FAO Reference Centre for Bivalve Mollusc Sanitation

Generic protocol – Enumeration of faecal coliform bacteria in seawater using the most probable number technique (based on APHA recommended procedures)

Author: Louise Stockley

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History of Procedure

Issue	Date	Section	Changes
1	26.03.21	All	First issue
2	15.05.26	Throughout	Minor corrections and improvements to text for clarity
		Throughout	Hyperlinks updated
		Title	Reference to the FDA Bacteriological Analytical Manual replaced by APHA recommended procedures as primary source of method protocol
		6	Media recipes reformatted
		8.4	Section renamed and reformatted
		8.6.1	Section rewritten and combined with Appendix 2 (now deleted)
		11	References updated with new editions where necessary. APHA recommended procedures added as primary source of method protocol
		Appendix 1	For a small number of tube combinations results in MPN/100ml have been changed to match the MPN calculator, rather than the values in APHA recommended procedures; these are indicated in footnotes

Contents

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 the American Public Health Association (APHA) Recommended Procedures for the Examination of Sea Water and Shellfish (4th edition (APHA, 1970), specifically Part III, Section A, Subsection 3.0), as recommended in the US National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish (US Food and Drug Administration, 2023). Minor modifications to the method have been introduced based on Chapter 4 of the Bacteriological Analytical Manual (US Food and Drug Administration, 2020) – these are detailed in footnotes. Where appropriate, the format has been modified to assist laboratories accredited to or following the principles of ISO/IEC 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, 2021). 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 NSSP (e.g. for trade purposes) should ensure that the relevant parts of the NSSP Guide are satisfied:

- Section II Chapter III: Laboratory
- Section IV, Chapter III, Laboratory

Note: other sections of the NSSP (US Food and Drug Administration, 2023) may also be relevant. Other requirements may be specified by the US Food and Drug Administration 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±0.5 °C for 24-48 hours. The presence of faecal coliforms is confirmed by inoculation of positive tubes in EC broth (ECB) and incubation at 44.5±0.2 °C for 24-48 hours. Determination of MPN is made using MPN tables or 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) and the NSSP Guide (US Food and Drug Administration, 2023) give three tubes per level as an alternative although five tubes are

recommended. These references also give 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)
- Incubators or recirculating water baths operating at 35±0.5 °C and 44.5±0.2 °C
- Thermometer with 0.1 °C subdivisions
- pH-meter with a resolution of 0.01 pH units
- Tubes (or bottles) suitable for preparing sample dilutions
- Tubes (or bottles) capable of containing 20 ml volume (plus airspace) and caps
- Tubes (or bottles) capable of containing 10 ml volumes (plus airspace) and caps
- Durham tubes (e.g. 6 x 50 mm) for detection of gas production
- 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
- Refrigerator operating at 5±3 °C

6 Media and Reagents

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. If any alternative products are used the laboratory must determine that they are fit for purpose.

- **Phosphate Buffered Dilution Water (PBDW)**
  - **Stock solution**
    - de-ionised water 500±5 ml
    - potassium dihydrogen phosphate (KH_2PO_4) 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 de-ionised 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.
  - **Working solution**

    Add 1.25±0.1 ml of stock PBDW solution to 1 litre de-ionized water. Dispense in 9±0.1 ml volumes in tubes. Cap the tubes and sterilise by autoclaving at 121 ° for 15 minutes. Store at 5±3 °C.

- **Lauryl Tryptose Broth (LTB)** [1]
  - **Double strength**
    - de-ionised water 1±0.01 litre
    - tryptose or trypticase 40±0.1 g
    - lactose 10±0.1 g
    - dipotassium hydrogen phosphate (K_2HPO_4) 5.5±0.1 g
    - potassium dihydrogen phosphate (KH_2PO_4) 5.5±0.1 g
    - sodium chloride (NaCl) 10±0.1 g
    - sodium lauryl sulfate 0.2±0.01 g

    Dispense 10±0.2 ml volumes in sterile tubes containing inverted Durham tubes. Cap the tubes 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**
    - de-ionised water 1±0.01 litre
    - tryptose or trypticase 20±0.1 g
    - lactose 5±0.1 g
    - dipotassium hydrogen phosphate (K_2HPO_4) 2.75±0.1 g
    - potassium dihydrogen phosphate (KH_2PO_4) 2.75±0.1 g
    - sodium chloride (NaCl) 5±0.1 g
    - sodium lauryl sulfate 0.1±0.01 g

