

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

Generic protocol

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

Issue 14: 25.05.17

19 pages

| | | | |
|-----------------------|--|-----------------|----------|
| Document approved by: | C6927a Project Manager – James Lowther | Review date: | n/a |
| Document checked by: | Rachel Hartnell | Classification: | Official |
| Document prepared by: | Louise Stockley | Location | |

Whereas every precaution has been taken in the preparation of this document, Cefas 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 Cefas must be removed if any alterations are made to this publication.

History of Procedure

| Issue | Date | Section | Changes |
|--------------|---|-------------------------|--|
| 11 | 20.01.15 | Appendix 2 | Amendments to MPN values – Added 5 0 0 0 tube combination. |
| 12 | 25.02.16 | 1, 2 and 11 | Update of ISO 16649-3 publication date. |
| | | 2 | Rewording of note regarding how the MPN tables were generated. |
| | | 6 | The addition of Peptone salt solution (PSS). |
| | | 7.2, 8.6 and 8.7 | Amendments to reference strains to be consistent with ISO 16649-3. The inclusion of link to identify WDCM numbers. |
| | | 8.5.2 | The addition of note stating all dilutions tested should be included when obtaining the MPN value. |
| | | Footnotes 1 - 10 | Included throughout the protocol. |
| | | 8.7 | Change of incubation time from 22±2 hrs to 21±3 hrs. Rewording of text regarding the streaking and stacking of TBGA/TBX plates. |
| | | 8.8 | Rewording to address issues when calculating the MPN value. The inclusion of the Excel spreadsheet MPN calculator for 3 and 4 dilutions. |
| 13 | 13.09.16 | Appendix 2 | Application and use of the MPN calculator |
| | | 5.0, 8.6 and 8.7 | Change sterile loop from 1µl to 10µl |
| | | 7.0 | Reference strains updated to include both strains as a minimum and those that are the laboratories free to choose. |
| | | 8.4 and 8.5.1 | Notes moved and added to footnote. |
| | | 8.6 | MMGB diluent volume added to text. |
| | | 8.7 | Update to reference strains. |
| | | 8.8.1 | Update to MPN calculator Version 4. |
| 14 | 25.05.17 | Appendix 2 | Update to the application of the MPN calculator |
| | | 2.0 note | The inclusion of the bivalve shellfish MPN calculator |
| | | 8.1 | Sample temperature criteria for samples received within 4 hours of sample collection. |
| | | 8.8 | Clear description for the inoculation of TBGA/TBX plates from incubated MMGB tubes |
| | | 8.8, footnote and 8.8.1 | Removal of old MPN calculator and replaced with the Shellfish MPN calculator |
| | | 11 | Update to References |
| 1.1 footnote | The inclusion of (for very small species such as the <i>Donax</i> spp. a minimum amount of 25g is permitted). | | |

Contents

| | | |
|--------|---|----|
| 1 | Introduction..... | 3 |
| 2 | Scope..... | 3 |
| 3 | Principle..... | 3 |
| 4 | Safety precautions..... | 3 |
| 5 | Equipment..... | 3 |
| 6 | Media..... | 4 |
| 7 | Microbiological reference cultures for performance testing and controls..... | 4 |
| 8 | Procedures..... | 4 |
| 8.1. | Sample transport and receipt..... | 4 |
| 8.2 | Sample storage..... | 5 |
| 8.3 | Sample selection..... | 5 |
| 8.4 | Sample preparation..... | 5 |
| 8.4.1 | The oyster cracker..... | 5 |
| 8.4.2 | Shucking with a knife..... | 5 |
| 8.5 | Dilution and homogenisation..... | 5 |
| 8.5.1 | Homogenisation in a blender (homogeniser)..... | 6 |
| 8.5.2 | Homogenisation in a stomacher..... | 6 |
| 8.6 | Inoculation and incubation of primary broth..... | 6 |
| 8.7 | Confirmation of <i>E. coli</i> | 6 |
| 8.8 | Calculation of <i>E. coli</i> most probable number (MPN)..... | 6 |
| 8.8.1 | The Shellfish MPN calculator..... | 7 |
| 8.8.2 | MPN tables..... | 7 |
| 8.9 | Reporting results..... | 7 |
| 9 | Uncertainty of test results..... | 8 |
| 10 | Quality control..... | 8 |
| 10.1 | Internal Quality Assurance (IQA)..... | 8 |
| 10.2 | Proficiency testing..... | 8 |
| 11 | References..... | 8 |
| 12 | Appendices..... | 10 |
| 12.1 | Appendix 1: Sub sample sizes of shellfish required for <i>E. coli</i> analysis..... | 10 |
| 12.2 | Appendix 2: <i>E. coli</i> Most Probable Number tables (MPN)..... | 11 |
| 12.2.1 | Table 1: Most probable number of organisms: table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g..... | 11 |
| 12.2.2 | Table 2: Most probable number of organisms: table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g, 5 × 0.001 g..... | 12 |
| 12.3 | Appendix 3: Application and use of the MPN calculator..... | 14 |
| 12.3.1 | Calculating the MPN value using 3 dilutions..... | 14 |
| 12.3.2 | Calculating the MPN value using 4 dilutions..... | 17 |

