FAO Reference Centre Generic Protocol

Enumeration of *Escherichia Coli* in bivalve molluscan shellfish by the most probable number (MPN) technique (based on ISO 16649-3)

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History of Procedure

Issue	Date	Section	Changes
1	29.06.20	All	First issue
2	26.03.21	2	Paragraph giving information on protocols application

Contents

1   INTRODUCTION

The consumption of sewage polluted bivalve molluscs may cause illness in the consumer through ingestion of pathogens (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 *Escherichia coli* (*E. coli*) are 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.

Codex Standard 292-2008 (2015 revision; Codex Alimentarius 2015) identifies the use of a most probable number (MPN) method specified in ISO 16649-3 ('Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide') or equivalent for the enumeration of *E. coli* in live or raw bivalve molluscs. The method is also recommended in the FAO/WHO Technical guidance for the development of the growing area aspects of bivalve mollusc sanitation programmes (FAO/WHO 2018) and is the reference method for the enumeration of *E. coli* in bivalve molluscs under EU food hygiene legislation for both classification (European Union, 2019) and end product testing purposes (European Union, 2015).

2   SCOPE

This protocol has been produced with reference to ISO 16649-3 and selected parts of other relevant ISO standards referenced in Section 11. The theoretical limit of detection is an MPN of 18 *E. coli* per 100 g of bivalve mollusc flesh. In the context of this protocol, *E. coli* produces acid from lactose at 37±1 ˚C and expresses β-glucuronidase activity at 44±1 ˚C.

Laboratories using this protocol to examine samples in support of a sanitation programme intended to comply with official controls for the application of food and feed law with regard to live bivalve molluscs for the European Union should ensure that the relevant parts of the following references are satisfied.

3   PRINCIPLE

This protocol for the enumeration of *E. coli* in bivalve molluscs is based on a two-stage, five-tube (or bottle) three-dilution MPN format (although additional dilutions can be used). The first stage of the method is a resuscitation step requiring inoculation of minerals modified glutamate medium (MMGM) with a series of diluted bivalve mollusc homogenate and incubation at 37±1 ˚C for 24±2 hours. The presence of *E. coli* is confirmed by sub-culturing tubes or bottles showing acid production onto agar containing 5-bromo-4-chloro-3-indolyl-β-D glucuronide and detecting β-glucuronidase activity. Additional dilutions may be required to give a required end-point.

Note: Strains of *E. coli* that do not grow at 44±1 ˚C and those that are β-glucuronidase negative, such as *E. coli* O157 and some other strains of pathogenic *E. coli,* will not be detected by the procedure given in this protocol.

4   SAFETY PRECAUTIONS

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before using this protocol. Homogenisation of shucked bivalve molluscs should be performed in a Class II safety cabinet to reduce the risk of infection from aerosol inhalation. 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)
- Drying cabinet or ventilated oven capable of being maintained between 25±1 °C and 50±1 °C
- Balance capable of weighing to ±0.1 g and reference weights for calibration
- pH-meter, with automatic or manual temperature compensation and having a resolution of 0.01 pH units and accurate to within ±0.1 pH unit at 25 °C. Reference solutions for calibrating the pH meter
- Local Exhaust Ventilation (LEV; for weighing powders during media preparation)
- Class II safety cabinet
- Thermometer with a resolution of 0.1 °C or better at the temperature being measured
- Sterile glass tubes or screw-topped bottles capable of holding 20 ml volumes (for liquid media)
- Sterile petri dishes (for solid media): diameter 90 mm; height 15-16 mm; vented
- Shucking knife, mechanical oyster shucker or other suitable equipment for opening bivalve molluscs
- Weighing dish capable of holding the largest size of bivalve mollusc to be tested plus released intravalvular fluid. Must be capable of being cleaned and sterilized. [For use with oyster cracker]

- Safety gloves – for example a chain mail glove (for the bivalve opening procedure)
- Waring blender and 1 litre blending jars or stomacher and stomacher bags
- Refrigerators at 3±2 ˚C and 5±3 ˚C
- Sterile container of at least 500 ml capacity (A larger capacity may be required when large bivalve molluscs are to be examined. Examples include *Panopea generosa* (geoducks), *Mya arenaria* (soft shell clams; sand gapers) and some types of razor clams
- Safety Bunsen burner or electric micro-incinerator
- Protective gloves – single use
- Incubator or recirculating water bath at 37±1 ˚C and 44±1 ˚C [A]
- Platinum or sterile disposable loops - 10 µl (or 1 µl; see Section 8.6)
- Pipettor - automatic or manual for use with 1 ml and 10 ml sterile pipette tips or 1 ml and 10 ml sterile open-ended graduated pipettes.
- Sterile measuring cylinder – 250 ml (a larger cylinder may be needed for some species)

6   MEDIA [B]

Media stored under refrigeration should be allowed to equilibrate at room temperature before use (ISO 11133). If necessary, dry the surface of plated media before use. For performance testing of media see Table 1.

Note: ISO 16649-3 contains further details for the preparation of MMGM and TBGA from the basic ingredients. Dehydrated media purchased commercially should be prepared according to the manufacturer's instructions.

