Measurement Uncertainty in Microbiology

LYNNE I. FORSTER
Lynne I. Forster Training & Consulting Services, PO Box 15847, New Lynn, Auckland, New Zealand

Testing laboratories wishing to comply with the requirements of ISO/IEC 17025:1999 need to estimate uncertainty of measurement for their quantitative methods. Many microbiological laboratories have had procedures available for monitoring variability in duplicate results generated by laboratory analysts for some time. These procedures, however, do not necessarily include all possible contributions to uncertainty in the calculations. Procedures for estimating microbiological method uncertainty, based on the Poisson distribution, have been published but, at times, the procedures can either underestimate uncertainty or require laboratories to undertake considerable experimental studies and more complex statistical calculations. This paper proposes procedures for estimating uncertainty of measurement in microbiology, whereby routine laboratory quality control data can be analyzed with simple statistical equations. The approaches used in these procedures are also applied to published data and examples, demonstrating that essentially equivalent results can be obtained with these procedures.

As is well understood, no measurement is perfect. It has an associated uncertainty arising from many factors. Measurement uncertainty is defined as being “a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand” (the particular quantity subject to measurement; 1).

In 1992, at its conference held in Ottawa, Canada, the International Laboratory Accreditation Conference (ILAC; now International Laboratory Accreditation Cooperation) suggested that a series of working groups be established to consider procedures for estimating uncertainty of measurement in different testing disciplines, one of which was microbiology.

At the following ILAC conference held in Hong Kong in 1994, the working group concerned summarized its findings as follows: “It is virtually impossible to know the exact microbial concentration of any sample, natural or artificial. In certain circumstances, assigned values based on consensus may be used but certified reference materials for running as controls alongside tests are not generally available and where these are available, it will be unlikely that they will be matrix matched ...” (2).

During the past few years, considerable attention has been paid to the estimation of measurement uncertainty in microbiology and how this can be undertaken. ISO/IEC 17025:1999 requires that a “laboratory shall at least attempt to identify all the components of uncertainty and make a reasonable estimation ...” (3).

Few suggestions have yet been made on how bias may be determined for microbiological analyses. The ILAC working group suggested that comparison with a reference procedure may not be sufficient to determine bias, as the reference method may have its own bias, which may not easily be determined. The working group also stated that interlaboratory comparison programs and proficiency testing schemes may not give information about bias in absolute terms but could be useful in determining the most likely number of organisms in a sample using particular methodologies. When uncertainties are estimated, those that cannot be evaluated statistically can be estimated only through a thorough knowledge of all the steps in the measurement process. The working group believed that quantification of at least some of these might well be possible, but the workload involved may well be prohibitive (2).

For many years, various microbiological texts and publications have included procedures for determining the precision criterion in performing microbiological analyses (4, 5). This involves laboratory analysts performing sample analyses in duplicate. The sets of duplicate results are collected and logarithmically transformed. The range between each of these transformed duplicate results is calculated and the average range determined and multiplied by 3.27 to give the precision criterion. It can be used as the benchmark for assessing ranges in subsequent transformed results. This has been described as either measuring analyst precision (4) or the precision of quantitative methods (5).

A number of more recent approaches for estimating uncertainty are based on the concept of the Poisson distribution (6–8). A Poisson distribution is defined as being “a fully random distribution of particle numbers when sampling a perfectly mixed suspension” (6). It is unique in that the standard deviation is equal to the square root of the mean of the counts obtained, i.e., the variance is numerically equal to the mean. In other words, the precision of a colony count method is governed by the magnitude of the count itself. Equations for calculating the confidence interval associated with a count at a...
95% confidence level, based on the Poisson distribution, are included in the publications described above.

In simple equations, the uncertainty associated with a count depends primarily on the total colony count, dilutions, and the number of replicate plates. All contributions to uncertainty are therefore not necessarily included in the estimation. Various publications have recognized that when samples are analyzed in replicate, variability is greater than fully random (in the Poisson sense), i.e., overdispersion may be observed (6, 7). Pure cultures of bacteria can be expected to follow a Poisson series, but mixed cultures may deviate from Poisson, especially when sublethal cell damage has occurred (9).

ISO/TR 13843 includes a worked example in which the overdispersion factor is estimated (6; Annex B). In this example, results from 12 laboratories are used to demonstrate the calculations involved. Each laboratory analyzed a sample of the same type, but of its own choice, and performed quadruplicate parallel counts on its homogenized sample suspensions. All laboratories used the same method. The results obtained were used to calculate the overdispersion factor by a number of statistical techniques including linear regression.

