National Reference Laboratory for bacteriological 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)

Note: For official control testing in the UK, either this document or the NRL generic protocol for enumeration of *Escherichia coli* in bivalve molluscan shellfish by the colony-count technique (based on ISO 16649-2) must be used.

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Issue number: 17

Date: March 2024

Centre for Environment Fisheries & Aquaculture Science

Cefas

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History of Procedure

Issue	Date	Section	Changes
13	14.06.17	Footnotes	Included throughout the document
		2.0 note	The inclusion of the bivalve shellfish MPN calculator
		5.0, 8.6, 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. Footnote amended.
		8.1	Sample temperature criteria for samples receipted within 4 hours of sample collection. Footnote added provides guidance on use of temperature data loggers.
		8.2	Inclusion of guidance relating to extended storage conditions.
		8.4 and 8.5.1	Notes moved and added to footnote.
		8.7	Footnote added relating to inoculating loops.
		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
		9	Inclusion of measurement uncertainty
		10, 10.1, 10.2 and 10.3	Update to the PHE trend analysis link, guidance on Internal quality controls, proficiency testing and trend monitoring.
		11	Update to References
		Appendix 1	Update to shellfish species and the inclusion of (for very small species such as the Donax spp. a minimum amount of 25g is permitted).
		Appendix 2	Update to the application of the MPN calculator
14	23.05.18	2	Removal of the 'TS' and update of the publication date for ISO 16649-3
		8.2	Update on sample storage specifications with upper limit (48 h) indicated
15	17.09.19	10.1	Foot note add stating recommended proficiency testing frequency requirements
16	03.10.23	Front page	Note added to identify that this NRL generic protocol should be used for official testing in the UK
		5	Equipment for dissection of gastropods and echinoderms added
		8.4	Harmonisation of sample preparation, addition of oyster cracker image and information on how to prepare gastropods and echinoderms
		8.5	Procedure for blending and stomaching harmonised with the NRL Salmonella protocol
		Appendix 1	Species list updated
		References	Updated References

17	20.03.24	Front page	Note amended to clarify that either this document or the NRL generic protocol for enumeration of E.coli using the colony-count method should be used for UK official testing

Contents

1. Introduction

The risks of infectious human diseases acquired from the consumption of bivalve molluscan shellfish (BMS) are internationally recognised. These health hazards are largely due to the phenomenon of filter-feeding whereby BMS concentrate and retain various bacterial and viral pathogens, often derived from sewage contamination of their growing waters. The risks of exposure to infectious agents are compounded by the traditional consumption of raw, or only lightly cooked, BMS. Historically, enteric bacteria, such as faecal coliforms, have been adopted as surrogate indicator organisms to assess the quality of shellfish flesh, and, consequently, to predict the risk of exposure to enteric pathogenic viruses.

In the UK, the criteria for laying down the microbiological standards for BMS and other marine shellfish including gastropods and echinoderms are set out in Retained Regulation (EC) 2017/625 (Anon, 2017) and Retained Regulation (EC) 2073/2005 (Anon, 2005) stipulating conditions for the production and placing on the market of live shellfish. In the UK *Escherichia coli* is used as an indicator of faecal contamination of shellfish.

2. Scope

This protocol has been produced with reference to ISO 16649-3 (Anon, 2015) and relevant parts of other 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±1˚C and expresses β-glucuronidase activity at 44±1˚C.

Note: In addition to BMS, this method is also applicable to analysis of other marine shellfish species including gastropods and echinoderms. In these cases alternative sample preparation methods should be used – these are detailed in section 8.4.

Note: The 5x3 and 5x4 MPN tables included in this document have been generated using the MPN calculator referenced in ISO 7218:2007 + Amd 1:2013. Values have been converted from MPN/g to MPN/100g. A shellfish-specific MPN calculator has been developed and empty and prepopulated versions are available on the NRL website (NRL Laboratory Protocols).