- Ethanol
- 0.1 % peptone (0.1 % P); formula per litre - de-ionised water 1±0.01 litre, peptone bacteriological 1±0.1 g. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121 °C for 15 minutes. Store at 5±3 °C in the dark.
- Peptone salt solution (PSS); formula per litre - de-ionised water 1±0.01 litre, peptone 1±0.1 g, sodium chloride 8.5±0.1 g, pH 7±0.2. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121 °C for 15 minutes. Store at 5±3 °C in the dark.
- Single strength Minerals Modified Glutamate Medium (MMGM); - formula per litre - de-ionised water 1±0.01 litre, ammonium chloride 2.5±0.1 g, sodium glutamate 6.4±0.1 g, minerals modified medium base 11.4±0.1 g. pH 6.7±0.1. Dispense aseptically in 10±0.2 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize by autoclaving at 116 °C for 10 minutes. If not used immediately after cooling to room temperature, Store at 5±3 °C in the dark.
- Double strength MMGM; - formula per litre - de-ionised water 1±0.01 litre, ammonium chloride 5±0.1 g, sodium glutamate 12.7±0.1 g, minerals modified medium base 22.7±0.1 g, pH 6.7±0.1. Dispense aseptically in 10±0.2 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize the medium by autoclaving at 116 °C for 10 minutes. If not used immediately after cooling to room temperature, Store at 5±3 °C in the dark.
- Tryptone bile glucuronide agar (TBGA; also marketed commercially as TBX); formula per litre - de-ionised water 1±0.01 litre, tryptone bile x-glucuronide medium 36.6±0.5 g, pH 7.2±0.2. Sterilize by autoclaving at 121 °C for 15 minutes. Cool to 50 °C and pour 18 ml to 20 ml of molten medium into sterile Petri dishes and allow to solidify. Store at 5±3 °C in the dark and protected from desiccation for up to four weeks unless results of the laboratory shelf life validation indicate a longer shelf-life. Plates should be dried before use to remove excess surface moisture (see ISO 11133 for further considerations in relation to drying). Drying is especially important where TBGA plates are to be subdivided for the inoculation of more than one tube from a single dilution.

[A] In countries where the ambient temperature exceeds the target incubation temperature, an incubator with both heating and cooling units may be required to maintain the required temperature range.

[B] Formulations provided in this generic 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.

7 MICROBIOLOGICAL REFERENCE CULTURES FOR PERFORMANCE TESTING AND CONTROLS

Table 1: Microbiological reference strains used for media performance testing

Media type	Function	Control strain	WDCM [a]	Characteristic reaction	Strain choice [c]
Mineral-modified glutamate medium (MMGM)	Productivity	*Escherichia coli*	00012 or 00013	Acid production. Colour change to yellow	1
	Selectivity	*Enterococcus faecalis*	00009 or 00087	No growth. No colour change	1
Tryptone bile glucuronide agar (TBGA)	Productivity (and characteristic reaction)	*Escherichia coli*	00202	Blue to blue-green colonies. B-glucuronidase positive (weak)	2
		Escherichia coli	00012 or 00013	Blue to blue-green colonies. B-glucuronidase positive	1
	Selectivity	*Enterococcus faecalis*	00009 or 00087	Total inhibition. No growth	1
	Specificity	*Pseudomonas aeruginosa* [b]	00025	White to beige colonies. B-glucuronidase negative	1
		Citrobacter freundii [b]	00006		

[a] Follow the link http://refs.wdcm.org/species.htm to obtain culture collection numbers for WDCM reference strains.
[b] The use of *P. aeruginosa* (WDCM 00025) or *C. freundii* (00006) is given in ISO 16649-3. The FAORC has noted that the *C. freundii* strain does not always produce growth on TBGA plates and therefore recommends the use of *P. aeruginosa* instead of *C. freundii* as a control for this medium.
[c] Reference strains given in ISO 16649-3 for use in performance testing. Strain choice: 1 - Laboratory must select 1 strain as a minimum from the list provided for each media type; 2 - Strain to be used as a minimum.

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

8 PROCEDURES

8.1. Sample transport and receipt

Samples must be received in an intact food-grade plastic bag, or equivalent, and properly packed in a cool box with ice packs – packed in this manner they should reach a temperature between 0 ˚C and 10 ˚C within 4 hours and then maintain this for at least 24 hours. The specific cool box, ice pack and transport condition combination should be validated to ensure that this can be achieved (https://www.cefas.co.uk/nrl/information-centre/nrl-laboratory-protocols/) or samples where less than 4 hours have elapsed between collection from the growing area and receipt at the laboratory, internal air temperature should be less than the temperature recorded at the time of sampling. Samples should be regarded as unsatisfactory if on receipt at the laboratory the sample is frozen, the container is leaking, the bivalve molluscs are covered in mud or immersed in water or mud/sand.

The sample transport criteria given here are extracted from ISO 6887-3. The use of alternate sample transport criteria may be acceptable, where verification studies have been undertaken and the results of those studies demonstrate that there is no significant effect on the quality of the test results. For samples being taken in support of a growing area sanitation programme, it is recommended that verification studies supporting the use of sample transport criteria outside of the ranges given in ISO 6887-3 are approved by the responsible authority. (See Section 4.3.6 and Annex 13 of the FAO/WHO technical guidance (http://www.fao.org/3/CA1213EN/ca1213en.pdf).

