VALIDATION OF MICROBIOLOGICAL METHODS FOR WATER AND ENVIRONMENT

REVISION OF ISO 13843

Eurachem Workshop Gent 2016

Method Validation in Analytical Science
Current Practices and Future Challenges

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Revision of ISO/TR 13843

Guidance on validation of microbiological methods

- **ISO/TC 147 – Water quality**
  - **ISO/TC 147/SC 4 Microbiological methods**
    - **ISO/TC 147/SC 4/WG21 Validation**

- Convenor : Colin Fricker
- Co-convenor : Olivier Molinier
- Objective of the revision of ISO 13843
  - more precise determination of the parameters useful for the characterization of microbiological methods
  - Re-titled : Water quality — Requirements for establishing performance characteristics of quantitative microbiological methods
  - achieve an ISO standard status

Current version of the document : DIS Ballot initiated (Draft International Standard)

Basic concepts

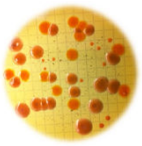
➢ **Characterization of microbiological methods**:

Exploratory process with the aim of establishing the likely set of performance characteristics of a new or a modified method, under a specific set of circumstances

The emphasis is on selective quantitative methods for all types of water

Analyte(s) ⇨

Measurands ⇨

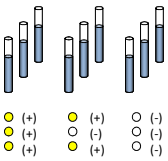


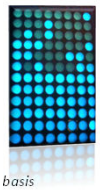
Indirect count on the basis of growth into colonies (CFU)

Particles (germs) in a homogenized suspension

↓

Random distribution…



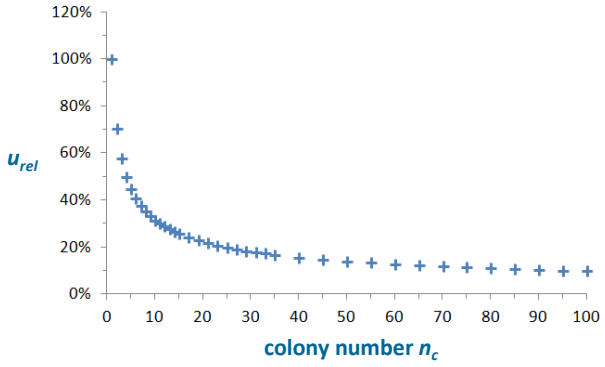


Indirect count on the basis of turbidity (MPN)

Intrinsic variablity of microbiological determinations

➢ **Random variation of particles numbers in the test portion**:

This unavoidable intrinsic variation is associated with distributions of particles (germs) in suspensions. For colony counting methods, it can be mathematically modeled by the Poisson distribution.



The relative standard deviation u_{rel} (expressed in %) of colony number n_c in a perfectly mixed suspension following the Poisson distribution

Operational variability

➢ **Every technical step of the method adds to the total variability of the measurement**:

Sub-sampling, mixing, dilution, inoculation, incubation, counting and possibly confirmation steps often cause higher than Poisson variation. This additional variation is called over-dispersion and is statistically taken into consideration using the negative binomial distribution model of variance :

$$s^2 = \overline{x} + u_o^2 \overline{x}^2$$

Poisson "Characteristics"

Combined effect of all the random over-dispersion factors

$\overline{x}$ is the mean number of colonies counted

s^2 is the variance of the number of colonies counted

u_o is the over-dispersion, the relative operational standard deviation

➢ **Quantifying over-dispersion**

$$u_o^2 = \frac{s^2 - \overline{x}}{\overline{x}^2}$$

Estimate of the operational variability

Detection level and limit of determination

➢ **Lower working limits of microbiological methods**

✓ The **detection level** is :

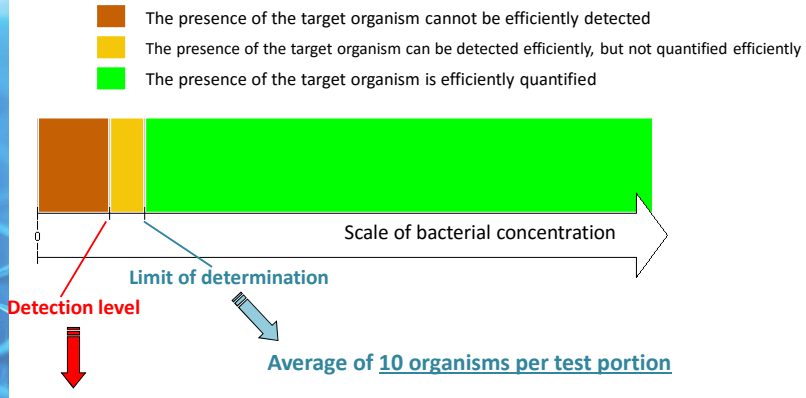
- ➢ The lowest analyte concentration that can be reliably detected (95 % probability of a positive result).
- ➢ The average count that conforms to this definition is **3 organisms per test portion, using Poisson distribution.**
  - ❖ Irrespective of the analytical technique, method, or target organism, the detection level defined in terms of probabilities varies very little. Only extreme degrees of over-dispersion might change the picture slightly