The data from ISO/TR 13843 can be developed further and used as an example of the additional calculations needed to determine the confidence interval for microbiological counts on a particular type of sample at a 95% confidence level using an overdispersion model. In this calculation:

\[ S = \sqrt{C + u^2C^2} \]

where \( S \) is the standard deviation, \( C \) is the colony count, and \( u \) is the overdispersion factor, calculated from the slope of the line relating the variance-to-mean ratio to concentration. In the ISO/TR 13843 example, \( u = 0.088 \).

At a 95% confidence interval, the count

\[ C \pm 2 \times \sqrt{C + u^2C^2} \]

If \( C = 105 \), the 95% confidence interval

\[ 105 \pm 2 \times \sqrt{105 + (0.00766 \times 105^2)} \]

\[ = 105 \pm 28, \text{i.e.,} 77 - 133 \]

Other approaches are available for estimating \( u \) (7).

A laboratory may use the ISO/TR 13843 procedure to estimate the uncertainty or the confidence interval for each of its methods and sample types by undertaking a series of replicate determinations. ISO/TR 13843 suggests replicates be in excess of quadruplicate for each sample and that more than 12 samples may be desirable for reliable results.

Many laboratories lack the resources of staff numbers and the time required to accumulate sufficient data for the ISO/TR 13843 approach. They will, however, be conversant with the concept of precision criterion, and a similar approach for estimating uncertainty from duplicate data could therefore be more readily adopted.

### Table 1. Calculation of intermediate precision for Heterotrophic Plate Counts of clean waters

<table>
<thead>
<tr>
<th>No.</th>
<th>Result 1</th>
<th>Result 2</th>
<th>LogR₁</th>
<th>LogR₂</th>
<th>(LogR₁ – LogR₂)</th>
<th>(LogR₁ – LogR₂)²</th>
</tr>
</thead>
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<tr>
<td>1</td>
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<td>127</td>
<td>2.0492</td>
<td>2.1038</td>
<td>0.0546</td>
<td>0.002981</td>
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<td>2</td>
<td>37</td>
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<td>1.5682</td>
<td>1.5911</td>
<td>0.0229</td>
<td>0.022900</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>23</td>
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<td>1.3617</td>
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<td>0.002841</td>
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<tr>
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<td>1.5682</td>
<td>0.0241</td>
<td>0.000581</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>59</td>
<td>1.8751</td>
<td>1.7708</td>
<td>0.1043</td>
<td>0.010878</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>23</td>
<td>1.3222</td>
<td>1.3617</td>
<td>0.0395</td>
<td>0.001560</td>
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<tr>
<td>7</td>
<td>229</td>
<td>220</td>
<td>2.3598</td>
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<td>0.001459</td>
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<td>12</td>
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<td>2.3364</td>
<td>2.3483</td>
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<td>0.000142</td>
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<tr>
<td>13</td>
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<tr>
<td>14</td>
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<td>1.4314</td>
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<td>2.2988</td>
<td>0.0376</td>
<td>0.001414</td>
</tr>
<tr>
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<td>130</td>
<td>210</td>
<td>2.1139</td>
<td>2.3222</td>
<td>0.2083</td>
<td>0.043389</td>
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<td></td>
<td>0.127775</td>
<td></td>
<td></td>
<td>0.127775</td>
</tr>
</tbody>
</table>

* Data provided by Palmerston North City Council (New Zealand).
The EURACHEM/CITAC Approach

In 1995, the 1st Ed. of the EURACHEM/CITAC publication *Quantifying Uncertainty in Analytical Measurement* was published; the 2nd Ed. was published in 2000. This protocol establishes general rules for the evaluation and expression of uncertainty in quantitative chemical analysis, based on the approach laid down in the ISO Guide to the Expression of Uncertainty in Measurement. In the evaluation of the measurement uncertainty of a method, the EURACHEM guide requires the analyst to look closely at all the possible sources of uncertainty within a method and states that “in practice, a preliminary study will quickly identify the most significant sources of uncertainty” which will be the dominating influences in the total uncertainty of the method.

Many of the following concepts and procedures in this EURACHEM guide apply equally well to microbiological testing:

(a) Specifying clearly what is being measured or specifying the measurand

(b) Identifying contributions to uncertainty in the method concerned

(c) Estimating the size of each identified contribution to uncertainty as a standard deviation

(d) If necessary, combining the values obtained for uncertainties

(e) Calculating the expanded uncertainty

An important point is made in the EURACHEM guide regarding empirical methods. In such methods, the analytical results obtained are dependent on the procedures used in the analysis. The method accordingly defines the measurand or, in other words, the “right” answer is not a property of the sample or of the target organisms, but of the method. Where such a method is in use within its defined field of application, the bias associated with the method is defined as being zero. That is, it is not meaningful to consider correction for bias intrinsic to these methods (10).

