



Food and Agriculture  
Organization of the  
United Nations



**Cefas**  
INTERNATIONAL  
CENTRES OF  
EXCELLENCE



Centre for Environment  
Fisheries & Aquaculture  
Science

## **FAO Reference Centre Generic Protocol**

Determination of faecal coliform bacteria in seawater by the the most probable number (MPN) technique (based on Bacteriological Analytical Manual - BAM)

**Author(s): Louise Stockley**

**Date in format: March 2021**

Whereas every precaution has been taken in the preparation of this document, the FAO Reference Centre (FAORC) cannot be held responsible for the accuracy of any statement or representation made nor the consequences arising from the use of or alteration to any information contained within. All references to the FAORC and Cefas must be removed if any alterations are made to this publication.

#### History of Procedure

Issue	Date	Section	Changes
1	26.03.21	All	First issue

## Contents

1	Introduction.....	3
2	Scope .....	3
3	Principle.....	3
4	Safety precautions.....	3
5	Equipment .....	3
6	Media and reagents .....	4
7	Microbiological reference cultures.....	4
8	Procedures.....	5
	8.1 Sample transport and receipt.....	5
	8.2 Sample storage .....	5
	8.3 Sample preparation.....	5
	8.4 Enumeration of faecal coliforms in seawater.....	5
	8.5 Confirmation of faecal coliforms.....	6
	8.6 Calculation of faecal coliform concentration.....	6
	8.6.1 The Water MPN calculator .....	6
	8.6.2 MPN tables.....	8
	8.7 Reporting results .....	8
9	Uncertainty of test results.....	8
10	Quality control.....	8
	10.1 Internal Quality Assurance (IQA).....	8
	10.2 Comparative testing .....	8
11	References.....	8
12	Appendices .....	10
	12.1 Most probable numbers (MPN) per 100 ml of sample, inoculating 5 replicate portions of geometrically decreasing volumes at 3 levels (based on Table 11 of APHA (1970)).....	10
	12.2 Application and use of the MPN calculator.....	11

## 1 INTRODUCTION

The consumption of sewage polluted bivalve molluscs may cause illness in the consumer through ingestion of pathogens transmitted by the faecal-oral route (such as *Salmonella*, norovirus and hepatitis A virus). The risks of exposure to these infectious agents are compounded by the traditional consumption of raw, or only lightly cooked bivalve molluscs. Enteric bacteria, such as faecal coliforms and *Escherichia coli* (*E. coli*) have traditionally been used as indicator organisms to assess the sanitary quality of bivalve molluscs and their growing waters and to predict the risk of exposure to enteric pathogenic microorganisms.

## 2 SCOPE

This protocol has been produced with reference to Recommended Procedures for The Examination of Sea Water and Shellfish (4th ed.) 1970. American Public Health Association. New York. 105pp. (specifically, Part III, Section A, Subsection 3.0). Where appropriate, the format has been modified to assist laboratories accredited to, or following the principles of ISO 17025.

This protocol is primarily intended for the enumeration of faecal coliforms in seawater samples taken in association with a bivalve mollusc growing area sanitation programme (see FAO/WHO 2018). Using the appropriate volumes/dilutions. It may also be used for the enumeration of faecal coliforms in freshwater and effluent samples taken in association with such a programme.

Laboratories using this protocol to examine samples in support of a sanitation programme that is intended to comply with the US National Shellfish Sanitation Program (NSSP; e.g. for trade purposes) should ensure that the relevant parts of the following references are satisfied:

- NSSP Section II Chapter III: Laboratory (FDA 2019)
- NSSP Section IV, Chapter II, Section 14: Approved NSSP Laboratory Tests: 1. Approved Methods for Microbiological Testing (FDA 2019)
- NSSP Section IV, Chapter II, Section 15: Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists. (FDA 2019)
- Recommended procedures for the examination of Sea Water and Shellfish (APHA 1970)

**Note:** Other sections of the NSSP (FDA 2019) may also be relevant. Other requirements may be specified by the FDA in relation to a Memorandum of Understanding with an exporting country.