✓ Alternatively, when a consensual relative standard deviation can be determined, the **limit of determination** can be used :

- ➢ It corresponds to the lowest analyte concentration where relative standard deviation equals the determined specified limit
- ➢ For colony count methods, ISO 8199 mentions a limit of determination of **10 organisms per test portion**, corresponding to a relative precision of around 32 %, in a fully random (Poisson) situation

Detection level and limit of determination

➢ **Lower working limits of microbiological methods**



Maximum upper limit

➢ **The upper end of the working range for which the method is useful**

➢ **Theory**

With colony count methods, precision theoretically improves steadily with the number of target colonies observed (Poisson distribution)

The working range of a method is often specified in the original description of the procedure or defined by manufacturers

➢ **« Real world »**

Depending on the type and size of colonies (target organisms, background flora), the agar plate or membrane filter can become « saturated » before the predefined limit

➢ **Objective**

Determining the region of colony numbers where counts per plate become too uncertain to base a valid determination on

➢ **Experimental design / Statistical tools**

- finely graded series of dilutions or volumes with replication of plating
- Data analysed for proportionality and over-dispersion of parallels assuming perfect randomness at every step : log-likelihood-ratio statistic G^2

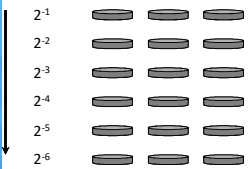
Maximum upper limit

➢ **The upper limit related to linearity (proportionality)**

Two-fold dilutions

Plating in triplicate

(e.g. 6 dilution steps)



Dilution	Parallel counts			Sum S_i	Mean $\bar{x}$	Relative Volume R_i	S_i/R_i
2^{-1}	121	204	162	487	162,3	32	15,22
2^{-2}	109	128	148	385	128,3	16	24,06
2^{-3}	111	114	97	322	107,3	8	40,25
2^{-4}	56	60	68	184	61,3	4	46,00
2^{-5}	36	29	24	89	29,7	2	44,50
2^{-6}	11	13	17	41	13,7	1	41,00

Statistically, the proportionality is verified in the four dilutions from 2^{-3} to 2^{-6} and not for the higher concentrations

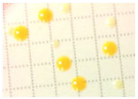
The point at which the linearity is not seen : upper limit of the method

✓Examination of a minimum of 20 samples is required

✓naturally contaminated samples, pure cultures or samples spiked with contaminated material

Categorical performance characteristics

➢ **Presumptive colonies versus confirmed colonies** :



The identification can be divided into 4 categories

		Presumptive count +	Presumptive count -	
Confirmed count	+	a (True +)	b (False -)	a+b
	-	c (False +)	d (True -)	c+d
		a+c	b+d	n

✓ Sensitivity = a / (a+b) - The fraction of the total positives correctly assigned in the presumptive count

✓ Specificity = d / (c+d) – The fraction of the total negatives correctly assigned in the presumptive count

✓ False positive rate = c / (a+c) - The proportion of positive results (e.g. typical colonies) that are subsequently shown to be due to non-target organisms

✓ False negative rate = b / (b+d) - The proportion of negative results (e.g. atypical colonies) shown to be target organisms

Categorical performance characteristics

➢ **Presumptive colonies versus confirmed colonies** :

The identification can be divided into 4 categories

True (+) — Presumptive count — False (-)

Confirmed count	+	-	
+	a	b	a+b
-	c	d	c+d
	a+c	b+d	n

False (+) — True (-)

➢ Selectivity = a / n - The ratio of the number of target colonies to the total number of colonies

➢ Type and number of samples :

- Minimum of 20 samples from different sources
- Samples containing target and non target organisms, ideally naturally contaminated
- All typical and atypical colonies are confirmed

Assessment of precision

➢**The characterization of a new microbiological method should provide the initial values of its precision estimates**

✓Applied to water microbiological methods, ISO 5725-1, ISO 5725-2 and ISO 5725-3 need some adaptations because the basic principles originally applied to continuous data and not to discrete data (counts)

✓The three most commonly used measures of precision are assessed :

➢Repeatability

A minimum of 3 series of 10 measurements – Preferably natural samples

➢Intra-laboratory reproducibility

Experimental design described in ISO 29201 (MU standard) – A minimum of 30 samples

➢Interlaboratory reproducibility

At least, 2 replications, a minimum of 8 labs and 3 sets of samples

Assessment of precision

➢**Worked example : determination of the repeatability performance of a microbiological method**

➢The question is : Does the observed variability between parallel counts comply with Poisson distribution?