3. Principle

This protocol used to enumerate *E. coli* in shellfish 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±1˚C for 24±2 hours. The presence of *E. coli* is subsequently confirmed by sub-culturing 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±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 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±2˚C
- Sterile glassware
- Safety/electric Bunsen system
- Protective gloves – single use
- Safety gloves – for example a chain mail glove
- Incubator at 37±1˚C and 44±1˚C
- Shucking knife, oyster cracker or other suitable equipment for opening and dissecting shellfish
- Electric top pan balance
- Loops - sterile, 10µl
- Pipette filler and graduated pipettes or automatic pipettor and pipette tips of a range of sizes e.g. 1ml and 10ml. For handling mixed shellfish homogenates (1:3 and 10-1 dilution) open-ended pipettes may be necessary due to high concentrations of particulate matter
- Sterile forceps
- Sterile scissors
- Sterile spatula
- Sterile hammer or equivalent tool
- Food grade plastic bags
- Absorbent paper towel

6. Media

- Ethanol
- 0.1% peptone (0.1% P)
- Peptone salt solution (PSS) (referred to as Maximum Recovery Diluent (MRD) in the UK)
- Minerals modified glutamate broth at single and double strength (MMGBx1, MMGBx2)
- Tryptone bile glucuronide agar (TBGA/TBX)

7. Microbiological reference cultures for performance testing and controls

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

Table 1: Microbiological reference strains used for control purposes

Media type	Control strain	WDCM [a]	Criteria	Characteristic reaction	Strain choice [c]
Mineral-modified glutamate medium (MMGB)	*Escherichia coli*	00012 or 00013	Acid production	Colour change to yellow	1
	Enterococcus faecalis	00009 or 00087	No growth	No colour change	1
Tryptone bile glucuronide agar (TBGA/TBX)	*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
	Enterococcus faecalis	00009 or 00087	Total inhibition	no growth	1
	Pseudomonas aeruginosa	00025	White to beige colonies	B-glucuronidase negative	1
	Citrobacter freundii [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 former EURL noted growth was not always present. We therefore recommend 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 placed 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.[1] For samples where less than 4 hours have elapsed between collection from the production area and receipt at the laboratory, the internal cool box air temperature (or between-shellfish sample temperature) should be less than the temperature recorded at the time of sampling. Samples from harvesting areas should have been rinsed (but not immersed) and drained at time of sampling. A sample 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 (CA).

Note: The UK NRL has carried out studies on the effect of extended storage time and elevated

[1] A temperature data logger may be used to monitor the sample temperature during transit. The data logger must be held in a central position within the cool box and not allowed to come into contact with the ice packs. If the logger has not been activated or is found to be in contact with the ice packs on arrival at the laboratory, then the temperature of the shellfish sample itself should be measured using an appropriately calibrated temperature probe.

temperatures on certain species of BMS. 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 microbiological analysis cannot be initiated within 24 hours of sample collection, an upper limit of 48 hours should be used.

8.3. Sample selection

Choose shellfish that are alive according to the following criteria:

- Reaction or movement of exposed flesh after touching using a sterile shucking knife.
- Shellfish 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

Any mud and sediment adhering to the shellfish should be removed prior to opening the shellfish 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 [2].

8.4.1. Preparation of BMS using the oyster cracker

Sterilise the blade of the oyster cracker before use (see Figure 1 and Figure 2). Place a single animal in a weighing dish on the platform underneath the blade. Lower the lever so that the blade engages the hinge of the shellfish, then fully pull the lever down so that the blade separates the shells. Using a sterilised shucking knife cut the muscle and scrape the meat of both shells into the sterilised container. Transfer any liquor collected in the weighing boat into the sterilised container.

[2] Alternative suitable equipment can be used to open bivalve shellfish.

