Centre for Environment Fisheries & Aquaculture Science

Cefas

European Union Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs

DETERMINING UNCERTAINTY OF MEASUREMENT FOR THE ENUMERATION OF E. COLI IN BIVALVE MOLLUSCS BY ISO 16649-3

Introduction

ISO/IEC 17025 states that "Testing laboratories shall have and shall apply procedures for estimating uncertainty of measurement.". However, it allows that this may be achieved by determination by rigorous determination or, where this is not possible, by reasonable estimation.

The present version of ISO/TS 19036 gives guidelines for the estimation of measurement uncertainty (MU) for quantitative determinations for the microbiological examination of food and animal feeding stuffs. However, that the scope of that technical specification states that "This Technical Specification is not applicable to enumeration using a most probable number technique…". No other ISO standard or technical specification presently addresses the determination of measurement uncertainty for Most Probable Number (MPN) methods. A draft revision to ISO TS 19036 does do so but, until the revision has been completed and published, there is presently no guidance to which National Reference Laboratories (NRLs) or Official Control Laboratories (OCLs) undertaking enumeration of E. coli in bivalve molluscs using the EU reference method (ISO 16649-3) can refer.

The present document presents interim guidance for such laboratories and has been developed by the European Union Reference Laboratory (EURL) in collaboration with members of the Good Practice Guide (GPG) Working Group. It is a stand-alone document and does not form a part of the GPG. The present version of ISO TS 19036 states that the approach used there is based on experimental results (with replication of the same analysis). This same approach will be followed in this document.

Either of the described approaches may be used for determining the overall uncertainty of the method. They are intended to yield the intra-laboratory reproducibility of the method. Approach 2 addresses more factors than does Approach 1 and is to be preferred, where practical.

The analysis of data from comparative testing is often put forward as a means of determining MU for a laboratory/method combination. This requires each separate sample to be analysed in at least duplicate. Ideally, the results of twenty such samples need to be analysed to give a robust estimate of MU. However, most bivalve matrix proficiency testing schemes (e.g. the EURL scheme) have too few samples a year to yield this number of samples over a reasonable time period. Although full participation in the PHE/Cefas Shellfish EQA Scheme would yield a reasonable number of results over a 3-year period, the scheme is based on the use of artificial samples and any derived MU will not represent the full variability of the method in a laboratory. If sufficient data is available from relevant comparative testing, it may be analysed following approach 2 given below.

Neither approach given in this document accounts for uncertainty due to sampling. They do take into account the uncertainty due to homogenisation which is not explicitly addressed in ISO TS 19036 (or the revision thereof). The EURL has evidence that the uncertainty associated with the homogenisation of bivalve molluscs can contribute significantly to overall uncertainty of the method (e.g. homogenisation of clams/cockles by stomaching) and it is therefore important to take this into account. In practice, that part of the overall uncertainty is confounded in the study plan with the uncertainty due to subsampling (via the testing of portions). However, it is usual to only test a proportion of animals from a submitted sample and so this aspect is also important to take into account.

Recommendations

Either:

1. Single sample approach

   See Figure 1 and Table 1.

   a. Identify a suitable production area from which to obtain material. The target level for the overall geometric mean of the results for sample material should ideally be in the range 140-1400 E. coli/100 g[1] for a 3-dilution MPN format (5 x 1g, 5 x 0.1g, 5 x 0.01) and in the range 140-14000 E. coli/100 g for the four dilution MPN format (5 x 1g, 5 x 0.1g, 5 x 0.01g, 5 x 0.001 g).
   b. Obtain a large sample of one species of mature bivalve molluscs taken at one location on one day. In order to assist homogeneity, the smaller the area over which the sample is obtained the better. Obtain enough bivalves to allow for division into 20 test portions (i.e. subsamples), each yielding the minimum number of animals per assay recommended for the species.
   c. Transport to the laboratory under controlled conditions and start the rest of the procedure as soon as possible and certainly within 24 hours.
   d. Ensure that the animals in the original sample are well mixed and randomly assign the animals to 20 test portions.
   e. Homogenise each test portion and then prepare dilutions and undertake MPN determinations by the same standard laboratory procedure used for routine samples.
   f. For <18 values assign a nominal value of 9, for >18000 values assign a nominal value of 36000, for >180000 values, assign a nominal value of 360000.
   g. Determine the log10transformed values for all subsamples.
   h. Determine the mean and standard deviation of the log10-transformed data.
   i. Take the antilog of the mean to yield the geometric mean – check that this falls in the target range. If not, the process should preferably be repeated.
   j. Determine the expanded log10 MU as ±2 x the standard deviation of the log10-transformed data. Give the log10 MU to two decimal places.