## 3 PRINCIPLE

This protocol for the enumeration of faecal coliforms in seawater is based on a most probable number (MPN) technique with an initial incubation in Lauryl Tryptose Broth (LTB) incubated at  $35\pm 0.5$  °C for  $24\pm 2$  hours. The presence of faecal coliform is confirmed by inoculation of positive tubes in EC broth (ECB) and incubation at  $44.5\pm 0.2$  °C for  $24\pm 2$  hours. Determination of MPN is made using an MPN calculator.

The MPN format given in this protocol is based on five tubes per inoculation level (volume and dilution). A minimum of three different inoculation levels is recommended but further levels may be required to determine an endpoint. APHA (1970) gives three tubes per level as an alternative although five tubes are recommended. APHA (1970) also gives the possibility of using multiple tubes at a single inoculation volume or dilution: for this approach a minimum of twelve replicate tubes is identified.

## 4 SAFETY PRECAUTIONS

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before performing this procedure. Laboratory procedures should conform to the recommendations given in the WHO Laboratory Biosafety Manual Third edition (WHO 2004) or relevant national legislation or guidelines.

## 5 EQUIPMENT

- Autoclave (or media preparator including an autoclave component)
- $35\pm 0.5$  °C air incubator
- Incubator or recirculating water bath at  $35\pm 0.5$  °C and  $44.5\pm 0.2$  °C
- Calibrated Immersion-type thermometer, 1-55 °C, about 55 cm long, with 0.1 °C subdivisions
- pH-meter, with automatic or manual temperature compensation and having a resolution of 0.01 pH units
- Dilution bottles made of borosilicate glass, with polyethylene caps equipped with Teflon liners. (Commercially prepared dilution bottles containing sterile Butterfield's phosphate buffer can also be used)

- Pyrex tubes or bottles capable of containing 20 ml volume (plus airspace) and cap
- Pyrex tubes or bottles capable of containing 10 ml volumes (plus airspace)
- 6 x 50 mm Pyrex tubes for detection of fermentation (Durham tubes)
- Pipettor - automatic or manual for use with 1 ml and 10 ml sterile pipette tips
- 10 µl inoculating loops (platinum or sterile disposable) or sterile applicator stick
- Refrigerators at 5±3 °C

## 6 MEDIA AND REAGENTS<sup>A</sup>

Media stored under refrigeration should be allowed to equilibrate at room temperature before use (ISO 11133). For performance testing of media see Table 1.