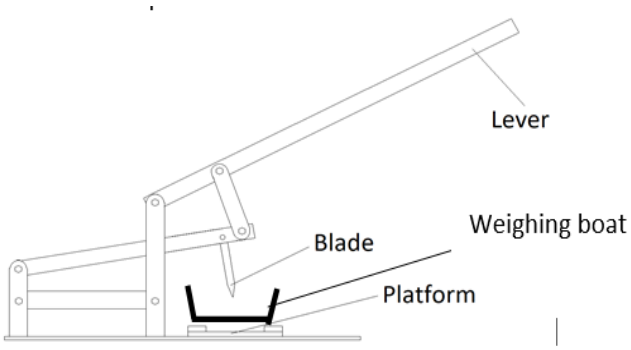

Figure 1: Oyster cracker apparatus

Figure 2: Opening an oyster using an oyster cracker

8.4.2. Preparation of BMS using a shucking knife

Open all selected shellfish as described below using a sterilised shucking knife or equivalent and empty meat and liquor into a sterile container. 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.

8.4.2.1. Oysters and 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 and cockles

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.

8.4.3. Preparation of gastropods

Place a single animal into the hinge section of the oyster cracker (see Figure 3) and pull the lever down to break open the shell. Transfer the material onto sterile absorbent paper towel on a tray and remove any shell debris using sterile forceps. Repeat the process with the required number of animals (see Appendix 1). Dice the animal flesh using sterile scissors before transferring to a sterile container.

Alternatively, the animals can be opened using a sterile hammer or equivalent tool, without damaging the flesh, by placing the animals in a food grade bag and covering with absorbent paper towel.

Figure 3: Opening a gastropod using an oyster cracker

8.4.4. Preparation of echinoderms (e.g. sea urchins)

Hold the echinoderm with the mouth parts facing upwards. Using sterile scissors and wearing a strong clean glove, cut out the mouth section (Figure 4) to expose the flesh inside. Transfer the flesh and internal fluid into a sterile container using a sterile spatula. Repeat the process with the required number of animals (see Appendix 1).

Figure 4: Opening an echinoderm using sterile scissors

8.5. Dilution and homogenisation

Weigh the sterilised container and calculate the weight of the contents by subtracting the weight of the pre-weighed container to the nearest gram.

Note: Complete either sections 8.5.1 or 8.5.2.

8.5.1. Homogenisation in a blender

Measure 2ml of sterile 0.1% P per 1g of shellfish (±2ml) using a measuring cylinder. Place contents of sterilised container into a 1 litre blender jar [3] with the 0.1% P from the measuring cylinder and homogenise at high speed for approximately 1 minute (4 bursts of 15 seconds with at least 5 seconds between bursts) in a class two microbiological laminar flow cabinet. Decant the contents (mixed shellfish homogenate (1:3 dilution)) back into the sterilised container.

Add 30±0.5ml of mixed shellfish homogenate to 70±1ml of 0.1% P to make a master 10^{-1} dilution, ensure that this master dilution is thoroughly mixed.

Note: it may be necessary to use an open-ended pipette to transfer mixed shellfish homogenate due to high concentrations of particulate matter.

8.5.2. Homogenisation using a stomacher

Place the contents of the sterilised container into at least three stomacher bags (e.g. place three stomacher bags inside each other to avoid small pieces of shell from puncturing the bags). Remove excess air from the bags and operate the stomacher for 2-3 minutes. Transfer 50g of homogenised shellfish into another set of 3 stomacher bags, add 100ml of 0.1% P and homogenise for a further 2-3 minutes. After homogenisation decant the contents into a new sterilised container and add 350ml 0.1% P to make a master 10^{-1} dilution, ensure that this master dilution is thoroughly mixed.

Note: Results from proficiency testing distributions of cockles have tended to give lower *E. coli* concentrations following stomaching than following blending. NRL advice is therefore to use blending for samples of cockles and other small species such as clams.

[3] If shellfish are particularly small, it may be necessary to use a smaller blender to achieve a consistent homogenate.

8.5.3. Preparation of further dilutions

Make a further decimal dilution (10^{-2}) using the master 10^{-1} dilution as prepared in 8.5.1 or 8.5.2 by adding 1ml of the 10^{-1} dilution to 9ml of 0.1% P or PSS. If required, prepare a 10^{-3} dilution by adding 1ml of the 10^{-2} dilution to 9ml of 0.1% P or PSS.