    Dispense 10±0.2 ml volumes in sterile tubes containing inverted Durham tubes. Cap the tubes 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)**
  - 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
  - dipotassium hydrogen phosphate (K_2HPO_4) 4±0.1 g
  - potassium dihydrogen phosphate (KH_2PO_4) 1.5±0.1 g
  - sodium chloride (NaCl) 5±0.1 g

  Dispense 5 to 10 ml volumes into tubes containing inverted Durham tubes. Cap the tubes and sterilize by autoclaving at 121°C for 15 minutes. The final pH of the medium should be 6.9±0.2 after sterilization. Store at 5±3 °C.

[1] 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.

7 Microbiological reference cultures

The FAORC recommends the use of positive and negative controls throughout the procedure. The strains and criteria included in Table 1 are recommended for use as a minimum.

Table 1: Microbiological reference strains

Media type	Function	Organism	WDCM[a]	Characteristic reaction
Lauryl Tryptose Broth	Productivity	*Escherichia coli*	00013	Turbid growth. Gas
	Selectivity	*Staphylococcus aureus*	00034	Inhibition or no growth
EC Broth	Productivity	*Escherichia coli*	00013	Turbid growth. Gas
	Selectivity	*Pseudomonas aeruginosa*	00025	No growth

[a] Follow the link WDCM Reference Strain Catalogue to obtain culture collection numbers for WDCM reference strains.

8 Procedure

8.1 Sample transport and receipt

Seawater samples should be placed in a 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 be 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 cannot be maintained within 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±2 ˚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' (undiluted) sample should be mixed by gently inverting several times before testing begins [2].

Note: if the sample is expected to be heavily polluted then preparation of decimal dilutions should be carried out by adding 1±0.1 ml of 'neat' sample to 9±0.1 ml of PBDW using a graduated pipette (10^{-1} dilution), adding 1±0.1 ml of 10^{-1} dilution to 9±0.1 ml of PBDW using a graduated pipette (10^{-2} dilution) and so on.

8.4 Presumptive test for faecal coliforms

Aseptically inoculate five tubes for each of three inoculation levels:

- inoculate five tubes containing 10±0.2 ml of double strength LTB with 10±0.2 ml of 'neat' sample
- inoculate five tubes containing 10±0.2 ml of single strength LTB with 1±0.1 ml of 'neat' sample
- inoculate five tubes containing 10±0.2 ml of single strength LTB with 0.1±0.01 ml of 'neat' sample.

Note: where a decimal dilution series has been prepared for a sample as described in section 8.3, inoculate five tubes containing 10±0.2 ml of single strength LTB with 1±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 µl sterile loop, inoculate separate double and single strength LTB tubes with the control strains (productivity and specificity) listed in Table 1. Inoculate a separate tube of double strength LTB with an equal volume of diluent (PBDW) and leave one tube uninoculated.

Incubate all inoculated tubes and controls at 35±0.5 ˚C for 24±2 hours.

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

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

[2] APHA (1970) specifies that: 'Sample and dilutions of sample should be shaken vigorously (25 times in a 12" [12 inch] arc in 7 seconds) before inoculation'

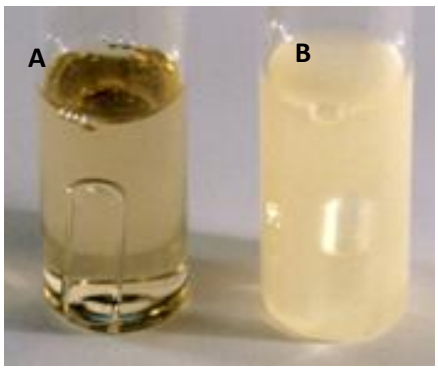

Figure 1. Negative (no growth; A) and positive (growth accompanied by gas production; B) tubes of LTB following incubation.