**Note:** Dehydrated media purchased commercially should be prepared according to the manufacturer's instructions.

- Phosphate Buffered Dilution Water (PBDW)
  - Stock solution: formula per litre - de-ionised water 500±0.1 ml, potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) 34±0.1 g. Adjust to pH 7.2±0.2 with 1 N NaOH (approximately 150 to 175 mL of 1 N NaOH may be required to adjust to pH 7.2). Add deionised water to make up to a final volume of 1 litre. Sterilise by autoclaving at 121 °C for 15 minutes. Store at 5±3 °C.
  - Final solution: add 1.25 ml of Stock PBDW to 1 litre de-ionized water. Dispense in 90±1 ml or 99±1 ml volume (as required) in tubes (or bottles). Cap the tubes (or bottles) and sterilise by autoclaving at 121 ° for 15 minutes. Store at 5±3 °C.
- Lauryl Tryptose Broth (LTB)<sup>B</sup>
  - *Double strength broth*; formula per litre - de-ionised water 1±0.01 litre, Tryptose or Trypticase 40±0.1 g, Lactose 10±0.1 g, K<sub>2</sub>HPO<sub>4</sub> (dipotassium hydrogen phosphate) 5.5±0.1 g, KH<sub>2</sub>PO<sub>4</sub> (potassium dihydrogen phosphate) 5.5±0.1 g, NaCl (sodium chloride) 10±0.1 g, Sodium lauryl sulfate 0.2±0.1 g, Dispense 10±0.2 ml volumes in sterile tubes (or bottles) containing inverted Durham tubes. Cap the tubes (or bottles) and sterilize by autoclaving at 121°C for 15 minutes. The final pH of the medium should be 6.8±0.2 after sterilization. Store at 5±3 °C.
  - *Single strength broth*; formula per litre - de-ionised water 1±0.01 litre, Tryptose or Trypticase 20±0.1 g, Lactose 5±0.1 g, K<sub>2</sub>HPO<sub>4</sub> (dipotassium hydrogen phosphate) 2.75±0.1 g, KH<sub>2</sub>PO<sub>4</sub> (potassium dihydrogen phosphate) 2.75±0.1 g, NaCl (sodium chloride) 5.0±0.1 g, Sodium lauryl sulfate 0.1±0.1 g, Dispense 10±0.2 ml volumes in sterile tubes or bottles containing inverted Durham tubes. Cap the tubes or bottles and sterilize by autoclaving at 121°C for 15 minutes. The final pH of the medium should be 6.8±0.2 after sterilization. Store at 5±3 °C.
- EC broth (ECB); formula per litre - de-ionised water 1±0.01 litre, Tryptose or trypticase 20±0.1 g, Lactose 5±0.1 g, Bile salts (No.3) 1.5±0.1 g, K<sub>2</sub>HPO<sub>4</sub> (dipotassium hydrogen phosphate) 4±0.1 g, KH<sub>2</sub>PO<sub>4</sub> (potassium dihydrogen phosphate) 1.5±0.1 g, NaCl (sodium chloride) 5±0.1 g. Dispense 5 to 10 ml volumes into tubes or bottles containing inverted Durham tubes. Cap the tubes or bottles and sterilize by autoclaving at 121°C for 15 minutes. The pH of the medium should be 6.9±0.2 after sterilization. Store at 5±3 °C.

## 7 MICROBIOLOGICAL REFERENCE CULTURES

**Table 1: Microbiological reference strains used in this protocol**

Media type	Function	Organism	WDCM <sup>a</sup>	
Lauryl Tryptose Broth	Productivity	<i>Escherichia coli</i>	00013	Turbid growth. Gas
	Selectivity	<i>Staphylococcus aureus</i>	00034	Inhibition or no growth
EC broth	Productivity	<i>Escherichia coli</i>	00013	Turbid growth. Gas
	Selectivity	<i>Pseudomonas aeruginosa</i>	00025	No growth

<sup>a</sup> Follow the link <http://refs.wdcm.org/species.htm> to obtain culture collection numbers for WDCM reference strains.

**Note:** These strains may also be used as internal quality controls (See Section 10.1).

<sup>A</sup> Formulations provided in this protocol are based upon the use of Oxoid Ltd listed products for illustrative purposes, alternative suppliers of media and reagents can be used according to the manufacturers' instructions. Laboratories should determine that alternative products are fit for purpose.

<sup>B</sup> If stored at 5±3°C the broth will become cloudy or form a precipitate. This should clear at room temperature, but gas formation is the criterion of growth not turbidity.

## 8 PROCEDURES

### 8.1 Sample transport and receipt

Seawater samples should be placed in light-proof box for transport. A separate second sample of seawater should be included to allow the sample arrival temperature to be recorded. The temperature of microbiological samples during transport should reach between 0°C and 10°C within 4 hours of sample packing and then maintained within this range until receipt at the laboratory. If cool packs are used samples should not come into direct contact with their surfaces. Samples should not be frozen. Microbiological analyses should be commenced within 24 hours of sampling. Sample containers should be closed and separated to prevent cross contamination among them.

If initiation of microbiological examination cannot be undertaken within 24 hours of sample collection, or if sample transport or storage temperatures are outside the recommended range, verification studies should be undertaken to support the use of those conditions.

If the laboratory receives a sample or samples that do not conform to the requirements specified in the relevant protocol under the sanitation programme, this should be reported to the submitting authority or agency and any other associated actions required by the protocol should be followed.