Note: The UK National Reference Laboratory for foodborne viruses and bacteriological contamination of bivalve molluscs has carried out studies on the effect of extended storage time and elevated temperatures on certain species of bivalve molluscs. The report of this work can be accessed using the following web link https://www.cefas.co.uk/nrl/information-centre/nrl-reports/

8.2 Sample storage

Upon receipt in the laboratory the temperature of the samples should be recorded. 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, due to logistical problems, microbiological analysis of samples taken in support of a growing area programme cannot be started within 24 hours of sample collection, a verification study should be undertaken to show that extended storage does not affect the microbiological content of the sample.

8.3 Sample selection

Choose bivalve molluscs that are alive according to the following points:

- Movement of exposed flesh to touch using a sterile shucking knife
- Bivalve molluscs are open and close of their own accord
- A tap on the shell causes closing or movement
- Tightly closed bivalve molluscs

Discard all dead bivalve molluscs and those with obvious signs of damage. Select the appropriate number depending on the species (Appendix 1). More bivalve molluscs can be used, if necessary, to produce the required volumes for each analysis.

8.4 Sample preparation

Any material adhering to the shell should be removed prior to opening by rinsing/scrubbing under cold, running tap water of potable quality. Bivalve molluscs should not be re-immersed in water as this may cause them to open. Open all selected bivalve molluscs as described below.

8.4.1 Mechanical oyster shucker

An example of a mechanical oyster shucker is shown in Figure 1. Sterilise the pin of the mechanical oyster shucker before use. Place a single animal in a sterilized weighing dish underneath the shucking device and rest the shucking lever at the hinge. Pull the handle down to separate the shells. Using a sterilised shucking knife, cut the muscle and scrape the meat of both shell halves into the sterilised container. Transfer any liquor collected in the weighing boat into the sterilised container.
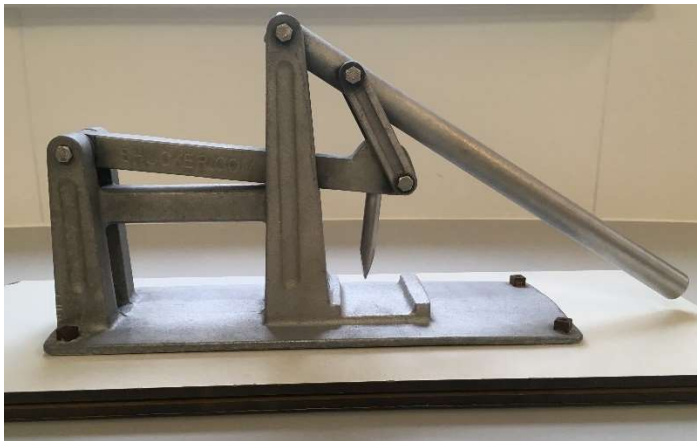

Figure 1. One type of mechanical oyster shucker

8.4.2 Shucking with a knife

Using a sterilised shucking knife, open all selected bivalve molluscs as described below. If sterilised by heating allow the knife to cool before use. When opening bivalve molluscs, ensure that the hand holding the shell is protected with a heavy-duty safety glove to prevent cuts.

8.4.2.1 Oysters and large clams

Insert the knife between the two shells towards the hinge end of the animal. Push the knife further into the animal and prise open the upper shell, allowing any liquor to drain into the sterilised container. Push the blade through the animal and sever the muscle attachments by sliding across the animal. Remove the upper shell and scrape the contents of the lower shell into the sterilised container.

8.4.2.2 Mussels, cockles and small clams

Insert the knife in between the shells of the animal and separate the shells with a twisting motion of the knife. Collect the liquor from the animal in the sterilised container, then cut the muscle between the shells and scrape the contents into the sterilised container.

8.5 Dilution and homogenisation

Weigh the sterile container and calculate the weight of the contents by subtracting the weight of the pre-weighed sterile container to the nearest gram. Add 2 ml of sterile 0.1 % P per 1 g of shucked bivalve molluscs using a sterilised measuring cylinder and measure to ±2 ml.

Note: ISO 6887-3 allows the use of PSS for the preparation of the primary dilution. FAORC advice is to use 0.1 % P instead at this stage to avoid the addition of extra NaCl.

Then complete either Section 8.5.1 or 8.5.2.

The FAORC recommends using a rotary blender and not a stomacher when preparing clam and cockle samples for *E. coli* enumeration. Results from proficiency testing distributions have shown that lower and/or more variable results may be obtained with some clam and cockle species after homogenisation using a stomacher.

8.5.1   Homogenisation in a rotary blender (homogeniser)

Place the contents of the sterilised container into a sterilised 1 litre blender jar and homogenise at high speed for approximately 1 minute (4 bursts of 15 seconds with at least 5 seconds between bursts) in a class II safety cabinet. Decant the contents back into the sterilised container.

Note: If animals are particularly small, it may be necessary to use a smaller blender to achieve a consistent homogenate.

8.5.2   Homogenisation in a stomacher

If a stomacher (peristaltic homogeniser) is used, the initial homogenisation should be carried out using a proportion of the volume of diluent calculated, and the resultant homogenate added to the rest of the final volume and thoroughly mixed.

Place the contents of the sterile container into the inner of at least three stomacher bags, placed inside each other, to avoid small pieces of shell from puncturing the bags. Remove excess air from the bag and operate the stomacher for 2 - 3 minutes.