It may well be that the majority of quantitative microbiological methods can be considered to be empirical methods, where results generated are dependent on the media used, times and temperatures of incubation, and inclusion or exclusion of resuscitative steps in the methods. Variations in the recovery of organisms resulting from the above factors have been well documented over the years (5, 9).

Each of the above steps specified in the EURACHEM guide for estimating uncertainty can be considered in turn.

**Specification**

In this step, what is being measured is clearly defined. The equation used to calculate the value of the measurand at the end of the method process is a good starting point. In microbiology, very simple equations are usually involved in the calculation of colony-forming units (CFUs) or specific organisms in a sample. These equations normally take into account the average of duplicate results, the dilution used, and the volume of the inoculum.

Specification can also include an overview or flowchart of the steps undertaken in the performance of the method. Initially, a laboratory may wish to consider contributions to uncertainty from the subsampling stage, which is normally a characteristic of a test method.

**Identification of Sources of Uncertainty**

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

The EURACHEM guide recommends the use of “cause and effect” diagrams for identifying contributions to uncertainty. A good starting point in the construction of a cause and
effect diagram is the equation used to calculate the measurand (i.e., CFUs or organisms being analyzed for) and consideration of each step of the analysis depicted in the flowchart. In this way, a clear picture of all potentially significant contributions to uncertainty is obtained.

Quantification of Contributions to Uncertainty

The contributions to uncertainty that have been identified above are usually examined to see which are accounted for by data already available in the laboratory. These contributions can then be grouped together in the cause and effect diagram.

Not all identified contributions to uncertainty will make a significant contribution to the total uncertainty. Unless there is a large number of them, contributions that are less than one third of the largest need not be quantified in detail. In microbiology, we can expect that the precision of the method itself forms the dominant contribution to the uncertainty estimate. In microbiology, it is usually possible to quantify the combined effect of most (if not all) sources of uncertainty, reducing the overall effort involved. Microbiological testing laboratories generally have a program whereby a certain number of samples are analyzed at least in duplicate. Duplicate data (for a particular test and for particular types of samples) collected over a period of time can be analyzed to determine the standard deviation. In ISO 5725, this is called the intermediate precision of the test method, if all possible sources of variation in the method are taken into account when duplicate analyses are performed (11). These sources of variation include storage effects, laboratory environmental effects, operator effects, effects of using different items of equipment, different batches of media, etc.

At this stage, publications suggest that estimations of uncertainty commence from the subsampling stage of an analysis, i.e., from the time samples are received in the laboratory for testing. Subsampling procedures are normally included in a test method, whereas external sampling is not.

The standard deviation or intermediate precision of a series of duplicate results, for a particular sample type, is calculated by:

\[ S = \left( \frac{\sum (y_{1i} - y_{2i})^2}{2t} \right)^{1/2} \]

where \((y_{1i} - y_{2i})\) is the difference between individual duplicate results; \(\sum (y_{1i} - y_{2i})^2\) is the sum of the squared differences between each set of duplicate results; and \(t\) is the number of test samples analyzed (note that other publications may use different symbols from those in ISO 5725).

Microbial distributions are not necessarily symmetrical, as counts are often characterized as having a skewed distribution because of many low values and a few high ones. The application of parametric statistical techniques generally includes the assumption that the data being analyzed is from a symmetrical or normal distribution. In microbiology, a common practice is to first transform data into logarithms before equations for calculating standard deviation, etc., are applied (4, 5, 9, 12–14).

An example of the calculation of intermediate precision from a series of duplicate Heterotrophic Plate Count results for clean water is included in Table 1. In Table 2, the data included in ISO/TR 13843 are reproduced and confidence intervals recalculated after the quadruplicate data from the 12 laboratories are transformed into logarithms. The result that the 95% confidence interval is 70–157 for a mean result of 105 can be compared with the confidence interval of 77–133 calculated with these same data, but by the ISO/TR 13843 procedure.

Using the data in Table 1:

\[ S^2 = \frac{\sum (\log R_1 - \log R_2)^2}{2t} = 0.127775/32 \]

\[ S = 0.0632 \]

\[ 2S = 0.1264 \]

For sample No. 1, the mean of the results = (2.0492 + 2.1038)/2 = 2.0765. At a 95% confidence level, the confidence interval = 2.0765 ± 0.1264 = 1.9501–2.2029. On antilogging, for a result of 120, the confidence interval is 90–160.

Combination of Uncertainty Values

It can be expected that all major contributions to uncertainty are accounted for in calculating intermediate precision. On occasion, minor contributions to uncertainty may be quantified separately as standard deviations and, if relevant, combined with the intermediate precision value.

