____ **%**

ANNEX D. D2. Report sheet. Multiple-plate instrument.

Sample: _____ Date: _____

Basics:

Plate	Presumptive count	Colonies tested	Colonies confirmed	Confirmed count
1				
2				
3				
4				
5				
6				
Sum				

Dilution factor F of final suspension: _____
Test portion volumes: _____ Sum V = _____
Sum of confirmed counts (if relevant): _____ X = _____

Systematic correction coefficients applied:
(Figures in parentheses refer to the clause of the main text)

		Coefficient	**w**	**w²**
personal yield K_H	(8.4)			
sample satability KS	(8.6)			
medium yield K_A	(8.7)			
coverage K_L	(8.9)			
dilution factor F	(8.2, 5.7)			

(When a coefficient is unknown or unused its value is taken as 1 and the uncertainty as 0.)

Test result: $y = K_H \cdot K_S \cdot K_A \cdot K_L \cdot F \cdot \frac{X}{V}$

Uncertainty components of the "particle detection instrument":

		w	**w²**
reading (T)	(4.5, 5.1.2)		
confirmed count (X)	(5.3)		
test portion (V)	(5.5)		

Relative uncertainty of the test result:

$$w_y = \sqrt{w_{K_H}^2 + w_{K_S}^2 + w_{K_A}^2 + w_{K_L}^2 + w_F^2 + w_T^2 + w_X^2 + w_V^2}$$

Final report:

Microbial content of the sample *y*: _____

Relative uncertainty, $100w_y$: _____ **%**

J1/1999	Nordic Intercomparison in Barometric Pressure
J2/1999	Automaattisten punnustenvaihtimien suunnittelu, toteutus ja käyttö
J3/1999	Intercomparison of Gauge Pressure Measurements between SP/FFA and MIKES in the Range 32 kPa ... 132 kPa
J4/1999	Ainemäärän kansallisen mittanormaalijärjestelmän toteuttamista ja organisaatiota koskeva selvitys
J5/1999	Mikrobiologisen metrologian tilanneselvitys ja kehittämissuunnitelma
J6/1999	Finnish National Standards Laboratories FINMET. Annual Report 1998
J7/1999	Lämpötilan vertailumittaus L10, S-tyypin termoelementin kalibrointi
J8/1999	Mekaanisten värähtelyiden mittausten kartoitus
J9/1999	Intercomparison of the Hydrometer Calibration Systems at the IMGC and the MIKES
J10/1999	National Basis for Traceability in Humidity Measurements
J1/2000	Intercomparison of Temperature Standards of Lithuania and Finland
J2/2000	Finnish National Standards Laboratories FINMET. Annual Report 1999
J3/2000	Mass Comparison M3
J4/2000	Mass and Volume Comparisons at MIKES
J5/2000	Nanometritason mittaukset, kartoitus
J6/2000	Nordic Intercomparison in Gauge Pressure Range 0 ... 2 MPa
J1/2001	Mikrobiologian kvantitatiivisten viljelymääritysten mittausepävarmuus
J2/2001	Finnish National Standards Laboratories. Annual Report 2000
J3/2001	Lämpötilan vertailumittaus L11, PT100-anturin sovitusmenetelmän kehittäminen
J4/2001	High Precision Roundness. Euromet Project 533. Final Report
J5/2001	Kaasun kosteuden mittaaminen
J6/2001	Intercomparison of Humidity Standards
J7/2001	Comparisons in the Pressure Range from 50 kPa to 350 kPa
J1/2002	Lämpötilan mittaus
J2/2002	Annual Report 2001
J3/2002	Uncertainty of quantitative Determinations derived by Cultivation of Microorganisms

Tilaukset toimistosihteeri Kirsi Tuomisto, puh. (09) 6167 457,
e-mail kirsi.tuomisto@mikes.fi.

MITTATEKNIIKAN KESKUS
CENTRE FOR METROLOGY AND ACCREDITATION
P.O. Box 239
FIN- 00181 HELSINKI
Tel. +358 9 616 761
Telefax +358 9 616 7467

ISBN 952-5209-68-7
ISSN 1235-5704