Note: Ideally the 10^{-3} dilution should be performed for all samples in order to allow a result of over 46000 (the upper threshold defined in the legislation for any classified sites) to be determined in the event of gross contamination episodes. However, if this is not possible then this dilution should at least be carried out for samples more likely to be contaminated according to the following criteria [4].

- Class B areas that have returned > 18000 results
- Seasonal Class B/C areas
- Class C and prohibited areas

Note: Dilutions beyond 10^{-3} will only be required in special circumstances (e.g. pollution incident investigations).

Note: For samples where more than 3 dilutions are tested, ensure all results from each dilution tested are 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} master dilution (equivalent to 1g of tissue per tube and referred to as the 'Neat' concentration). Inoculate five bottles containing 10±0.2ml of single strength MMGB with 1±0.1ml of the 10^{-1} master dilution (equivalent to 0.1g of tissue per tube and referred to as '10^{-1}' concentration). Inoculate five bottles containing 10±0.2ml of single strength MMGB with 1±0.1ml of the 10^{-2} diluted homogenate (equivalent to 0.01g of tissue per tube and referred to as '10^{-2}' concentration) and subsequently repeat with any further dilutions.

For controls, inoculate a bottle containing 10±0.2ml of single strength MMGB with *E. coli* WDCM 00012 or 00013 and a separate bottle containing 10±0.2ml of single strength MMGB with *E. faecalis* WDCM 00009 or 00087 using 10µl sterile loop [5]. Include an uninoculated bottle containing 10±0.2ml of single strength MMGB as a negative control.

Incubate all test and control 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. Confirm the presence of *E. coli* in all tubes showing acid production by subculturing onto TBGA/TBX plates within 4 hours using a 10µl sterile loop, streaking with the objective to obtain single colonies. Subculture each MMGB control

[4] NRL advice

[5] A 1µl loop can be used instead of a 10µl loop as given in ISO 16649-3. The NRL has shown the change in loop size has no practical consequences on *E. coli* recoveries.

prepared in 8.6 onto a TBGA/TBX plate. Incubate the TBGA/TBX 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 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 incubation examine the TBGA/TBX plates for the presence of blue to blue-green colonies. Record the results as positive for any shade of blue or blue-green colonies and negative for colonies of any other colour or for no growth.

8.8. Calculation of *E. coli* Most Probable Number

To determine the MPN, record as negative all MMGB tubes 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/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 (normally 5) equals the sum of tubes that are positive and negative at that dilution. The number of positive and total tubes at each dilution is used to calculate the MPN value, either by using the Shellfish MPN calculator [6] (see section 8.8.1) or by referring to the MPN tables given in Appendix 2 (see section 8.8.2).

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. The Shellfish MPN calculator displays the probability category in column 14. Only values determined from combinations that are shown as category 1 or 2 should be recorded. Results for tube combinations that are shown as category 3 values should be recorded as 'Void'. The MPN tables in Appendix 2 only give results for category 1 and 2 combinations; any other combination will be category 3 and results for these tube combinations should be recorded as 'Void'.

8.8.1. The Shellfish MPN calculator

Three copies of the Shellfish MPN calculator are available from the NRL website (NRL Laboratory Protocols). Two have been prepopulated in the general data section with 3 and 4 dilution factors respectively, and a number of 5 MMGB tubes inoculated at each dilution added in the input data section. The third version, suitable for use with more than 4 dilutions, has no information added.

To use the Shellfish MPN calculators attached to this protocol;

A. Download and open a copy of the appropriate MPN calculator (depending on the number of dilutions tested) from the website.

Note: MPN values must be determined using the results obtained from all dilutions tested

[6] The Shellfish MPN calculator has been developed specifically for use in bivalve shellfish official control laboratories. The Excel spreadsheet MPN calculator cited in ISO 7218 (http://standards.iso.org/iso/7218/) can 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).

even if lower dilutions are completely negative

B. Click on the 'Enable content' button at the top of the spreadsheet.

C. If required, enter relevant data in the general data section. The no. of samples (column 3) and max. no. of dilutions cells (column 4) must be populated if using the generic version of the calculator (these cells are prepopulated in the 3 and 4 dilution factor versions). Name of experiment and date of experiment cells (columns 1 and 2) do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files.