Add 30±0.5 ml of mixed homogenate from 8.5.1 or 8.5.2 to 70±1 ml of 0.1 % P using a 10 ml open-ended pipette to make a master 10^{-1} dilution. Thoroughly mix by vigorous shaking of the bottle. Make further decimal dilutions to 10^{-2} in 0.1 % P by adding 1±0.1 ml of the 10^{-1} dilution to 9±0.1 ml of 0.1 % P using a 1ml open-ended pipette. If samples are expected to be heavily polluted (Category C or above) further decimal dilutions may be necessary.

Any diluent left in opened bottles or flasks at the end of the working day should be discarded.

Note: For samples where more than 3 dilutions are tested, ensure the results from each dilution tested is used to calculate the MPN value in Section 8.8.

8.6   Inoculation and incubation of MMGM

MMGM tubes or bottles should be inoculated within 45 minutes of preparing the initial sample suspension (ISO 7218 Section 9.2.1). Inoculate five tubes (or bottles) containing 10±0.2 ml of double strength MMGM with 10±0.2 ml of the 10^{-1} diluted homogenate (equivalent to 1 g of tissue per tube). Inoculate five tubes (or bottles) containing 10±0.2 ml of single strength MMGM with 1±0.1 ml of the 10^{-1} diluted homogenate (0.1g). Inoculate five tubes (or bottles) containing 10±0.2 ml of single strength MMGM with 1±0.1 ml of the 10^{-2} diluted homogenate (0.01 g) and repeat with any further dilutions.

Prepare a set of controls for each batch of MMGM inoculated, using a 10 μl sterile loop, inoculate separate double and single strength MMGM 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 (0.1% P or PSS) and leave one tube (or bottle) uninoculated.

Incubate all inoculate tubes (or bottles) at 37±1 ˚C for 24±2 hours.

8.7   Confirmation of *E. coli*

After incubation examine the MMGM tubes (or bottles) for the presence of acid. Acid production is denoted by the presence of any yellow coloration throughout the medium or change from the negative control (see Figure 2). Confirm the presence of *E. coli* in each tube (or bottle) showing acid production by sub-culturing onto the surface of a TBGA plate (whole plate) within 4 hours, using a 10 μl sterile loop, streaking with the objective to obtain single colonies. Also subculture all MMGM control tubes (or bottles) onto separate TBGA plates for each set of tests undertaken. Incubate all TBGA plates at 44±1 ˚C for 21±3 hours in stacks no more than six plates high and separated from each other and the incubator walls by at least 25 mm.

Figure 2. MMGM at three dilutions after incubation showing change of colour

Note: TBGA plates can be subdivided into 5 segments, with each segment being inoculated from a single presumptive positive tube (or bottle). It is recommended to use separate plates for each dilution level even if there are less than 5 tubes (or bottles) to subculture. When using subdivided plates, it is recommended to use a 1 µl loop for subculture to help avoid overgrowth of target colonies. Comparative work carried out by the FAORC showed that sub-culturing MMGM tubes using a 1µl loop onto TBGA plates divided into 5 segments did not affect the MPN value.

Following incubation, examine the TBGA for the presence of blue or blue-green colonies. Record the results as positive for any shade of blue or blue-green colonies, negative for colonies of any other colour and no growth. See Figure 3, image A for the results of positive and negative reference controls sub-cultured onto individual TBGA plate and Image B using a subdivided plate for separate tubes from a single dilution.

Figure 3. Image A: Positive (blue-green colonies) and negative (cream colonies) subcultures on TBGA. Image B: Results of positive and negative MMGM tubes sub-cultured on a subdivided TBGA plate.

8.8 Determination of *E. coli* most probable number (MPN)

To determine the MPN, record as negative all MMGM tubes (or bottles) that either do not produce acid after incubation or are negative (i.e. no blue or blue-green colonies) following subculture and incubation on TBGA plates. Record as positive MMGM tubes (or bottles) that produce acid after incubation and are positive (i.e. presence of blue or blue-green colonies) following subculture and incubation on TBGA plates. The total number of tubes (or bottles) at each dilution should equal the sum of tubes (or bottles) that are positive and negative at that dilution. The value for the positive tubes (or bottles) at each dilution are used to determine the MPN by means of either the bivalve mollusc MPN calculator (see Section 8.8.1) or MPN tables (see Section 8.8.2).

The number of positive tubes (or bottles) at each dilution is used to look up the corresponding MPN value in the tables given in Appendix 2 and is the value entered in Column 4 of the bivalve mollusc MPN calculator. See Section 8.8.1 for the use of the bivalve mollusc MPN calculator and Section 8.8.2 for use of the MPN tables.

Note: MPN tube (or bottle) combination falls into one of three probability categories. 95 % of observed tube or bottle 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 bivalve mollusc 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'.

8.8.1 The bivalve mollusc MPN calculator

The bivalve mollusc MPN calculator has been developed specifically for use in bivalve mollusc testing.

Below are three versions of the bivalve mollusc MPN calculator. Two have been pre-populated with the 3 and 4 dilution factors respectively, volume of dilution inoculated per tube (or bottle) in grams and the number of MMGM tubes (or bottles) inoculated as five per dilution. The third version of the calculator has not been populated with these parameters and can be used when it is necessary to use different values for these.

| Shellfish MPN calculator - 3 dil..xls | Shellfish MPN calculator - 4 dil..xls | Shellfish MPN calculator - Blank.xls |

To calculate the MPN value, input all data obtained from each dilution examined into the bivalve mollusc MPN calculator attached to this protocol as described below and Appendix 3.