### 8.2 Sample storage

Upon receipt in the laboratory the temperature of the samples should be recorded. To avoid contaminating the test sample water temperatures should be taken from the separate water sample container. Samples should be examined immediately - if storage in the laboratory is necessary then samples should be stored at  $3\pm 2^{\circ}\text{C}$  and should be processed within 24 hours of collection. If initiation of the microbiological analysis cannot be within 24 hours of sample collection, data should be generated to show that extended storage does not affect the microbiological content of the sample.

### 8.3 Sample preparation

The 'Neat' sample should be mixed <sup>c</sup> by gently inverting several times before testing begins. If dilutions are necessary, prepare decimal dilutions in PBSW by adding  $1\pm 0.1$  ml of 'neat' sample to  $9\pm 0.1$  ml of PBSW using a graduated pipette ( $10^{-1}$  dilution).

**Note:** Further decimal dilutions can be made in the same way if the sample is expected to be heavily polluted.

### 8.4 Enumeration of faecal coliforms in seawater

Where a sample does not require dilutions to be examined, aseptically inoculate five tubes (or bottles) containing  $10\pm 0.2$  ml of double strength LTB with  $10\pm 0.2$  ml of 'Neat' sample. Inoculate five tubes (or bottles) containing  $10\pm 0.2$  ml of single strength LTB with  $1\pm 0.1$  ml of the 'Neat' sample and inoculate five tubes (or bottles) containing  $10\pm 0.2$  ml of single strength LTB with  $0.1\pm 0.01$  ml of the 'Neat' sample.

**Note:** Where a sample requires further dilutions to be examined, inoculate five tubes (or bottles) containing  $10\pm 0.2$  ml of single strength LTB with  $1\pm 0.1$  ml of each dilution prepared. These should be incubated and examined as described below.

Prepare a set of controls for each batch of LTB inoculated, using a  $10\ \mu\text{l}$  sterile loop, inoculate separate double and single strength LTB tubes (or bottles) with the control strains (productivity and specificity) listed in Table 1. Inoculate a separate tube (or bottle) of double with an equal volume of diluent (PBSW) and leave one tube (or bottle) uninoculated.

Incubate all inoculate tubes (or bottles) at  $35\pm 0.5^{\circ}\text{C}$  for  $24\pm 2$  hours.

After incubation examine the LTB tubes (or bottles) for the presence of growth accompanied by gas present in the Durham tube (See Figure 1). These tubes (or bottles) denote a presumptive positive result and should be subjected to confirmation (Section 8.5).

---

<sup>c</sup> APHA (1970) specifies that: 'Sample and dilutions of sample should be shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation'



Figure 1. A negative (no growth) and positive (growth accompanied by gas production) tubes of LTB following incubation.

All negative LTB tubes (or bottles) should be re-incubated and examined after a total incubation time of  $48 \pm 3$  hours. Any tubes (or bottles) showing a positive result (growth accompanied by gas production) after  $48 \pm 3$  hours of incubation should also be subjected to confirmation (see section 8.5). Tubes not showing growth accompanied by gas production after  $48 \pm 3$  hours are recorded as 'Negative'.

### 8.5 Confirmation of faecal coliforms

To confirm the presence of faecal coliforms, gently mix each tube (or bottle) giving a presumptive positive result at 24 and/or 48 hours and aseptically transfer an aliquot to a separate tube (or bottle) of ECB using a sterile  $10 \mu\text{l}$  loop or applicator stick. Also subculture all LTB control tubes (or bottles) into separate ECB tubes (or bottles).

Incubate all inoculated ECB tubes (or bottles) at  $44.5 \pm 0.2$  °C for  $24 \pm 2$  hours.

After incubation examine the ECB tubes (or bottles) for the presence of growth accompanied by gas production. All negative ECB tubes (or bottles) should be re-incubated and examined after a total incubation time of  $48 \pm 2$  hours. Any ECB tubes (or bottles) not showing growth accompanied by gas production after  $48 \pm 2$  hours of incubation, record the final result as 'Negative'. Any ECB tubes (or bottles) showing the presence of growth accompanied by gas production after  $24 \pm 2$  and/or  $48 \pm 2$  hours, record the final result as 'Positive' result.