1 INTRODUCTION

The consumption of sewage polluted bivalve molluscan shellfish (BMS) may cause illness in the consumer through ingestion of faecally borne 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 BMS. Enteric bacteria, such as *Escherichia coli* (*E. coli*) are used as indicator organisms to assess the sanitary quality of shellfish and their growing waters and to predict the risk of exposure to enteric pathogenic microorganisms.

In the European Union (EU), the criteria for laying down the microbiological standards for BMS are set out in Regulation (EC) 854/2004 (Anon 2004) and Regulation (EC) 2073/2005 (Anon 2005) stipulating conditions for the production and placing on the market of live BMS. Across the EU *E. coli* in shellfish flesh is used as an indicator of faecal contamination of BMS. The EU reference method for the determination of *E. coli* in BMS is 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' (Anon 2015).

2 SCOPE

The procedure has been produced with reference to ISO 16649-3 (Anon 2015) and relevant parts of ISO standards referenced in section 11. The theoretical limit of detection is a most probable number (MPN) of 18 *E. coli* per 100g of shellfish flesh. In the context of this test *E. coli* produces acid from lactose at $37\pm 1^\circ\text{C}$ and expresses β -glucuronidase activity at $44\pm 1^\circ\text{C}$.

Note: The 5x3 and 5x4 MPN tables included in this procedure have been generated using the MPN calculator referenced in ISO 7218:2007/Amd1:2013. Values have been converted from MPN/g to MPN/100g. A bivalve shellfish MPN calculator has been developed and is included in this protocol.

3 PRINCIPLE

The method used to enumerate *E. coli* in BMS is a two-stage, five-tube three-dilution MPN method. The first stage of the method is a resuscitation step requiring inoculation of minerals modified glutamate broth (MMGB) with a series of diluted shellfish homogenates and incubation at $37\pm 1^\circ\text{C}$ for 24 ± 2 hours. The presence of *E. coli* is confirmed by subculturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indolyl- β -D-glucuronide and detecting β -glucuronidase activity.

Note: Strains of *E. coli* that do not grow at $44\pm 1^\circ\text{C}$ 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 this method.

4 SAFETY PRECAUTIONS

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before performing this procedure. Homogenisation of shellfish should be performed in a Class II safety cabinet to reduce the risk of infection from aerosol inhalation. *E. coli* should be handled in accordance with ACDP category 2 guidelines.

5 EQUIPMENT

- Waring blender and jars or stomacher and stomacher bags
- Class II safety cabinet
- Refrigerator at $3\pm 2^\circ\text{C}$
- Sterile glassware
- Safety/electric Bunsen system
- Protective gloves – single use
- Safety gloves – for example a chain mail glove
- Incubator at $37\pm 1^\circ\text{C}$ and $44\pm 1^\circ\text{C}$
- Shucking knife, Oyster cracker or other suitable equipment for opening shellfish
- Electric top pan balance
- Loops - sterile, 10 μ l
- Pipette - automatic or manual for use with 1ml and 10ml pipette tips