Note: The Excel spreadsheet MPN calculator cited in ISO 7218 (http://standards.iso.org/iso/7218/) can also be used to calculate the MPN value. The MPN value calculated using the MPN calculator cited in ISO 7218 is reported as MPN/g and must be converted to MPN/100 g. Also, the values given for all negative or all positive tubes (or bottles) by the MPN calculator cited in ISO 7218 do not follow the traditional approach given in Section 8.9 of this protocol, whereas the bivalve mollusc MPN calculator does.

To use the bivalve mollusc MPN calculator attached to this protocol:

A. Select the relevant Excel spreadsheet, depending on the number of dilutions tested, by double clicking on the link. The Excel spreadsheet has previously been populated with the required information except the number of positive tubes or bottles.
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 number of positive tubes or bottles at each dilution in column 4.
D. 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 100 g.

For example:

i. Using the three standard dilutions, 5 tubes (or bottles) were positive at the first dilution, 3 were positive at the second dilution and 0 positive (all negative) at the third dilution.
ii. Open the standard 3 dilution bivalve mollusc MPN calculator spreadsheet.
iii. For the Sample 1 input data table:
   a. Enter 5 against the 0.1 dilution factor/10 g volume row.
   b. Enter 3 against the 0.1 dilution factor/1 g volume row.
   c. Enter 0 against the 0.01 dilution factor/1g volume row.
iv. Press the 'calculate results' button in the top right of the spreadsheet or press 'Ctrl + m'.
v. The MPN for sample 1 is given in the "Results of the MPN calculations" table as 790 per 100 g.

8.8.2 MPN tables

Tables 1 and 2 of Appendix 2 list MPN tube/bottle combinations for 3 and 4 dilutions respectively. To determine the MPN value, refer to the MPN table which contains the number of dilutions examined:

- For dilutions of neat, 10^{-1} and 10^{-2} use MPN Table 1
- For dilutions of neat, 10^{-1}, 10^{-2} and 10^{-3} use MPN Table 2

Find the line in the relevant table that contains the number of positive tubes (or bottles) for each dilution of the sample and thus read off the MPN value for that combination of results. The tables only contain category 1 and 2 combinations. Combinations of results that do not appear in the tables are category 3 (see Section 8.9).

Note: The entries in the MPN tables given in Appendix 2 are consistent with those obtained with the bivalve mollusc MPN calculator.

8.9   Reporting results

Results should be reported as the MPN per 100 g of bivalve mollusc flesh and intra-valvular liquid. Estimated MPN values given for tube (or bottle) combinations of 0, 0, 0 and 5, 5, 5 should be assigned a value of <18 and >18 000 per 100 g of bivalve mollusc flesh and intra-valvular liquid respectively (see note). Only values determined from combinations identified as category 1 or 2 should be reported. Category 3 combinations should be reported as 'Void'. Where a standard set of five tubes at each of three or four dilutions have been used (that is, with the first set of tubes have been inoculated with the equivalent of 1 g of sample), sets showing all negative tubes (or bottles) should be reported as MPN <18/100 g. Where a different series of dilutions has been used, sets showing all negative tubes (or bottles) should be reported as less than the lower nominal limit of detection (per 100 g) for that series.

Note: In ISO 7218:2007/Amd 1 2013, the MPN value given for samples yielding all negative tubes (or bottles) (e.g. a combination of 0, 0, 0) is zero and the MPN value given for all positive tubes (or bottles) (e.g. a combination of 5, 5, 5) is infinity. In bivalve mollusc sanitation programmes, it has been common practice, for samples yielding all negative tubes (or bottles) to be reported as containing *E. coli* at less than the nominal lower limit of detection for the range of dilutions used and for samples yielding all positive tubes (or bottles) have been reported as containing *E. coli* at a concentration above the upper nominal limit of detection for the range of dilutions used. This approach provides additional information of value in assessing data within a bivalve mollusc sanitation programme. The output of the bivalve mollusc MPN calculator and the MPN tables given in Appendix 2 of this protocol follow this practice.

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 controls

Sterility controls should be set up for each container of diluent (0.1% P or PSS) that is used, at the beginning and end of each set of samples that is examined.

Positive and negative controls should be included with each set of examinations (that is, those processed at approximately the same time) and for each medium type (double strength MMGM, single strength MMGM, TBGA). The control strains listed in Table 1 (Section 7) may be used for this purpose. Controls should not be inoculated onto the same TBGA plates as subcultures of MMGM tubes from samples. An uninoculated tube or bottle of double strength MMGM, single strength MMGM and/or plate of TBGA should also be incubated with each set as a sterility control. Where more than one batch of medium has been used for the sample examinations, positive, negative and sterility controls should be included for each batch.

10.2   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 *E. coli* procedures continue to be 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 *E. coli* in samples prepared using reference materials, should be appropriate to the usual range of *E. coli* concentrations expected in routine samples.

10.3   Comparative testing

It is recommended that laboratories undertaking microbiological examination of samples as part of a bivalve mollusc sanitation programme take part in comparative testing (also known as proficiency 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. Examples of comparative testing for bivalve molluscs are the FAO Reference Centre proficiency testing scheme (see https://www.cefas.co.uk/icoe/seafood-safety/services/proficiency-testing-and-quality-assurance/) and the PHE/Cefas Shellfish EQA scheme (for further information contact foodeqa@phe.gov.uk).