D. If required, enter the total number of tubes at each dilution in the input data section (column 3; these cells are prepopulated in the 3 and 4 dilution factor versions). Sample designation and target organism cells do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files.

E. For each sample, enter the number of positive tubes at each dilution in the input data section (column 4).

F. Press the 'calculate results' button in the top right of the spreadsheet or press 'Ctrl + M'. The calculated MPN value is shown in column 8 as MPN per 100g, and the probability category is shown in column 14.

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

8.8.2. MPN tables (Appendix 2)

All 3 and 4 dilution MPN tube combinations in probability categories 1 and 2 have been provided in Tables 1 and 2 in Appendix 2. To find the MPN value, refer to the appropriate MPN table for the number of dilutions examined:-

- For 3 dilutions (neat, 10^{-1} and 10^{-2}) use Table 1.

- For 4 dilutions (neat, 10^{-1}, 10^{-2} and 10^{-3}) use Table 2.

Note: The values in the MPN tables provided in Appendix 2 were generated by using the Excel spreadsheet MPN calculator documented in ISO 7218:2007 + Amd 1:2013 (http://standards.iso.org/iso/7218/) and converting the results to MPN/100g.

8.9. Reporting results

Results should be reported as MPN per 100g of flesh and intra-valvular liquid. Tube combinations of 0, 0, 0 and 5, 5, 5 should be assigned estimated values of <18 and >18000 MPN per 100g of flesh and intra-valvular liquid respectively (see note). Likewise, tube combinations of 0, 0, 0, 0 and 5, 5, 5, 5 should be assigned estimated values of <18 and >180000. Only values determined from combinations that give a category 1 or 2 profile should be reported. Results for samples with tube combinations that give a category 3 profile should be reported as 'Void'. Negative samples should be reported as <18 MPN per 100g.

Note: According to ISO 7218:2007 + Amd 1:2013 (both the Excel spreadsheet MPN calculator and MPN tables in Annex C (normative)), tube combinations of 0, 0, 0 (or 0, 0, 0, 0) and 5, 5, 5 (or 5, 5, 5, 5) should be given estimated MPN 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 these combinations as per the MPN tables in Appendix 2.

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-comparability 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.

The theoretical standard deviation of a $\log_{10}$ MPN result is approximately 0.24, provided that results do not show "extreme" tube combinations (Anon, 2010). In practice, the observed $\log_{10}$ standard deviations (SD) of several replicate examinations of a single sample are usually larger than this. The expanded uncertainty of the *E. coli* method described in this procedure has been estimated at 0.66 ($\log_{10}$-transformed data), this is calculated as 2 x the measured SD of the results of a structured analysis carried out by the former EURL for monitoring bacteriological and viral contamination of bivalve molluscs (Lee & Silk, 2012).

It should be noted that the variability of the estimated SD itself (and thus the expanded uncertainty) tends to be greater with smaller numbers of replicates. ISO 19036 gives guidance on the estimation of measurement uncertainty for quantitative determinations in food microbiology.

10. Quality control

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

10.1. Proficiency testing

In order to comply with the requirements of Retained Regulation (EC) No. 2017/625, laboratories undertaking microbiological examination of official control samples of shellfish for *E. coli* and/or *Salmonella* spp. are expected to take part in the UKHSA/Cefas Shellfish EQA scheme (for further information contact foodeqa@ukhsa.gov.uk) and NRL proficiency test distributions [7]. These schemes provide an independent assessment of a laboratory's performance against other participants and can help improve the performance of the laboratory.

[7] It was agreed at the Laboratory network meeting in 2018 that laboratories analysing OC samples should take part in at least 2 EQA (UKHSA/Cefas) schemes and 1 whole animal distribution per year.