6 MEDIA ^{A, B, C}

- Ethanol
- 0.1% peptone (0.1% P); formula per litre - de-ionised water 1±0.01 litre, peptone bacteriological 1.0±0.1g.
- Peptone salt solution (PSS); formula per litre - de-ionised water 1±0.01 litre, peptone 1.0±0.1g, sodium chloride 8.5±0.1g, pH 7.0±0.2.
- Minerals modified glutamate broth (MMGBx1, MMGBx2) - Single strength - de-ionised water 1±0.01 litre, ammonium chloride 2.5±0.1g, sodium glutamate 6.4±0.1g, minerals modified medium base 11.4±0.1g. Double strength - de-ionised water 1±0.01 litre, ammonium chloride 5.0±0.1g, sodium glutamate 12.8±0.1g, minerals modified medium base 22.8±0.1g, pH 6.7±0.1.
- Tryptone bile glucuronide agar (TBGA/TBX); formula per litre - de-ionised water 1±0.01 litre, tryptone bile glucuronide agar 36.5±0.5g, pH 7.2±0.2.

7 MICROBIOLOGICAL REFERENCE CULTURES FOR PERFORMANCE TESTING AND CONTROLS

Table 1: Microbiological reference strains used for control purposes

| Media type | Control strain | WDCM ^a | Criteria | Characteristic reaction | Strain choice ^c |
|---|--|-------------------|-----------------------------|---------------------------------|----------------------------|
| Mineral-modified glutamate medium (MMGB) | <i>Escherichia coli</i> | 00012 or 00013 | Acid production | Colour change to yellow | 1 |
| | <i>Enterococcus faecalis</i> | 00009 or 00087 | No growth | No colour change | 1 |
| Tryptone bile glucuronide agar (TBGA/TBX) | <i>Escherichia coli</i> | 00202 | Blue to blue-green colonies | B-glucuronidase positive (weak) | 2 |
| | <i>Escherichia coli</i> | 00012 or 00013 | Blue to blue-green colonies | B-glucuronidase positive | 1 |
| | <i>Enterococcus faecalis</i> | 00009 or 00087 | Total inhibition | no growth | 1 |
| | <i>Pseudomonas aeruginosa</i> | 00025 | White to beige colonies | B-glucuronidase negative | 1 |
| | <i>Citrobacter freundii</i> ^b | 00006 | | | |

^a Follow the link (<http://www.phe-culturecollections.org.uk/products/bacteria/WDCMStrains.aspx>) to obtain NCTC number for WDCM reference strains.

^b Following the sub-culturing of *C. freundii* onto TBGA/TBX plates, the EURL noted growth was not always present. The EURL recommends the use of *P. aeruginosa* over *C. freundii*.

^c Reference strains given in ISO 16649-3 for use in performance testing. Strain selection: 1 - Laboratory must select 1 strain from the list provided; 2 - Strain to be used as a minimum.

8 PROCEDURES

8.1. Sample transport and receipt

Samples must be received in an intact food grade plastic bag 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. For samples where less than 4 hours have elapsed between collection from the production 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 shellfish 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 Official Controls, 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 relevant Competent Authority.

Note: The UK NRL has carried out studies on the effect of extended storage time and elevated temperatures on certain species of BMS. The report of this work can be accessed using the following web link

^A Allow media to equilibrate at room temperature before use.

^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

^C For performance testing of media and reagents see Table 1.

<https://www.cefas.co.uk/nrl/information-centre/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 $3\pm 2^{\circ}\text{C}$, 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 selection

Choose shellfish that are alive according to the following points:

- Movement of any kind if any exposed flesh reacts to touch using a sterile shucking knife.
- Shellfish are open and close of their own accord.
- A tap on the shell causes closing or movement.
- Tightly closed shellfish.

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

8.4 Sample preparation

Mud and sediment adhering to the shell should be removed prior to opening by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be re-immersed in water as this may cause them to open. Open all selected shellfish as described below with a flame sterilised shucking knife or equivalent and empty meat and liquor into a sterile container ^D. If sterilised by heating allow the knife to cool before using. When opening shellfish ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

8.4.1 The oyster cracker

Sterilise the pin of the oyster cracker before use. Place a single animal in a weighing boat 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.

8.4.2 Shucking with a knife

Using a sterilised shucking knife, open all selected shellfish as described below:

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 ^{E F}

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 2ml of sterile 0.1% P or PSS per 1g of shellfish using a measuring cylinder and measure to $\pm 2\text{ml}$.