8.5 Confirmation of faecal coliforms

To confirm the presence of faecal coliforms, gently mix each tube giving a presumptive positive result at 24 and/or 48 hours and aseptically transfer an aliquot to a separate tube of ECB using a sterile 10 µl loop or applicator stick. Also subculture all LTB control tubes into separate ECB tubes.

Incubate all inoculated ECB tubes and controls at 44.5±0.2 °C for 24±2 hours.

After incubation examine the ECB tubes for the presence of growth accompanied by gas production. All negative ECB tubes should be re-incubated and examined after a total incubation time of 48±2 hours[3]. For any ECB tubes not showing growth accompanied by gas production after 48±2 hours of incubation, record the final result as 'Negative'. For any ECB tubes showing the presence of growth accompanied by gas production after 24±2 and/or 48±2 hours, record the final result as 'Positive'.

8.6 Calculation of faecal coliform concentration

To determine the Most Probable Number (MPN) first determine the number of negative and positive tubes at each inoculation level for each sample.

Record as negative:

i. All LTB tubes that were negative after 48±3 hours of incubation.

ii. All LTB tubes where the subcultured ECB tube was negative after 48±2 hours of incubation.

Record as positive all LTB tubes where the subcultured ECB tube gave a positive result (growth within 48±2 hours of incubation).

The sum of positive and negative tubes will equal the total number of tubes at each level.

The MPN value is determined using the number of positive tubes 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 combinations fall into one of three probability categories; 95 % of observed tube

[3] Re-incubation of ECB tubes that are negative after 24 hours is part of the method detailed in Chapter 4 of the Bacteriological Analytical Manual (US Food and Drug Administration, 2020). In APHA (1970), ECB tubes that are negative after 24 hours are considered as negative.

combinations fall into category 1 with 4 % and 1 % in categories 2 and 3 respectively. 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 Using the MPN calculator

A clean copy of the MPN calculator, developed by ISO/TC 34/SC 9 (the International Organization for Standardization committee for microbiology of the food chain), and suitable for use with seawater samples is available from the FAORC website (Method Guidance and Calculation Spreadsheets).

To use the MPN calculator;

A. Download and open a copy of the MPN calculator for seawater samples from the website.

B. If necessary, click on the 'Enable content' button at the top of the spreadsheet.

C. If required, enter relevant data in the general data section. The no. of test series (i.e. no. of samples; column C) and max. no. of dilutions cells (column D) must be populated; max. no. of dilutions should be set at 3 if following this protocol (see example in Figure 2), unless additional dilutions are used.

Note: name of experiment and date of experiment cells (columns A and B) do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files.

A	B	C	D
General data and data for generating the input tables			
Name of experiment	Date of experiment	No. of test series	Max. no. of dilutions
Experiment 1	08/05/2026	1	3

Figure 2. Example completion of general data section of MPN calculator.

D. Enter the dilution factor (column A; 1 for each inoculation level if following this protocol), volume in ml (column B; 10, 1 and 0.1 for the three inoculation levels if following this protocol) and the total number of tubes (column C; 5 for each inoculation level if following this protocol) in the input data section for each sample (see example in Figure 3).

Note: sample designation and target organism cells do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files.

Note: where a decimal dilution series has been prepared for a sample as described in section 8.3, appropriate dilution factors and volumes in ml should be entered (see example in Figure 4 where five tubes have been prepared using 1 ml sample at each of four dilutions)

A	B	C	D
Input data			
Test series 1 / Matrix 1 - Designation:		Sample X	
Target organism / Test medium:		Faecal coliforms	
Dilution factor *d*	Volume in ml or g *w*	No. of tubes *n*	No. of positive tubes *x*
1.0	10.0	5	5
1.0	1.0	5	0
1.0	0.1	5	0

Figure 3. Example completion of sample input data section of MPN calculator (5 tubes at each of 3 inoculation levels).

A	B	C	D
Test series 1 / Matrix 1 - Designation:		Sample Y	
Target organism / Test medium:		Faecal coliforms	
Dilution factor *d*	Volume in ml or g *w*	No. of tubes *n*	No. of positive tubes *x*
1.0	1.0	5	5
0.1	1.0	5	5
0.01	1.0	5	5
0.001	1.0	5	2

Figure 4. Example completion of sample input data section of MPN calculator (5 tubes at each of 4 dilutions).