10.2. Trend monitoring

To ensure continuing intra-comparison of test results, trend analysis should be undertaken by regularly reviewing laboratory performance in the Shellfish EQA scheme. Results should be assessed against the participants' median and plotted graphically showing performance over time. This enables recognition of unusual trends in performance compared to other laboratories and allows for appropriate follow-up action. An Excel spreadsheet for this purpose is given at the Shellfish EQA Scheme web page (https://www.gov.uk/government/publications/shellfish-scheme-trend-analysis).

In addition, laboratories should keep a check on the number of void (invalid) tube combinations seen over time. These are tube combinations that are not category 1 or 2 (and thus, if using the appropriate dilutions, do not appear in the tables given in Appendix 2). It is expected that less than 1 % of samples tested will show void tube combinations. If a higher proportion of void MPN tube combinations is observed, this should be investigated. An advice document has been produced by the NRL (Anon, 2008).

10.3. 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.

11. References

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Anon 2017. ISO 6887-3:2017 + Amd 1:2020. 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.

Anon 2017. ISO 6887-1:2017. Microbiology of food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 1: General rules for the preparation of the initial suspension and decimal dilutions. International Organization for Standardization, Geneva.

12. Appendices

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

The following sample sizes are recommended for inclusion in the homogenisation step (the recommended number for sampling is 10 % greater to allow for morbidity in a proportion of animals on receipt at the laboratory).

Type	Common name [8]	Scientific name [9]	Sample size [10]
Scallops	Mediterranean scallop	*Pecten jacobaeus*	10 - 12
	King (Great Atlantic) scallop	*Pecten maximus*	10 - 12
	Queen scallop	*Aequipecten (Chlamys) opercularis*	15 - 30
	Variegated scallop	*Mimachlamys (Chlamys) varia*	10 - 18
Oysters	Pacific oyster	*Magallana (Crassostrea) gigas*	10 - 18
	Portuguese oyster	*Magallana (Crassostrea) angulata*	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
Ark Clams	Ark clam	*Barbatia barbata*	15 - 25
	Noah's ark shell	*Arca noae*	15 - 30
Clams	Smooth clam	*Callista chione*	10 - 30
	Striped venus clam	*Chamelea gallina*	40 - 80
	Rayed artemis	*Dosinia exoleta*	18 - 35
	Hard clam	*Mercenaria mercenaria*	12 - 18
	Palourde (Grooved carpet shell)	*Ruditapes (Tapes/Venerupis) decussatus*	18 - 35
	Manila clam	*Ruditapes (Tapes) philippinarum*	18 - 35
	Banded carpet shell	*Polititapes (Venerupis) rhomboides*	20 - 25
	Pullet carpet shell	*Venerupis corrugata (senegalensis)*	20 - 25
	Warty venus clam	*Venus verrucosa*	15 - 30
	Atlantic surf clam (Thick trough shell)	*Spisula solida*	30 - 50
	Cut trough shell	*Spisula subtruncata*	70 - 90
	Bean clam	*Donax spp.*	30 - 50
	Wedge shell clam	*Donax trunculus*	40 - 80
	Common cockle	*Cerastoderma edule*	30 - 50
	Soft shell clam (Sand gaper)	*Mya arenaria*	10 - 12

[8] Species of relevance provided by European NRLs.
[9] Scientific names are those used by the World Register of Marine Species at the time of writing, however commonly used alternatives for genus or species names are shown in brackets.
[10] Number of shellfish to be tested for *E. coli.* Retained Regulation (EC) No. 2073/2005 specifies a minimum of 10 animals of any species should be examined. Sample sizes given in this table have been provided by European NRLs. The weight of shellfish flesh and liquor should be at least 50g for the *E. coli* method (for very small species such as *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.