^D Alternative equipment can be used to open bivalve shellfish.

^E ISO 6887-3 allows the use of PSS for the preparation of the primary dilution. EURL advice is to use 0.1% P instead at this stage in order to avoid the addition of extra NaCl.

^F Samples should be examined within 45 minutes after the end of preparation of the initial suspension and the end of the inoculum comes into contact with the final culture medium.

Note: Complete either sections 8.5.1 or 8.5.2.

8.5.1 Homogenisation in a blender (homogeniser)

Place the contents of the sterilised container into a 1 litre blender jar ^G 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 microbiological laminar flow cabinet. Decant the contents back into the sterilised container.

8.5.2 Homogenisation in a stomacher ^H

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 at least three stomacher bags, to avoid small pieces of shell from puncturing the bags. Remove excess air from the bag. Operate the stomacher for 2-3 minutes.

Add 30±0.5ml of mixed homogenate to 70±1ml of 0.1% P using a 10ml 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 1ml of the 10⁻¹ dilution to 9ml of 0.1% P. If samples are expected to be heavily diluted (Category C or above) further decimal dilutions may be necessary.

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 primary broth

Inoculate five bottles containing 10±0.2ml of double strength MMGB with 10±0.2ml of the 10⁻¹ diluted homogenate (equivalent to 1g of tissue per tube). Inoculate five bottles containing 10±0.2ml of single strength MMGB with 1±0.1ml of the 10⁻¹ diluted homogenate (0.1g). Inoculate five bottles containing 10±0.2ml of single strength MMGB with 1±0.1ml of the 10⁻² diluted homogenate (0.01g) and repeat with any further dilutions. Inoculate individual bottles containing 10±0.2ml of single strength MMGB for *E. coli* WDCM 00012 or 00013 and a separate single strength MMGB with *E. faecalis* WDCM 00009 or 00087 using a 10µl loop ^I. Include an uninoculated bottle of single strength MMGB as a negative control. Incubate inoculated bottles of MMGB at 37±1°C for 24±2 hours.

8.7 Confirmation of *E. coli*

After incubation examine the MMGB tubes 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. Confirm the presence of *E. coli* in all tubes showing acid production by subculture onto TBGA/TBX plates within 4 hours using a 10µl sterile loop ^J, streaking with the objective to obtain single colonies. Subculture each MMGB control inoculated in 8.6 onto a TBGA/TBX plate. Incubate TBGA/TBX at 44±1°C for 21±3 hours in stacks no more than six plates high ^K and separated from each other and the incubator walls by at least 25mm.

Note: TBGA/TBX plates can be divided into 5 segments, if plates have previously been dried, with each segment being inoculated from a single presumptive positive tube using a 1µl sterile loop. It is preferable to use separate plates for the individual dilution levels even if there are less than 5 tubes to subculture.

After the incubation period examine the TBGA/TBX 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.

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

To calculate the MPN, record as negative all MMGB tubes that either do not produce acid after incubation or

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

^H The EURL recommends using a Rotary blender and not a stomacher when preparing clam and cockle samples for *E. coli* enumeration following results obtained from proficiency testing distributions.

^I A 1µl loop can be used instead of a 10µl loop as given in ISO 16649-3. The EURL has shown the change in loop size has no practical consequences.

^J Using a 1µl loop rather than a 10µl loop, as given in ISO 16649-3 avoids overgrowth of target colonies when segments of a plate are used. Comparative work carried out by the EURL showed sub-culturing MMGB tubes using a 1µl loop onto TBGA/TBX plates divided into 5 segments did not affect the MPN value.

^K In accordance with ISO 7218, plates can be stacked no more than 6 high.

are negative (i.e. no blue or blue-green colonies) following subculture and incubation on TBGA/TBX plates. Record as positive MMGB tubes that produce acid after incubation and are positive (i.e. presence of blue or blue-green colonies) after subculture and incubation on TBGA/TBX plates. The total number of tubes at each dilution should equal the sum of tubes that are positive and negative at that dilution. This total should correspond to the value entered in Column 3 of the Shellfish MPN calculator¹. The number of positive tubes 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 Shellfish MPN calculator. See Section 8.8.1 for the use of the Shellfish MPN calculator and Section 8.8.2 for use of the MPN tables.