2. Multiple sample approach

   See Figure 2 and Tables 2 & 3.

   a. Obtain at least ten samples over a period of time. The number of animals per sample must contain at least twice the minimum number of animals per assay recommended for the species (see the EURL Generic E. coli protocol).
   b. Randomly assign the animals in each sample to each of two test portions.
   c. Homogenise each test portion and then prepare dilutions and undertake MPN determinations by the same standard laboratory procedure used for routine samples.

[1] These ranges have been defined on an arbitrary basis but are intended to ensure that few, if any, subsamples yield < or > results and also other extreme tube combinations where the contribution of distributional uncertainty tends to be greater than usual.

- Where possible, account for known factors that may add to the MU, e.g. by having the separate subsamples tested by separate analysts, inoculated media incubated in separate water baths or incubators, etc.

d. For <18 values assign a nominal value of 9, for >18000 values assign a nominal value of 36000, for >180000 values, assign a nominal value of 360000.
e. Exclude the results of any samples where the geometric means of the duplicate tests do not fall in the range 140-1400 E. coli/100 g[2] for a 3-dilution MPN format (5 x 1g, 5 x 0.1g, 5 x 0.01) and in the range 140-14000 E. coli/100 g for the four dilution MPN format (5 x 1g, 5 x 0.1g, 5 x 0.01g, 5 x 0.001 g).
f. Enter the values in an Excel spreadsheet, with samples represented in separate rows and duplicate test portions in separate columns.
g. Log10-transform the values.
h. Use the ANOVA:single factor analysis tool in the Excel Data Analysis add-in to run a one-way ANOVA test (ensure that "Grouped by rows" is checked).
i. Take the square root of the within-groups mean square.
j. Determine the expanded log10 MU as ±2 x the resulting value. Give the log10 MU to two decimal places.

Use of the expanded measurement uncertainty value

The MU value determined by the laboratory should be compared to a value determined from an appropriate validation study or provided by the EURL[3]. Where the laboratory MU markedly exceeds such a reference value, the laboratory should review and, where necessary, revise the laboratory procedure in order to reduce the MU to approach, or meet, the expected level.

It is not presently foreseen in either Regulation 854/2004 (with respect to classification monitoring) or Regulation 2073/2005 (with respect to end-product testing) that MU will be applied in determining compliance with the specified criteria. Therefore, MU should not be applied to the reported value. However, where agreed with the Competent Authority, the MU may be reported as a footnote to the laboratory report for information purposes only.

References

EURL 2015. Generic Protocol: Enumeration of Escherichia coli in bivalve molluscan shellfish by the most probable number (MPN) technique (based on ISO 16649-3). Issue 11. Available at: https://eurlcefas.org/media/13823/issue_11_eurl_generic_sop_e-coli_final_20_01_15.pdf

ISO/IEC 17025. General requirements for the competence of testing and calibration laboratories. Geneva: International Organisation for Standardisation.

ISO 16649-3. Microbiology of the food chain — Horizontal method for the enumeration of beta-glucuronidase positive Escherichia coli — Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide. Geneva: International Organisation for Standardisation.

ISO/TS 19036. Microbiology of food and animal feeding stuffs — Guidelines for the estimation of measurement uncertainty for quantitative determinations. Geneva: International Organisation for Standardisation.

[2] These ranges have been defined on an arbitrary basis but are intended to ensure that few, if any, subsamples yield < or > results and also other extreme tube combinations where the contribution of distributional uncertainty tends to be greater than usual.

[3] EURL stated MU = $\log_{10}$ 0.66 derived from a systematic, structured field study to determine variation of *E. coli* in bivalve shellfish MU determined as 2XSD. (Lee R. and Silk R. (2013), Sources of variation of *Escherichia coli* concentrations in bivalve molluscs. *J. Water Health* **11** (1) 78-83.