<table>
<thead>
<tr>
<th>Operator 1</th>
<th>Log result</th>
<th>Operator 2</th>
<th>Log result</th>
<th>Operator 3</th>
<th>Log result</th>
</tr>
</thead>
<tbody>
<tr>
<td>80000</td>
<td>4.9031</td>
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<td>5.2304</td>
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<td>90000</td>
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<td>80000</td>
<td>4.9031</td>
<td>80000</td>
<td>4.9031</td>
</tr>
</tbody>
</table>

a Data supplied by Watercare Laboratory Services (Auckland, New Zealand).
Calculation of the Expanded Uncertainty

The final stage is to multiply the (combined) standard uncertainty by a chosen coverage factor $k$, in order to obtain an expanded uncertainty.

The expanded uncertainty $U$ is required to provide an interval which may be expected to encompass a large fraction of the distribution of values which could reasonably be attributed to the measurand, i.e., an interval within which the value of the measurand is believed to lie, with a high level of confidence. For most purposes, a coverage factor of 2 is chosen (confidence level of approximately 95%). Note that the value obtained is still expressed as a logarithm and must be converted to the actual range.

Using the data in Table 2:

$$S^2 = \frac{1}{N-k} \left( n_i-1 \right) \sum_{i=1}^{k} S_i^2$$

$$S^2 = (3 \times 0.092033)/(48 – 12), S = 0.0876, 2S = 0.1752$$

where $S$ = overall standard deviation for the method; $S_i$ = sample standard deviation of the replicates for the $i$th sample, using $(n – 1)$ as the denominator; $n_i$ = number of replicates for the $i$th sample; $N$ = total number of analyses (number of samples x number of replicates); $k$ = total number of samples.

For a mean sample count of 105, the confidence interval is $2.0212 \pm 0.1752$, which is $1.8460–2.1964$, which is 70–157.

**Most Probable Number (MPN) Determinations**

It is traditional in MPN analyses to refer to MPN tables to obtain a test result and the associated 95% confidence limits. These have been established statistically, assuming that microorganisms are distributed in accordance with the Poisson distribution. That is, complete randomness of particle distribution in a liquid medium is assumed. MPN tables, however, may not necessarily include all contributions to uncertainty.

Labs should therefore establish if the confidence limits quoted in MPN tables are reasonable estimates of uncertainty for their circumstances. One way of doing this is to establish if replicate determinations indicate a similar level of uncertainty as indicated in the relevant MPN table. A series of duplicate results can be analyzed and the intermediate precision calculated as described previously or another approach can be used, as below.

In Table 3, the results from 3 operators in a laboratory, each analyzing, in quintuplicate, an effluent sample for fecal coliforms, are analyzed statistically. A plot of the results after transformation on normal probability paper (Figure 1) suggests that these transformed data are consistent with a normal distribution (15). The similarity of this calculated uncertainty to that in the relevant MPN table would indicate that no other significant sources of uncertainty are present and that, with subsequent analyses, a laboratory can use the confidence limits specified in the MPN tables.

Using the data in Table 3: mean = $4.9755$ (ca 95 000); standard deviation = 0.1848. At a 95% confidence level, the result is $4.9755 \pm 2 \times 0.1848 = 4.6059–5.3451$; i.e., 40 000–220 000 (taking antilogs).

For a result of 90 000, the 95% confidence interval in MPN tables is approximately 30 000–290 000.

**Reporting Uncertainty of Measurement**

Traditionally, results from microbiological analyses are presented unaccompanied by any form of uncertainty estimation. This situation, however, may change in the future. The information could be reported as a confidence interval or as confidence limits, once the expanded uncertainty data have been antilogged (9). For example: Result: $x$ (units) with a confidence interval of $y$ to $z$, or, $x$ (units) with confidence limits of $y$ and $z$.

**Conclusions**

Two procedures for estimating uncertainty of measurement in microbiology are presented in this paper. In these approaches, laboratory quality control results for both Heterotrophic Plate Count and MPN determinations are analyzed statistically, using simple standard deviation and intermediate precision equations.

In these 2 procedures, results are first transformed into logarithms. Figure 1 shows a plot of MPN results from Table 3 on normal probability paper, suggesting that these transformed data are consistent with the assumption of normality.

With general quantitative methods, intermediate precision can be calculated from a series of duplicate results (not less than 15) when all possible method variations are taken into account in performing the duplicates. In the case of MPN determinations, if a laboratory can demonstrate that its estimated measurement uncertainty is within the confidence limits published in MPN tables, the appropriate tabulated values can be quoted with subsequent analytical results, as this observation suggests no other significant sources of uncertainty are present.
References

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(7) NMKL (1999) Measurement of Uncertainty in Microbiological Examination of Foods, Nordic Committee on Food Analysis, Oslo, Norway
(8) BS 5763 (1996) Microbiological Examination of Food and Animal Feeding Stuffs, Part 0—General Laboratory Practice, British Standards Institute, London, UK