Note: MPN tube combinations fall 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 Shellfish 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 Shellfish MPN calculator

Attached to this generic protocol are three copies of the Shellfish MPN calculator. Two have been prepopulated with the 3 and 4 dilution factors respectively, volume in ml/g and No. of MMGB tubes inoculated. To calculate the MPN value, input all data obtained from each dilution examined into the Shellfish MPN calculator within this generic protocol as described below (See spreadsheets attached to this protocol).

To use the Shellfish MPN calculator attached to this protocol;

- A. Select an excel spreadsheet attached by double clicking on the excel spreadsheet depending on the number of dilutions tested. The Excel spreadsheet has previously been populated with the required information except the number of positive tubes.
- B. Single click on the 'Enable content' button at the top of the spreadsheet.
- C. Enter the number of positive tubes at each dilution in column 4.
- D. Press the 'calculate results' button in the top right of the spreadsheet or press 'Ctrl + M'. The MPN value is calculated in column 8 as MPN per 100g.

8.8.2 MPN tables (Appendix 3)

MPN tube combinations for 3 and 4 dilutions giving category 1 and 2 values only have been provided in Table 1 and 2 of Appendix 3 using the Excel calculator referred to above. To calculate 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.

Note: The Excel spreadsheet MPN calculator documented in ISO 7218:2007/Amd1:2013 was used to generate the MPN tables provided in Appendix 3 (<http://standards.iso.org/iso/7218/>) and have been converted to MPN/100g.

8.9 Reporting results

Results should be reported as the MPN per 100g of flesh and intra-valvular liquid. Estimated MPN values given for tube combinations of 0, 0, 0 and 5, 5, 5 should be assigned values of <18 and >18 000 per 100g of flesh and intra-valvular liquid respectively (see note). Only values determined from combinations that give a category 1 or 2 profile should be reported. Category 3 values should be reported as 'Void'. Negative samples should be reported as MPN < 18/100g.

Note: Estimated MPN values given for tube combinations of 0, 0, 0 and 5, 5, 5 included in ISO 7218:2007/Amd 1 2013 (both the Excel spreadsheet MPN calculator and Annex C (normative)) are given values of 0 and infinity respectively. At the 2014 annual workshop of NRLs for monitoring bacteriological and viral contamination of bivalve molluscs it was agreed that it was technically and scientifically justified in microbiology to report

¹ The Shellfish MPN calculator has been developed specifically for use in bivalve shellfish testing. 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/100g (see Appendix 3).

combinations of 0, 0, 0 and 5, 5, 5 as per the MPN tables in appendix 3. See NRL resolutions 2014 <https://eur1cefafas.org/public-documents/workshops-of-nrls/workshop-resolutions.aspx>

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 routine *E. coli* procedures continue to be efficient and effective. An example for assessing quantitative methods is the use of Lenticule™ discs.

10.2 Proficiency testing

Laboratories undertaking microbiological examination of Official Control samples of shellfish are expected to take part in a scheme to provide an independent assessment of a laboratories performance against other participants. These schemes can help improve the performance of the laboratory. An example of this is the PHE/Cefas Shellfish EQA scheme (for further information contact foodeqa@phe.gov.uk).

11 REFERENCES

Anon 2004 Regulation (EC) No 854/2004 of the European parliament and the council, 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Off. J. Eur. Communities L 226, 25.6.04:83-127.

Anon 2005 Commission Regulation (EC) No 2073/2005 of the European parliament and the council, 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Communities L338, 22.12.05:1-26.

Anon 2006. ISO/TS 19036:2006. Microbiology of food and animal feeding stuffs -- Guidelines for the estimation of measurement uncertainty for quantitative determinations. International Organization for Standardization: Geneva, Switzerland.

Anon 2007 ISO 7218:2007. Microbiology of food and animal feeding stuffs – general requirements and guidance for microbiological examinations. International Standards Organisation, Geneva.

Anon 2010. ISO 22117:2010. Microbiology of food and animal feeding stuffs – Specific requirements and guidance for proficiency testing by interlaboratory comparison. International Organization for Standardization: Geneva, Switzerland.

Anon 2010. Commission Regulation (EC) No. 505/2010 of 14 June 2010 amending annex II to Regulation (EC) No 854/2004 of the European parliament and the Council laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Off. J. Eur. Communities L 149, 15.06.10:1-2.