Sample	Repeated measurements (plates)									
1	63	65	77	59	69	61	55	65	33	90
2	47	60	40	57	24	39	57	52	35	54
3	21	16	20	24	21	34	23	26	18	14

Tabulation of the counts in 3 repeatability experiments

Detection of (significant ?)over-dispersion

Sample	Arithmetic mean	Variance	Observed value of χ^2_{r-1}	Critical value (α = 5%)	Relative operational variance u_0^2
1	63,7	216,4556	30,5824	16,9190	0,0376
2	46,5	136,2778	26,3763	16,9190	0,0415
3	21,7	31,7889	13,1843	16,9190	0,0214

Order of magnitude of over-dispersion

Average relative operational variance = 0,0335 (its square root in % : 18,3%)

Relative recovery

➢**Method comparison**

✓Method performance consists of many aspects

➢ e.g. one method may be superior in specificity but inferior in recovery

True value of the measurand remains unknown…

For recovery comparisons, it is necessary to apply two methods in parallel on the **same samples** when developing an in-house method, and also when collecting information to justify the use of an alternative method

➢ ISO 17994:2014 - Water quality - Requirements for the comparison of the relative recovery of microorganisms by two quantitative methods

✓a wide range of samples

✓number of laboratories allowing the expansion of the sample range over large geographical areas

✓comparison must be based on confirmed counts generated by confirming all colonies

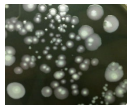
Robustness

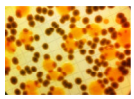
- For most microbiological methods **time** and **temperature** of incubation are parameters of importance

- duplicate samples of either seeded or naturally contaminated samples should be run at the extremes of the parameter being studied
  - e.g. - a method procedure states the temperature range as (36 ± 2) °C and the incubation period as 18 h to 24 h.
- Robustness data : a matrix using the maximum and minimum incubation temperatures for the maximum and minimum duration of incubation
- A minimum of 30 data points should be collected for each parameter

Uncertainty of counting

- **Reliability of the counts**
- Repeated counting of the colonies of the same plates within a short time





- individual (or personal) uncertainty of counting colonies: e.g. - A technician familiar with the microbiological method should read different plates twice within a short time interval (e.g. less than one hour)
- intra-laboratory uncertainty of counting colonies - Five technicians participated in a colony counting session - Standard agar plates were picked from the available determinations and were read by each participant

- For a reliable general estimate, at least 30 plates should be available
- The plates for repeated counting should be selected at random ignoring plates with less than 20 colonies and not selecting unusual ones
- Estimates of counting uncertainty expressed as relative standard deviation

Specifications

- **Some guidelines**
  - ✓ **Sensitivity**: Generally greater than 90 %;
  - ✓ **Specificity**: Generally greater than 80 %;
  - ✓ **Selectivity**: Results are not valid if selectivity is less than 10 %
  - ✓ **Uncertainty of counting**: Individual counting uncertainty (one person) remains normally below u_{rel} = ± 0,03 (3%) . Intra laboratory uncertainty of counting is generally less than u_{rel} = 0,05 (5%). Intra-laboratory uncertainty of counting greater than 0,1 (10%) is a certain sign of problems or difficulties
  - ✓ **Repeatability** (parallel plating): Variation is within the Poisson distribution. If not, the extent of over-dispersion should be given
  - ✓ **Maximum upper limit**:
    - For membrane filtration methods the range typically quoted is 0 cfu to 80 cfu while for plate count methods using a 90 mm Petri dish, the range may be 0 cfu to 300 cfu

A clause of performance characteristics will be added to all ISO standards that deal with water microbiology

Routine labs - Verification

- **Implementation of a method developed elsewhere**
- data likely to be generated by the laboratory with a given procedure and any given sample type
- verification uses selected and simplified forms of the same procedures used in method characterization, but possibly extended over a longer time
  - Aspects of the method performance that are of interest to the laboratory
  - Samples : 5 samples minimum (20-80 target organisms / test portion). Naturally contaminated materials wherever possible / spiked with surface water or sewage effluent if appropriate
  - ***Minimum***
    - Categorical performance characteristics : sensitivity, specificity, selectivity, false positive rate and false negative rates
    - Determination of repeatability (3 data sets-10 replicates)
    - Uncertainty of counting


Thank you
Prof. Seppo Niemelä
TC 147 / SC4 / WG21
Prof. Colin Fricker
Eurachem / belab
Thank you for your attention