E. For each sample, enter the number of positive tubes at each inoculation level in the input data section (column D; see examples in Figures 3 and 4).

F. Press the 'calculate results' button in the top right of the spreadsheet or press 'Ctrl + M'. The calculated MPN value is shown in column H as MPN per ml, and the probability category is shown in column N (see example in Figure 5).

F	G	H	I	J	K	L	M	N
Results of the MPN calculations								
Test series / Matrix		MPN	$\log_{10}$ MPN	SD	95% confidence limits		Rarity	Category
No.	Designation			$\log_{10}$ MPN	Lower	Upper	Index	
1	Sample X	0.23	-0.64	0.24	0.076	0.70	0.769	1

Figure 5. Example results output section of MPN calculator.

G. Multiply the obtained result (column H) by 100 to obtain results for each sample in MPN per 100 ml (e.g. for the example in Figure 5 the result would be expressed as 23 per 100 ml).

Note: if any data in the input data section is changed, the MPN value will need to be recalculated by re-pressing the 'calculate results' button or 'Ctrl + M'.

Note: the MPN values obtained with the calculator for a small number of tube combinations differ slightly from the values given in Table 11 of APHA (1970) as detailed in Table 2 in Appendix 1. 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).

8.6.2 Using the MPN tables

The MPN table provided in Table 2 (Appendix 1) lists the tube combinations for a test with 5 tubes at each of 3 inoculation levels. To find the MPN value, find the line in the table that contains the number of positive tubes for each inoculation level and thus read off the MPN value for that combination of results.

Note: the MPN table only contains category 1 and 2 combinations. Combinations of results that do not appear in the tables are category 3 (see above).

Note: if more than three inoculation levels have been used, it is recommended that the MPN calculator is used so that the information from all the levels is used to determine the MPN value.

8.7 Reporting results

Results should be rounded to 2 significant figures if necessary and reported in units of MPN per 100 ml faecal coliforms. Only values determined from combinations in probability categories 1 or 2 should be reported. Results for samples with tube combinations in probability category 3 should be reported as 'Void'.

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, in-house comparability testing between analysts and external intercomparison exercises, which would highlight any uncertainties within the test methods.

10 Quality control

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

10.1 Proficiency 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.

10.2 Internal quality controls

It is recommended that regular (e.g. monthly) monitoring is carried out using samples with known levels of target organisms 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.

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.

APHA, 1970. Recommended Procedures for the Examination of Sea Water and Shellfish, 4th Edition.

FAO/WHO, 2021. Technical guidance for the development of the growing area aspects of bivalve mollusc sanitation programmes, second edition. Food and Agriculture Organisation of the United Nations/World Health Organization.

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

US Food and Drug Administration, 2020. Bacteriological Analytical Manual Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria. October 2020 Edition.

US Food and Drug Administration, 2023. National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish: 2023 Revision.

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

Appendix 1: MPN table

Table 2: MPN table for 5 tube x 3 inoculation level method (5 x 10 ml, 5 x 1 ml, 5 x 0.1 ml)

Note: values in the MPN/100 ml column are those produced by the MPN calculator available from the FAORC website. For a small number of tube combinations these differ slightly from the values given in Table 11 of APHA (1970) (see footnotes to table). 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).

No. positive tubes			MPN/100 ml	Probability category
10 ml	1 ml	0.1 ml		
0	0	0	0[a]	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	63[b]	1
5	2	0	49	1
5	2	1	70	1
5	2	2	94[c]	1

No. positive tubes			MPN/100 ml	Probability category
10 ml	1 ml	0.1 ml		
5	3	0	79	1
5	3	1	110	1
5	3	2	140	1
5	3	3	170[d]	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	∞[a]	1

[a] Value not given in Table 11 of APHA (1970)

[b] 64 according to Table 11 of APHA (1970)

[c] 95 according to Table 11 of APHA (1970)

[d] 180 according to Table 11 of APHA (1970)

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Centre for Environment Fisheries & Aquaculture Science

Cefas

Pakefield Road, Lowestoft, Suffolk, NR33 0HT

The Nothe, Barrack Road, Weymouth, DT4 8UB

www.cefas.co.uk | +44 (0) 1502 562244