Anon 2013. ISO 7218:2007/Amd1:2013. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - Amendment 1. International Standards Organisation, Geneva.

Anon 2014. EURL publication, Microbiological Monitoring of bivalve mollusc harvesting areas, guide to good practice: Technical application. Available to download from: <https://eur1cefafas.org/public-documents/official-control-guides.aspx>

Anon 2016. ISO 16649-3:2016. 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 Standards Organisation, Geneva.

Anon 2017. ISO 6887-3:2017. 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 Standards Organisation, Geneva.

This protocol was produced by the EURL for monitoring bacteriological and viral contamination of bivalve molluscs

12 APPENDICES

12.1 Appendix 1: Sub sample sizes of shellfish required for *E. coli* analysis

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

| Name ^{M N} | Latin name ^O | Sample size ^P |
|---|---|--------------------------|
| Saint James Scallop | <i>Pecten jacobaeus</i> | 10 - 12 |
| King scallops | <i>Pecten maximus</i> | 10 - 12 |
| Razor clams | <i>Ensis spp.</i> | 10 - 12 |
| Soft shell clams (Sand Gapers) | <i>Mya arenaria</i> | 10 - 12 |
| Northern horse mussels | <i>Modiolus</i> | 10 - 12 |
| Abalone | <i>Haliotis spp</i> | 10 - 12 |
| Whelks | <i>Buccinum undatum</i> | 10 - 15 |
| Variegated scallop | <i>Chlamys varia</i> | 10 - 18 |
| Oysters | <i>Crassostrea gigas and Ostrea edulis</i> | 10 - 18 |
| Noah's Ark shells | <i>Arca noae</i> | 10 - 25 |
| Mediterranean mussels | <i>Mytilus galloprovincialis</i> | 10 - 30 |
| Smooth clams | <i>Callista chione</i> | 10 - 30 |
| Purple or green Sea urchins – Adults (juvenile) | <i>Paracentrotus lividus</i> | 10 – 30 (40 – 80) |
| Hard clams | <i>Mercenaria</i> | 12 - 18 |
| Dog winkles | <i>Thais haemastoma</i> | 15 - 25 |
| Ark clams | <i>Barbatia barbata</i> | 15 - 25 |
| Bearded horse mussels | <i>Modiolus barbatus</i> | 15 - 30 |
| Queen scallops | <i>Aequipecten opercularis or Chlamys opercularis</i> | 15 - 30 |
| Warty venus clams | <i>Venus verrucosa</i> | 15 - 30 |
| Mussels | <i>Mytilus spp.</i> | 15 - 30 |
| Manila clams | <i>Tapes philippinarum</i> | 18 - 35 |
| Palourdes (Grooved carpet shell clams) | <i>Tapes decussatus (Venerupis decussata)</i> | 18 - 35 |
| Rayed artemis | <i>Dosinia exoleta</i> | 18 - 35 |
| Clam | <i>Venerupis rhomboides</i> | 20 - 25 |
| Pullet carpet shell | <i>Venerupis senegalensis</i> | 20 - 25 |
| Cockles | <i>Cerastoderma edule</i> | 30 - 50 |
| Turbinate monodont | <i>Phorcus turbinatus</i> | 30 - 50 |
| Atlantic surf clams (Thick trough shells) | <i>Spisula solida</i> | 30 - 50 |
| Periwinkles | <i>Littorina littorea</i> | 30 - 50 |
| Bean clams | <i>Donax spp.</i> | 30 - 50 |
| Wedge shell clams | <i>Donax trunculus</i> | 40 - 80 |
| Striped venus clams | <i>Chamelea gallina</i> | 40 - 80 |
| Cut trough shells | <i>Spisula subtruncata</i> | 70 - 90 |

^M Species of relevance provided by EU NRLs.

^N The list given in Appendix 1 includes bivalve molluscs, echinoderms, tunicates and marine gastropods. Commission Regulation (EU) No 505/2010 amending 854/2004 does not foresee a requirement for classification of marine gastropods.