### 8.6 Calculation of faecal coliform concentration

To determine the MPN:

- **Record as negative**
  - i. All LTB tubes (or bottles) that were negative after  $48 \pm 3$  hours of incubation.
  - ii. All LTB tubes (or bottles) that were sub-cultured into ECB and a negative result was observed.
- **Record as positive** all LTB tubes (or bottles) that were positive and the subculture on to ECB was positive.
- The total number of tubes (or bottles) at each level should equal the sum of tubes (or bottles) that were positive at that dilution and all those tubes (or bottles) that were negative at that level.

The MPN value is determined using the number of positive tubes (or bottles) at each level. See Section 8.6.1 for the use of the MPN calculator and Section 8.6.2 for use of the MPN tables.

**Note:** MPN tube (or bottle) combination falls into one of three probability categories. 95% of observed tube combinations fall into category 1 with 4% and 1% in categories 2 and 3 respectively. Both the category and MPN result can be determined using the Water MPN calculator and should be determined using the results obtained from all dilutions tested. Only values determined from combinations that give a category 1 or 2 profile should be recorded. Category 3 values should be recorded as 'Void'.

#### 8.6.1 The Water MPN calculator

Attached to this protocol is a copy of an MPN calculator that is referenced in Jarvis, *et al.* (2010). To calculate the MPN value, input the data into the MPN calculator attached as described below and Appendix 2.

**Note:** It is important to note that this calculator is different to those provided for use with the microbiological examination of bivalve molluscs.

To use the MPN calculator attached to this protocol:

- A. Open the Excel spreadsheet by double clicking on the link.

- B. If Excel macros are not already enabled on the computer, single click on the 'Enable content' button that appears at the top of the spreadsheet.
- C. Enter the following minimum amount of information:
  - The number of test series in row 7 column 3 (each test series usually relates to one sample).
  - The maximum number of dilutions used in the tests in row 7 column 4.
- D. The input tables for the specified number of test series should appear (a separate table for each test series).
- E. For each test series:
  - Enter the dilution factor for each row in column 1 (this will be 1 if all tubes (or bottles) contain undiluted sample)
  - Enter the sample volume in column 2 (this will usually be 10 ml for the first set of tubes (or bottles), 1 ml for the second set of tubes (or bottles), 0.1 ml for the third set of tubes (or bottles))
  - Enter the total number of tubes (or bottles) examined for each set of tubes (or bottles) in column 3 (this will usually be 5 for the standard 5 tube (or bottle) 3 dilution (set) MPN)
  - Enter the number of positive tubes (or bottles) for each set of tubes (or bottles) in column 4.
- F. Press the 'calculate results' button in the top right of the spreadsheet or press 'Ctrl + m'. The calculated MPN value is then displayed in column 8 as MPN per 1 ml.
- G. Multiply the obtained result by 100 to obtain MPN/100 ml.

**Note:** The MPN values obtained with the calculator for a small number of tubes (or bottles) combinations differ slightly from the values given in the table in Appendix 1 and thus also from Table 11 of APHA (1970). The laboratory should determine whether this is acceptable to any appropriate regulatory agency(ies) (e.g. the US Food and Drug Administration for exports to the US).