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.

Codex Alimentarius 2015. Standard for Live and Raw Bivalve Molluscs: Codex Stan 292-2008 (Adopted in 2008. Amendment: 2013. Revision: 2014 and 2015). Food and Agriculture Organisation of the United Nations/World Health Organization.

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

ISO 7218. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - Amendment 1. International Organization for Standardization: Geneva, Switzerland.

ISO 16649-3. Microbiology of food chain - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. International Organization for Standardization: Geneva, Switzerland.

ISO 6887-3. Microbiology of the food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 3: Specific rules for the preparation of fish and fishery products. 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

12   APPENDICES

12.1 Appendix 1: Sub-sample sizes of bivalve molluscs required for *E. coli* examination

Suggested minimum sample sizes given in this table have been provided by several laboratories. ISO 6887-3 specifies the use of a minimum of ten animals. The weight of bivalve mollusc flesh and liquor should be at least 50 g for the *E. coli* method (for very small species such as the *Donax* spp. a minimum amount of 25 g can be used for practical reasons). For species not given in the table, sufficient bivalve molluscs should be opened to achieve this minimum weight of flesh and liquor, with the provision that a minimum of ten animals should be used even for very large species. In general, the more bivalve molluscs that are included in the initial homogenate, the less the result will be influenced by the inherent animal-to-animal variation in *E. coli* concentration.

The following sub-sample sizes are recommended for inclusion in the homogenisation step:

Group	Common Name	Latin name	Sample size
Scallops	Saint James Scallop	*Pecten jacobaeus*	10 - 12
	King (Great Atlantic scallop)	*Pecten maximus*	10 - 12
	Queen scallop	*Aequipecten opercularis or Chlamys opercularis*	15 - 30
	Variegated scallop	*Chlamys varia*	10 - 18
Oysters	Pacific oyster	*Crassostrea gigas*	10 - 18
	Portuguese oyster	*Crassostrea angulate*	10 - 18
	European flat oyster	*Ostrea edulis*	10 - 18
Mussels	Blue or common mussel	*Mytilus edulis*	15 - 30
	Mediterranean mussel	*Mytilus galloprovincialis*	10 - 30
	Northern horse mussel	*Modiolus modiolus*	10 - 12
	Bearded horse mussel	*Modiolus barbatus*	15 - 30
Clams	Smooth clam	*Callista chione*	10 - 30
	Hard clam	*Mercenaria mercenaria*	12 - 18
	Ark clam	*Barbatia barbata*	15 - 25
	Warty venus clam	*Venus verrusosa*	15 - 30
	Palourdes (Grooved carpet shells)	*Tapes decussatus (Venerupis decussata)*	18 - 35
	Rayed artemis	*Dosinia exoleta*	18 - 35
	Banded carpet shell	*Venerupis (Polititapes) rhomboides*	20 - 25
	Pullet carpet shell	*Venerupis senegalensis*	20 - 25
	Atlantic surf clam (Thick trough shell)	*Spisula solida*	30 - 50
	Bean clam	*Donax spp.*	30 - 50
	Wedge shell clam	*Donax trunculus*	40 - 80
	Striped venus clam	*Chamelea gallina*	40 - 80
	Cut trough shell	*Spisula subtruncata*	70 - 90
	Noah's Ark shells	*Arca noae*	10 - 25
	Manila clam	*Tapes philippinarum*	18 - 35
	Soft shell clam (Sand gaper)	*Mya arenaria*	10 - 12
	Pacific geoduck	*Panopea abrubta*	10 - 12
Cockles	Common edible cockle	*Cerastoderma edule*	30 - 50
Razor clams (razorfish, razorshells)	Common razor shell	*Ensis ensis*	10 - 12
	Razor shell	*Ensis magnus (Ensis arcuatus)*	10 - 12
	Atlantic razor clam; Jacknife clam	*Ensis directus*	10 - 12
	Pod razor	*Ensis siliqua*	10 - 12
	Grooved razor shell	*Solen marginatus*	10 - 12

Note: Laboratories are encouraged to submit suggestions on minimum sample sizes for other species to the FAORC. Please note that these recommendations must conform to the minimum requirement of 10 animals given in ISO 6887-3.

12.2   Appendix 2: Example *E. coli* Most Probable Number tables (MPN)

12.2.1   Table 1: Example - Most probable number of organisms: table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g.

1g	0.1g	0.01g	MPN/100g	Category
0	0	0	<18 [C]	1
0	1	0	18	1
1	0	0	20	1
1	0	1	40	2
1	1	0	40	1
1	2	0	61	2
2	0	0	45	1
2	0	1	68	2
2	1	0	68	1
2	1	1	92	2
2	2	0	93	1
3	0	0	78	1
3	0	1	110	1
3	1	0	110	1
3	1	1	140	2
3	2	0	140	1
3	2	1	170	2
3	3	0	170	2
4	0	0	130	1
4	0	1	170	1
4	1	0	170	1
4	1	1	210	1
4	2	0	220	1
5	0	0	230	1
4	2	1	260	2
4	3	0	270	1
4	4	0	340	2
5	0	1	310	1
5	1	0	330	1
5	1	1	460	1
5	1	2	630	2
5	2	0	490	1
5	2	1	700	1
5	2	2	940	1
5	3	0	790	1
5	3	1	1100	1
5	3	2	1400	1
5	3	3	1700 [D]	2
5	4	0	1300	1
5	4	1	1700	1
5	4	2	2200	1
5	4	3	2800	1
5	4	4	3500	2
5	5	0	2400	1
5	5	1	3500	1
5	5	2	5400	1
5	5	3	9200	1
5	5	4	16000	1
5	5	5	>18000 [E]	1

[C] 0 according to ISO 7218: Amd1. The use of <18 is considered good practise for labs analysing bivalve shellfish.