^O Sample sizes given in this table have been provided by NRLs. The weight of shellfish flesh and liquor should be at least 50g for the *E. coli* method (for very small species such as the *Donax* spp. a minimum amount of 25g is permitted). For species not given in the table, sufficient shellfish should be opened to achieve this minimum weight of flesh and liquor, with the provision that a minimum of ten animals should be used for very large species. In general, the more shellfish that are included in the initial homogenate, the less the final result will be influenced by the inherent animal-to-animal variation in *E. coli* concentration.

^P Number of shellfish to be tested for *E. coli*. Regulation (EU) No. 2073/2005 specifies a minimum of 10 animals of any species should be examined.

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

12.2.1 Table 1: 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 ^Q | 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 ^R | 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 ^S | 1 |

^Q 0 according to ISO 7218: Amd1. The use of <18 was agreed at the annual workshop of NRL (2014).^R 1800 according to ISO 7218: Amd1 but 1700 according to the ISO MPN calculator. 1700 confirmed by ISO statistical group.^S ∞ according to ISO 7218: Amd1. The use of >18,000 was agreed at the annual workshop of NRL (2014).

12.2.2 Table 2: 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 [†] | 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 |

[†] 0 according to ISO 7218: Amd1. The use of <18 was agreed at the annual workshop of NRL (2014).

Table 2 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 ^u | 1 |

^u ∞ according to ISO 7218:Amd 1. The use of >180,000 was agreed at the annual workshop NRL (2014)

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'.

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.
- '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}).

| 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 | | | 2 | 3 |
| 8 | Note: A test series / matrix consists of the different dilutions for one target organism / test medium. | | | |
| 9 | | | | |
| 10 | Note: 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 | | | | |
| Input data | | | | |
| 12 | | | | |
| 13 | Test series 1 / Matrix 1 - Designation: | | | |
| 14 | Target organism / Test medium: | | | |
| 15 | Dilution factor | Volume in ml or g | No. of tubes | No. of positive tubes |
| 16 | d | w | n | x |
| 17 | | | | |
| 18 | | | | |
| 19 | | | | |
| 20 | | | | |
| 21 | Test series 2 / Matrix 2 - Designation: | | | |
| 22 | Target organism / Test medium: | | | |
| 23 | Dilution factor | Volume in ml or g | No. of tubes | No. of positive tubes |
| 24 | d | w | n | x |
| 25 | | | | |
| 26 | | | | |
| 27 | | | | |

Number of samples tested

Number of dilutions examined

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 No. | Designation | MPN | \log_{10} MPN | SD \log_{10} MPN | 95% confidence limits | | Rarity Index | Category |
| | | | | | Lower | Upper | | |
| 1 | Example 1 | 22 | 1.3 | 0.20 | 8.8 | 56. | 0.30 | 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 - $22 \times 100 = 2\,200$ MPN /100g.
- For example 2 - 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 <i>n</i> | No. of positive tubes <i>x</i> |
| 5 | 0 |
| 5 | 0 |
| 5 | 0 |

| Example 2 | |
|--------------------------|-----------------------------------|
| E.coli | |
| No. of tubes <i>n</i> | No. of positive tubes <i>x</i> |
| 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 | | Rarity Index | Category |
| | | | | | Lower | Upper | | |
| 1 | Example 1 | 0 | | | 0 | 0.66 | 1.000 | 1 |
| 2 | Example 2 | ∞ | | | 65. | ∞ | 1.000 | 1 |

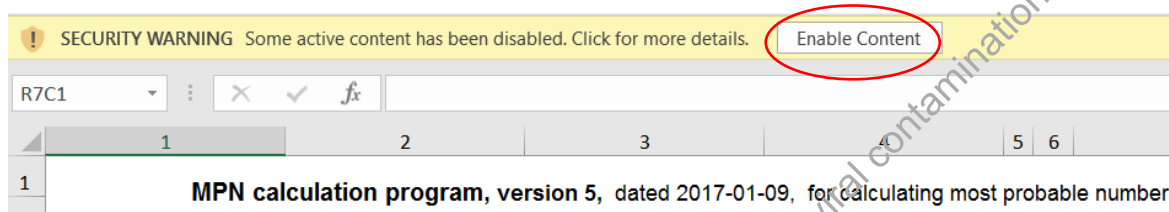
This protocol was produced by the EURL for monitoring bacteriological and viral contamination of bivalve molluscs