For example:

- a) The test format was:
  - I. First set: 10 ml of neat sample into each of 5 tubes (or bottles) of double strength LTB.
  - II. Second set: 1 ml of neat sample into each of 5 tubes (or bottles) of single strength LTB.
  - III. Third set: 0.1 ml of neat sample into each of 5 tubes (or bottles) of single strength LTB.
 With confirmation of positive tubes (or bottles) using ECB.
- b) The confirmed results were:
  - I. First set: 5 tubes (or bottles) were positive.
  - II. Second set: 3 tubes (or bottles) were positive.
  - III. Third set: 0 tubes (or bottles) were positive (that is, all were negative).
- c) Open the attached MPN calculator spreadsheet.
- d) Enter the following minimum amount of information:
  - I. If required, enter the name of experiment in row 7 column 1 and the date of experiment in row 7 column 2.
  - II. Enter the number 1 in row 7 column 3 (assuming only one test was undertaken on one sample).
  - III. Enter 3 in row 7 column 4 as the maximum number of dilutions used (equivalent to the maximum number of sets of tubes (or bottles) used).
- e) One sample data input table should appear (if 1 was entered in step d.ii).
- f) For the sample data input table:
  - I. If required:
    - Enter the sample number or name in row 13 column 3 (the row number will be different if this is not the first and only sample tested)
    - Enter the target organism (for example, Faecal coliforms) and test medium (for example, LTB and EC medium) in row 13 Column 4 (again, the row number will be different if this is not the first and only sample tested)
  - II. In column 1, enter 1 for the dilution factor (d) for each of the three sets
  - III. In column 2, enter 10 ml for the volume (w) for set 1; 1 ml for the volume for set two, 0.1 ml for the volume for set 3
  - IV. In column 3, enter 5 as the number of tubes (n) for each set
  - V. In column 4:
    - Enter 5 as the number of positive tubes (or bottles) (x) for set one.



- Enter 3 as the number of positive tubes (or bottles) (x) for set two.
  - Enter 0 as the number of positive tubes (or bottles) (x) for set three.
- g) Press the 'calculate results' button in the top right of the spreadsheet or press 'Ctrl + m'
- h) The MPN for the sample is given in the "Results of the MPN calculations" table as 0.79 per 1 ml.
- i) Multiply by 100 to obtain the result per 100 ml:  $0.79 \times 100 = 79$  per 100 ml.

### 8.6.2 MPN tables

Table 1 (Appendix 1) lists the MPN tube (or bottle) combinations for a 3-dilution set of five tubes (or bottles) using the same sample concentration. To determine the MPN value, find the line that contains the appropriate number of positive tubes (or bottles).

**Note:** This table has been derived from Table 11 in APHA (1970) but excludes all the tube (or bottle) combinations conforming to category 3 (which are deemed unlikely to occur with a properly conducted test).

**Note:** If more than three levels (sets of tubes) have been used, it is recommended that the MPN calculator is used so that the information from all the levels are used to determine the MPN value.

## 8.7 Reporting results

Results should be reported as "Faecal Coliform MPN /100 ml". Results should be given to two significant figures (the table given in Appendix 1 only contain values given to two significant figures but some MPN calculators may give output containing more significant figures).

## 9 UNCERTAINTY OF TEST RESULTS

Uncertainty inherent in any test method, i.e. instruments, media, analyst performance etc can be assessed by the repeatability and reproducibility of test results. These should be monitored through control tests analysed alongside sample tests, through in-house comparability testing between analysts and through external inter comparison exercises, which would highlight any uncertainties within the test methods.

The inclusion of the measurement of uncertainty (MU) or confidence limits (CL) for the result is not required but it is recommended that laboratories determine, as part of their quality procedure, the MU for the reported results and provide this information on request.

## 10 QUALITY CONTROL

Quality control systems offer a laboratory a mechanism to control preventable inaccuracies within a procedure.

### 10.1 Internal Quality Assurance (IQA)

It is recommended that regular (e.g. monthly) monitoring using known levels of target organism are examined to ensure that routine Faecal coliform procedures continue to be efficient and effective. Certified reference materials containing a target number of microorganisms (for example Lenticule discs) are available from commercial sources for use in internal quality assurance procedures. The target concentration of faecal coliforms in samples prepared using reference materials, should be appropriate to the usual range of faecal coliforms concentrations expected in routine samples. The target concentration of faecal coliforms in samples prepared using reference materials should be appropriate to the usual range of faecal coliforms expected in routine samples.