[D] 1800 according to ISO 7218: Amd1 but 1700 using the ISO MPN calculator (confirmed by ISO statistics working group).

[E] ∞ according to ISO 7218: Amd1. The use of >18000 is considered good practise for labs analysing bivalve shellfish.

12.2.2   Table 2: Example - Most probable number of organisms: table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g, 5 × 0.001 g.

1g	0.1g	0.01g	0.001g	MPN/100g	Category
0	0	0	0	<18 [F]	1
0	1	0	0	18	1
1	0	0	0	20	1
1	0	1	0	40	2
1	1	0	0	40	1
1	2	0	0	61	2
2	0	0	0	45	1
2	0	1	0	68	2
2	1	0	0	68	1
2	1	1	0	92	2
2	2	0	0	93	1
3	0	0	0	78	1
3	0	1	0	110	1
3	1	0	0	110	1
3	1	1	0	140	2
3	2	0	0	140	1
3	2	1	0	170	2
3	3	0	0	170	2
4	0	0	0	130	1
4	0	1	0	170	1
4	1	0	0	170	1
4	1	1	0	210	1
4	2	0	0	220	1
5	0	0	0	230	1
4	2	1	0	260	2
4	3	0	0	270	1
4	4	0	0	330	2
5	0	1	0	310	1
5	1	0	0	330	1
5	1	0	1	450	2
5	1	1	0	450	1
5	1	2	0	620	2
5	2	0	0	490	1
5	2	0	1	690	2
5	2	1	0	690	1
5	2	1	1	920	2
5	2	2	0	930	1
5	3	0	0	780	1
5	3	0	1	1100	1
5	3	1	0	1100	1
5	3	1	1	1400	2
5	3	2	0	1400	1
5	3	2	1	1700	2
5	3	3	0	1700	2
5	4	0	0	1300	1
5	4	0	1	1700	1
5	4	1	0	1700	1
5	4	1	1	2100	1
5	4	2	0	2200	1
5	4	2	1	2600	2
5	4	3	0	2700	1
5	4	4	0	3400	2
5	5	0	0	2300	1

[F] 0 according to ISO 7218: Amd1. The use of <18 is considered good practise for labs analysing bivalve shellfish.

Table 2 Example continued: Most probable number of organisms: table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g, 5 × 0.001 g.

1g	0.1g	0.01g	0.001g	MPN/100g	Category
5	5	0	1	3100	1
5	5	1	0	3300	1
5	5	1	1	4600	1
5	5	1	2	6300	2
5	5	2	0	4900	1
5	5	2	1	7000	1
5	5	2	2	9400	1
5	5	3	0	7900	1
5	5	3	1	11000	1
5	5	3	2	14000	1
5	5	3	3	17000	2
5	5	4	0	13000	1
5	5	4	1	17000	1
5	5	4	2	22000	1
5	5	4	3	28000	1
5	5	4	4	35000	2
5	5	5	0	24000	1
5	5	5	1	35000	1
5	5	5	2	54000	1
5	5	5	3	92000	1
5	5	5	4	160000	1
5	5	5	5	>180000 [G]	1

[G] ∞ according to ISO 7218:Amd 1. The use of >180000 is considered good practise for labs analysing bivalve shellfish.

12.3   Appendix 3: Application and use of the MPN calculator

12.3.1   Calculating the MPN value using 3 dilutions

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'.

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.
- 'Max. no. of dilutions' – The value recorded is based on the number of dilutions examined. E.g. For the example given below 3 dilutions were examined (N, 10^{-1} and 10^{-2}).

5. Populate the data table as follows:

- Column 1 - Insert the homogenate concentration used to inoculate each row of MMGB tubes.
- Column 2 - Insert the volume of inoculum added.
- Column 3 – Insert the number of MMGB tubes inoculated at each dilution. (For the *E. coli* generic protocol 5 tubes are inoculated for each dilution).
- Column 4 – Insert the number of positive tubes on TBGA/TBX at each dilution.

	Input data			
12				
13	Test series 1 / Matrix 1 - Designation:	Example 1		
14	Target organism / Test medium:	E.coli		
15	Dilution factor	Volume in ml or g	No. of tubes	No. of positive tubes
16	*d*	*w*	*n*	*x*
17	0.1	10.0	5	5
18	0.1	1.0	5	4
19	0.01	1.0	5	2
20				
21	Test series 2 / Matrix 2 - Designation:	Example 2		
22	Target organism / Test medium:	E.coli		
23	Dilution factor	Volume in ml or g	No. of tubes	No. of positive tubes
24	*d*	*w*	*n*	*x*
25	0.1	10.0	5	4
26	0.1	1.0	5	3
27	0.01	1.0	5	1

Homogenate concentration used to inoculate MMGB tubes	Volume of inoculum added	Number of MMGB tubes inoculated	Number of positive tubes on TBGA/TBX

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							
	Test series / Matrix	MPN	log$_{10}$ MPN	SD log$_{10}$ MPN	95% confidence limits		Rarity Index	Category
No.	Designation				Lower	Upper		
1	Example 1	22.	1.3	0.20	8.8	56.	0.304	1
2	Example 2	3.3	0.51	0.18	1.4	7.5	0.010	3

Note: An MPN that falls into category 3 is unreliable and should not be used. The respective rows are marked in blue.