Razor Clams and Geoducks	Pacific geoduck	*Panopea generosa*	10 - 12
	Sword razor	*Ensis ensis*	10 - 12
	Razor shell	*Ensis magnus (arcuatus)*	10 - 12
	Atlantic razor clam (Jacknife clam)	*Ensis leei*	10 - 12
	Pod razor	*Ensis siliqua*	10 - 12
	Grooved razor shell	*Solen marginatus*	10 - 12
Gastropods	Abalone	*Haliotis spp.*	10 - 12
	Turbinate monodont	*Phorcus turbinatus*	30 - 50
	Whelk	*Buccinum undatum*	10 - 15
	Dog winkle	*Stramonita (Thais) haemastoma*	15 -25
	Common periwinkle	*Littorina littorea*	30 - 50
Echinoderms	Purple sea urchin	*Paracentrotus lividus*	10 - 30 (adults), 40 - 80 (juveniles)

12.2. Appendix 2: *E. coli* MPN tables

12.2.1. Table 1: MPN 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 [11]	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 [12]	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 [13]	1

[11] 0 according to ISO 7218. The use of <18 was agreed by the NRL annual laboratory meeting (2014).
[12] 1800 according to ISO 7218 but 1700 according to the ISO MPN calculator. 1700 confirmed by ISO working group on statistics for food microbiology.
[13] ∞ according to ISO 7218. The use of >18000 was agreed by the NRL annual laboratory meeting (2014).

12.2.2. Table 2: MPN table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g, 5 x 0.001g.

1g	0.1g	0.01g	0.001g	MPN/100g [14]	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

[14] See footnotes to Table 1.

Table 2 continued: MPN table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g, 5 x 0.001g.

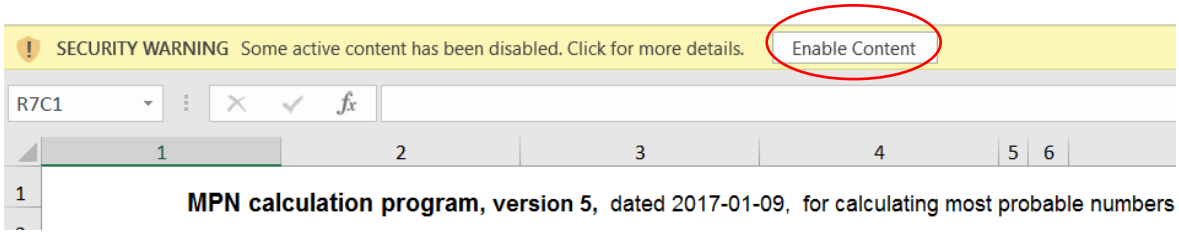
1g	0.1g	0.01g	0.001g	MPN/100g	Category
5	5	0	1	3100	1
5	5	1	0	3300	1
5	5	1	1	4600	1
5	5	1	2	6300	2
5	5	2	0	4900	1
5	5	2	1	7000	1
5	5	2	2	9400	1
5	5	3	0	7900	1
5	5	3	1	11000	1
5	5	3	2	14000	1
5	5	3	3	17000	2
5	5	4	0	13000	1
5	5	4	1	17000	1
5	5	4	2	22000	1
5	5	4	3	28000	1
5	5	4	4	35000	2
5	5	5	0	24000	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	1