### 10.2 Comparative testing

It is recommended that laboratories undertaking microbiological examination of samples as part of a bivalve mollusc sanitation programme should take part in comparative laboratory testing relevant to the sample type(s) and method(s). Participation may be required under the sanitation programme (by national legislation or official procedures) or specified by importing countries. Comparative testing provides an indication of performance relative to other participating laboratories and can help improve the performance of the laboratory.

## 11 References

**Regulations, guidance documents and international standards may be superseded, revised or updated over time. The user of this protocol should make sure that they refer to the most recent version.**

FDA 2019. NSSP Guide for the Control of Molluscan Shellfish: 2019 Revision. [National Shellfish Sanitation Program \(NSSP\) Guide for the Control of Molluscan Shellfish: 2019 Revision \(fda.gov\)](#)

FDA 2017. Enumeration of *Escherichia coli* and the Coliform Bacteria. In: FDA Bacteriological Analytical Manual

(BAM). <https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm>

FAO/WHO 2018. Technical guidance for the development of the growing area aspects of bivalve mollusc sanitation programmes.

ISO 11133. Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media. International Organization for Standardization: Geneva, Switzerland.

WHO 2004. Laboratory biosafety manual – third edition. Geneva: World Health Organization. 178pp. <http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf>

Jarvis *et al* (2010). Reconsideration of the derivation of Most Probable Numbers, their standard deviations, confidence bounds and rarity values. *Journal of Applied Microbiology* 109, 1660 – 1667.

## 12 APPENDICES

## 12.1 Most probable numbers (MPN) per 100 ml of sample, inoculating 5 replicate portions of geometrically decreasing volumes at 3 levels (based on Table 11 of APHA (1970))

No. Positive Tubes			MPN/100 ml	CATEGORY
10 ml	1 ml	0.1 ml		
0	0	0	<1.8	1
0	1	0	1.8	1
1	0	0	2.0	1
1	1	0	4.0	2
1	2	0	6.1	1
2	0	0	4.5	2
2	0	1	6.8	1
2	1	0	6.8	2
2	1	1	9.2	1
2	2	0	9.3	2
3	0	0	7.8	1
3	0	1	11	1
3	1	0	11	1
3	1	1	14	1
3	2	0	14	2
3	2	1	17	1
3	3	0	17	2
4	0	0	13	2
4	0	1	17	1
4	1	0	17	1
4	1	1	21	1
4	2	0	22	1
4	2	1	26	2
4	3	0	27	1
4	4	0	34	2
5	0	0	23	1
5	0	1	31	1
5	1	0	33	1
5	1	1	46	1
5	1	2	64	1
5	2	0	49	1
5	2	1	70	1
5	2	2	95	1
5	3	0	79	1
5	3	1	110	1
5	3	2	140	1
5	3	3	180	2
5	4	0	130	1
5	4	1	170	1
5	4	2	220	1
5	4	3	280	1
5	4	4	350	2
5	5	0	240	1
5	5	1	350	1
5	5	2	540	1
5	5	3	920	1
5	5	4	1600	1
5	5	5	>1600	1

**Notes:**

1. The tables given in APHA (1970) give MPN values for all possible tube or bottle combinations including those that are considered to be unlikely to occur under valid analytical conditions.
2. Values given in this table are taken from Table 11 of APHA (1970). More recent MPN calculators give slightly different MPN values for some tube or bottle combinations.


## 12.2 Appendix 2 – Application and use of the MPN calculator

1. Open the link <http://standards.iso.org/iso/7218/> (ISO 7218 – Amendment 1, section 10.5.6.3).
2. The ISO Standard Maintenance portal screen will appear. Click once the underlined name 'MPN calculator Excel programme ver5'.

### ISO Standards Maintenance Portal

[all / iso / 7218](#)

Type Name

 [MPN calculation Excel programm\\_ver5.xls](#)

3. On clicking the 'MPN calculator' link, an MPN calculator program excel spreadsheet will open. Click on the 'Enable Content' button at the top of the spreadsheet to enable all macros.