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.

- For example 1 - the MPN/100g can be used.
- For example 2 - the result is rejected and reported as a 'Void' result. A repeat sample must be requested.

8. The MPN calculator calculates the MPN per gram (MPN/g). *E. coli* results are reported as MPN per 100g (MPN/100g). Multiply the value given in column 8 by 100 to give the results in MPN/100g.

For the examples given above

- For example 1 - 22 x 100 = 2 200 MPN /100g.
- For example 1 - A repeat sample would need to be requested as it has a category number of 3.

9. For samples that give a tube combination of 0, 0, 0 or 5, 5, 5 the MPN calculator will calculate a value of 0 and infinity respectively (see below). An estimated MPN value given for these tube combinations of 0, 0, 0 and 5, 5, 5 should be assigned values of <18 and >18 000 per 100g.

data	
Example 1	
E.coli	
No. of tubes n	No. of positive tubes x
5	0
5	0
5	0

Example 2	
E.coli	
No. of tubes n	No. of positive tubes x
5	5
5	5
5	5

Results of the MPN calculations								
No.	Test series / Matrix Designation	MPN	$\log_{10}$ MPN	SD $\log_{10}$ MPN	95% confidence limits Lower	Upper	Rarity Index	Category
1	Example 1	0			0	0.66	1.000	1
2	Example 2	∞			65.	∞	1.000	1

12.3.2   Calculating the MPN value using 4 dilutions

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'.

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.
   - 'Max. no. of dilutions' – The value recorded is based on the number of dilutions examined. E.g. For the example given below 4 dilutions were examined (N, 10^{-1}, 10^{-2} and 10^{-3}).

5. Populate the data table as follows:

    - Column 1 - Insert the homogenate concentration used to inoculate each row of MMGB tubes.
    - Column 2 - Insert the volume of inoculum added.
    - Column 3 – Insert the number of MMGB tubes inoculated at each dilution. (For the *E. coli* generic protocol 5 tubes are inoculated for each dilution).
    - Column 4 – Insert the number of positive tubes on TBGA/TBX at each dilution.

	Input data			
12				
13	Test series 1 / Matrix 1 - Designation:		Example 1	
14	Target organism / Test medium:		E.coli	
15	Dilution factor	Volume in ml or g	No. of tubes	No. of positive tubes
16	d	w	n	x
17	0.1	10.0	5	5
18	0.1	1.0	5	5
19	0.01	1.0	5	4
20	0.001	1.0	5	2
21				
22	Test series 2 / Matrix 2 - Designation:		Example 2	
23	Target organism / Test medium:		E.coli	
24	Dilution factor	Volume in ml or g	No. of tubes	No. of positive tubes
25	d	w	n	x
26	0.1	10.0	5	5
27	0.1	1.0	5	4
28	0.01	1.0	5	3
29	0.001	1.0	5	1

Homogenate concentration used to inoculate MMGB tubes	Volume of inoculum added	Number of MMGB tubes inoculated	Number of positive tubes on TBGA/TBX

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							
	Test series / Matrix	MPN	log₁₀ MPN	SD log₁₀ MPN	95% confidence limits		Rarity Index	Category
No.	Designation				Lower	Upper		
1	Example 1	220.	2.3	0.20	88.	560.	0.304	1
2	Example 2	33.	1.5	0.18	14.	75.	0.010	3

Note: An MPN that falls into category 3 is unreliable and should not be used. The respective rows are marked in blue.

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.

    - For example 1 - the MPN/100g can be used.
    - For example 2 - the result is rejected and reported as a 'Void' result. A repeat sample must be requested.

8. The MPN calculator calculates the MPN per gram (MPN/g). *E. coli* results are reported as MPN per 100g (MPN/100g). Multiply the value given in column 8 by 100 to give the results in MPN/100g.

    For the examples given above

    - For example 1 - 220 x 100 = 22 000 MPN /100g.
    - For example 1 - A repeat sample would need to be requested as it has a category number of 3.

9. For samples that give a tube combination of 0, 0, 0, 0 or 5, 5, 5, 5 the MPN calculator will calculate a value of 0 and infinity respectively (see below). An estimated MPN value given for these tube combinations of 0, 0, 0, 0 and 5, 5, 5, 5 should be assigned values of <18 and >180 000 per 100g.

data	
Example 1	
E.coli	
No. of tubes	No. of positive tubes
n	x
5	5
5	5
5	5
5	5

Example 2	
E.coli	
No. of tubes	No. of positive tubes
n	x
5	0
5	0
5	0
5	0

Results of the MPN calculations								
No.	Test series / Matrix Designation	MPN	$\log_{10}$ MPN	SD $\log_{10}$ MPN	95% confidence limits Lower	Upper	Rarity Index	Category
1	Example 1	∞			650.	∞	1.000	1
2	Example 2	0			0	0.66	1.000	1

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