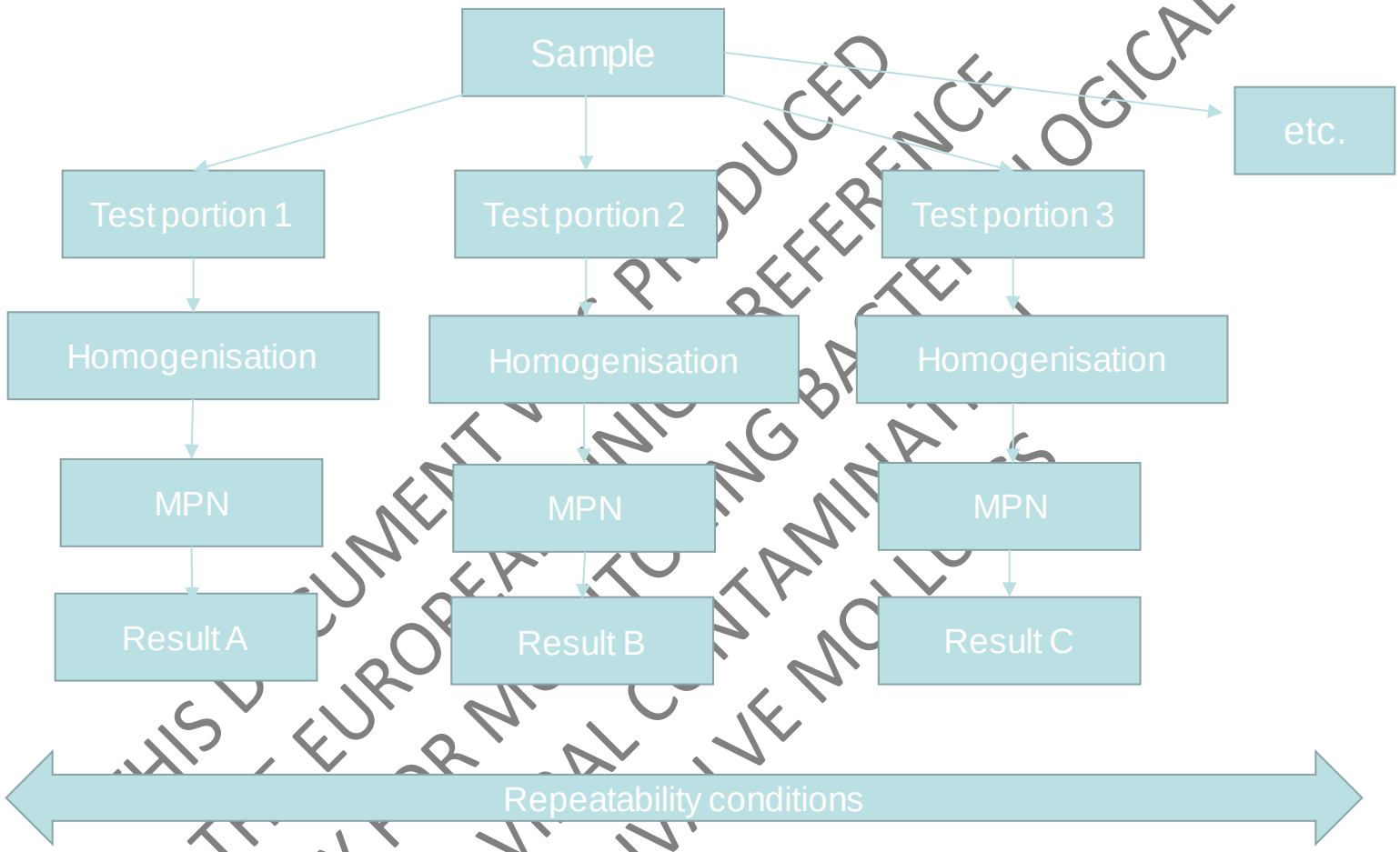

Figure 1. Schematic of the single sample approach

Table 1. Example spreadsheet for a single sample estimation of MU (4-dilution MPN)

Results of replicate examinations

E. coli/100 g	Log E. coli/100g				
1700	3.2304	Number of results	20		
2600	3.4150				
930	2.9685	Log mean	3.276165	Geometric mean	1889
310	2.4914				
450	2.6532	Log SD	0.400206	Expanded log uncertainty	±0.80
920	2.9638				
6300	3.7993				
1100	3.0414				
450	2.6532				
930	2.9685				
3300	3.5185				
4600	3.6628				
2700	3.4314				
2700	3.4314				
4600	3.6628				
2100	3.3222				
3100	3.4914				
7900	3.8976				
1700	3.2304				
4900	3.6902				