4. Enter the number of test series and dilutions in the relevant cells. Once the cells are filled, the required number of data boxes will automatically appear below (one for each test series specified).

- 'Name of experiment' – The name given for the work performed.
- 'Date of experiment' – The date the work was carried out.
- 'No. of test series' – The value recorded will determine how many sets of sample tables will be generated automatically. A total of 30 samples can be inputted into the spreadsheet at one time. Each test series relates to one sample.
- 'Max. no. of dilutions' – The value recorded is based on the number of dilutions used in the tests.

General data and data for generating the input tables				
5				
6	Name of experiment	Date of experiment	No. of test series	Max. no. of dilutions
7	My sample	31/08/2018	1	3
8	<b>Note:</b> A test series / matrix consists of the different dilutions for one target organism / test medium.			
9				
10	<b>Note:</b> The dilution factor $d$ is the dilution ratio used for inoculating the tubes of that row, i.e. 1.0 (undiluted), 0.1 (diluted 1 in 10), etc. The volume $w$ is the volume of the dilution added to each tube in that row.			
11				

Number of dilutions examined

Number of samples tested

5. Populate the data table as follows:

- Enter the dilution factor for each row in column 1 (this will be 1 if all tubes (or bottles) contain undiluted sample)
- Enter the sample volume in column 2 (this will usually be 10 ml for the first set of tubes (or bottles), 1 ml for the second set of tubes (or bottles), 0.1 ml for the third set of tubes (or bottles))
- Enter the total number of tubes (or bottles) examined for each set of tubes in column 3 (this will usually be 5 for the standard 5 tube (or bottle) 3 dilution (set) MPN)
- Enter the number of positive tubes (or bottles) for each set of in column 4.

Input data				
12				
13	Test series 1 / Matrix 1 - Designation:		My sample	
14	Target organism / Test medium:		Faecal coliforms/LTB and EC medium	
15	Dilution factor	Volume in ml or g	No. of tubes	No. of positive tubes
16	$d$	$w$	$n$	$x$
17	1.0	10.0	5	5
18	1.0	1.0	5	3
19	1.0	0.1	5	0

6. Press 'Ctrl M' or press Calculate results (green button). The MPN value (MPN/g) will be calculated in column 8 and the category in column 14.

Results of the MPN calculations								
No.	Test series / Matrix Designation	MPN	log <sub>10</sub> MPN	SD log <sub>10</sub> MPN	95% confidence limits		Rarity Index	Category
					Lower	Upper		
1	My sample	0.79	-0.10	0.25	0.25	2.5	1.000	1

MPN/g

Category number

7. Check the category of each sample tested and accept only MPN results that give a category 1 or category 2 profile. Reject all MPN results that fall within a category 3. .
8. The MPN calculator calculates the MPN per gram (MPN/g). Faecal coliform results are reported as MPN per 100 ml (MPN/100 ml). Multiply the value given in column 8 by 100 to give the results in MPN/100 ml.

For the examples given above

- For example 1 -  $0.79 \times 100 = 79$  per 100 ml.



## World Class Science for the Marine and Freshwater Environment

We are the government's marine and freshwater science experts. We help keep our seas, oceans and rivers healthy and productive and our seafood safe and sustainable by providing data and advice to the UK Government and our overseas partners. We are passionate about what we do because our work helps tackle the serious global problems of climate change, marine litter, over-fishing and pollution in support of the UK's commitments to a better future (for example the UN Sustainable Development Goals and Defra's 25 year Environment Plan).

We work in partnership with our colleagues in Defra and across UK government, and with international governments, business, maritime and fishing industry, non-governmental organisations, research institutes, universities, civil society and schools to collate and share knowledge. Together we can understand and value our seas to secure a sustainable blue future for us all, and help create a greater place for living.



© Crown copyright 2020

---

Pakefield Road, Lowestoft, Suffolk, NR33 0HT

The Nothe, Barrack Road, Weymouth DT4 8UB

[www.cefasc.co.uk](http://www.cefasc.co.uk) | +44 (0) 1502 562244