12.3. Appendix 3: Application and use of Excel MPN calculator (ISO 7218)

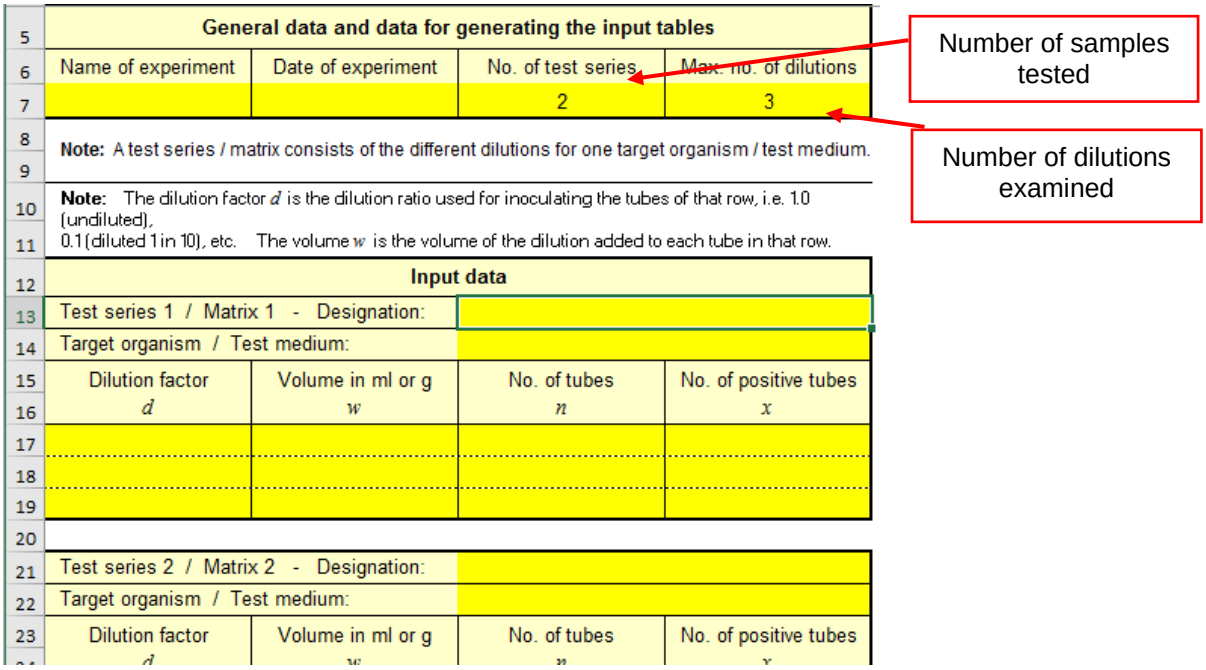
1. Open the link http://standards.iso.org/iso/7218/.

2. The ISO Standards 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 relevant data in the general data section. The no. of test series (column 3, equivalent to number of samples, maximum of 30) and max. no. of dilutions cells (column 4) must be completed. Name of experiment and date of experiment cells (columns 1 and 2) do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files. Once the cells are filled, the required number of data boxes will automatically appear below (one for each test series/sample specified).



5. Populate the input data table as follows (test series designation and target organism cells do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files):-

- Column 1 - Insert the homogenate concentration/dilution factor used to inoculate the MMGB tubes at each dilution (0.1 for the neat and 10^{-1} dilutions, 0.01 for the 10^{-2} dilution, 0.001 for

the 10^{-3} dilution if used).

- Column 2 - Insert the volume of inoculum added per tube at each dilution (10 for the neat dilution, 1 for all subsequent dilutions).
- 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 (after confirmation using TBGA/TBX) at each dilution.

12	**Input data**			
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

Volume of inoculum added

Number of MMGB tubes

Number of positive tubes on TBGA/TBX

6. Press 'Ctrl M' or press Calculate results (green button). The MPN value **in MPN per g** will be calculated in column 8 and the probability category for the combination shown 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 probability category of each sample tested and accept only MPN results that give a category 1 or category 2 profile. Reject all MPN results show as probability category 3.

- For example 1 - the result (category 1) can be used.
- For example 2 - the result (category 3) is rejected and reported as a 'Void' result. A repeat sample must be requested.

8. The MPN calculator displays results in units of MPN per gram (MPN/g). *E. coli* results for shellfish must be reported as MPN per 100g (MPN/100g). Multiply the value given in column 8 by 100 to give the results in MPN per 100g.

For the examples given above

- For example 1 - 22 x 100 = 2200 MPN /100g.
- For example 2 - A repeat sample would need to be requested as the tube combination gives a probability category of 3.

9. For samples that give a tube combinations of 0, 0, 0 and 5, 5, 5 (3 dilutions) or 0, 0, 0, 0 and 5, 5, 5, 5 (4 dilutions) the MPN calculator will provide a value of 0 and infinity respectively (see below). As explained in section 8.9 results for these tube combinations should be reported as <18 (0, 0 ,0 or 0, 0, 0, 0), >18000 (5, 5, 5) and >180000 MPN per 100g (5, 5, 5, 5) respectively.

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	95% confidence limits Upper	Rarity Index	Category
1	Example 1	0			0	0.66	1.000	1
2	Example 2	∞			65.	∞	1.000	1

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