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}).

| 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 | | | 2 | 4 |
| 8 | Note: A test series / matrix consists of the different dilutions for one target organism / test medium. | | | |
| 9 | | | | |
| 10 | Note: 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 | | | | |
| Input data | | | | |
| 12 | | | | |
| 13 | Test series 1 / Matrix 1 - Designation: | | | |
| 14 | Target organism / Test medium: | | | |
| 15 | Dilution factor | Volume in ml or g | No. of tubes | No. of positive tubes |
| 16 | d | w | n | x |
| 17 | | | | |
| 18 | | | | |
| 19 | | | | |
| 20 | | | | |
| 21 | | | | |
| 22 | Test series 2 / Matrix 2 - Designation: | | | |
| 23 | Target organism / Test medium: | | | |
| 24 | Dilution factor | Volume in ml or g | No. of tubes | No. of positive tubes |
| 25 | d | w | n | x |
| 26 | | | | |
| 27 | | | | |
| 28 | | | | |
| 29 | | | | |

Number of samples tested

Number of dilutions examined

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 | <i>d</i> | <i>w</i> | <i>n</i> | <i>x</i> |
| 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 | <i>d</i> | <i>w</i> | <i>n</i> | <i>x</i> |
| 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 No. | Designation | MPN | log ₁₀ MPN | SD log ₁₀ MPN | 95% confidence limits | | Rarity Index | Category |
| | | | | | 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 2 - 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.

| Example 1 | |
|--------------------------|-----------------------------------|
| E. coli | |
| No. of tubes <i>n</i> | No. of positive tubes <i>x</i> |
| 5 | 5 |
| 5 | 5 |
| 5 | 5 |
| 5 | 5 |

| Example 2 | |
|--------------------------|-----------------------------------|
| E. coli | |
| No. of tubes <i>n</i> | No. of positive tubes <i>x</i> |
| 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 | | Rarity Index | Category |
| | | | | | Lower | Upper | | |
| 1 | Example 1 | ∞ | | | 650. | ∞ | 1.000 | 1 |
| 2 | Example 2 | 0 | | | 0 | 0.66 | 1.000 | 1 |

This protocol was produced by the EURL for monitoring bacteriological and viral contamination of bivalve molluscs

About us

Cefas is a multi-disciplinary scientific research and consultancy centre providing a comprehensive range of services in fisheries management, environmental monitoring and assessment, and aquaculture to a large number of clients worldwide.

We have more than 500 staff based in 2 laboratories, our own ocean-going research vessel, and over 100 years of fisheries experience.

We have a long and successful track record in delivering high-quality services to clients in a confidential and impartial manner.
(www.cefas.defra.gov.uk)

Cefas Technology Limited (CTL) is a wholly owned subsidiary of Cefas specialising in the application of Cefas technology to specific customer needs in a cost-effective and focussed manner.

CTL systems and services are developed by teams that are experienced in fisheries, environmental management and aquaculture, and in working closely with clients to ensure that their needs are fully met.
(www.cefastechnology.co.uk)

Head office
Centre for Environment,
Fisheries & Aquaculture Science
Pakefield Road, Lowestoft,
Suffolk NR33 0HT UK

Tel +44 (0) 1502 56 2244
Fax +44 (0) 1502 51 3865
Web www.cefas.defra.gov.uk

Customer focus

With our unique facilities and our breadth of expertise in environmental and fisheries management, we can rapidly put together a multi-disciplinary team of experienced specialists, fully supported by our comprehensive in-house resources.

Our existing customers are drawn from a broad spectrum with wide ranging interests. Clients include:

- international and UK government departments
- the European Commission
- the World Bank
- Food and Agriculture Organisation of the United Nations (FAO)
- oil, water, chemical, pharmaceutical, agro-chemical, aggregate and marine industries
- non-governmental and environmental organisations
- regulators and enforcement agencies
- local authorities and other public bodies

We also work successfully in partnership with other organisations, operate in international consortia and have several joint ventures commercialising our intellectual property

**Centre for Environment,
Fisheries & Aquaculture Science**
Weymouth Laboratory,
Barrack Road, The Nothe, Weymouth,
Dorset DT4 8UB

Tel +44 (0) 1305 206600
Fax +44 (0) 1305 206601