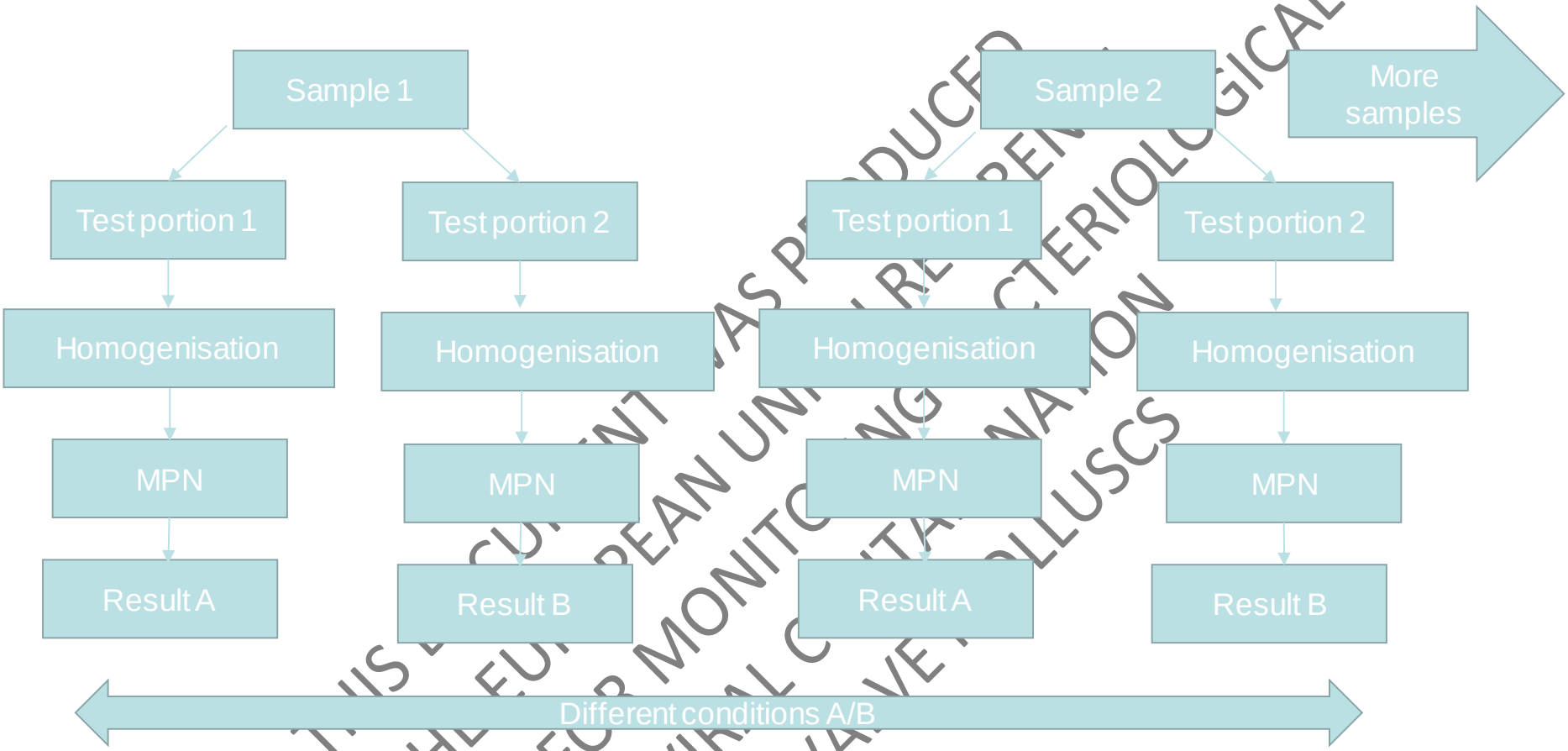

Figure 2. Schematic of the multiple sample approach

Table 2. Example data set for the multiple sample approach (4-dilution MPN)

Sample No.	Replicate 1	Replicate 2	Log replicate 1	Log replicate 2	Mean log of replicates	Geometric mean of replicates
1	92	450	1.96378783	2.65321251	2.3085002	203
2	3300	4600	3.51851394	3.66275783	3.5906359	3896
3	270	270	2.43136376	2.43136376	2.4313638	270
4	1100	330	3.04139269	2.51851394	2.7799533	602
5	2200	1400	3.34242268	3.14612804	3.2442754	1755
6	68	230	1.83250891	2.36172784	2.0971184	125
7	2700	450	3.43136376	2.65321251	3.0422881	1102
8	270	620	2.43136376	2.79239169	2.6118777	409
9	920	450	2.96378783	2.65321251	2.8085002	643
10	620	4900	2.79239169	3.69019608	3.2412939	1743

Table 3. Example output for multiple sample approach

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	2	4.617	2.3085	0.237653
Row 2	2	7.181272	3.590636	0.010403
Row 3	2	4.862728	2.431364	0
Row 4	2	5.559907	2.779953	0.136701
Row 5	2	6.488551	3.244275	0.019266
Row 6	2	4.194237	2.097118	0.140036
Row 7	2	6.084576	3.042288	0.30276
Row 8	2	5.223755	2.611878	0.065171
Row 9	2	5.617	2.8085	0.048229
Row 10	2	6.482588	3.241294	0.403026

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.961747	9	0.440194	3.229017	0.040964	3.020383
Within Groups	1.363245	10	0.136324			
Total	5.324992	19				

Square root of within groups MS =		0.369221
Therefore, expanded MU =	±	0.74