FAO Reference Centre for Bivalve Mollusc Sanitation

Generic protocol – Enumeration of *Escherichia coli* in bivalve molluscan shellfish by the colony-count technique (based on ISO 16649-2)

Author: James Lowther

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Centre for Environment, Fisheries & Aquaculture Science

Cefas

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History of Procedure

Issue	Date	Section	Changes
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1 Introduction

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

In the European Union (EU), the criteria for laying down the microbiological standards for bivalve molluscs are set out in Regulations 2017/625 (European Union, 2017) and 2073/2005 (European Union, 2005) for classification and end product testing respectively. In these regulations *E. coli* is used as an indicator of faecal contamination of shellfish and the reference method for enumeration of *E. coli* is stated as the most probable number (MPN) technique based on ISO 16649-3. These regulations further stipulate that alternative methods may be used if they are validated according to the criteria in ISO 16140.

In 2010, the RIKILT Institute of Food Safety, Wageningen University, the Netherlands undertook a validation study (Jacobs-Reitsma and Pol-Hofstad, 2010) to demonstrate the suitability of a colony-count (pour plate) protocol for enumeration of *E. coli* that it had developed based upon ISO 16649-2 as an alternative method to the MPN. This validation was carried out according to ISO 16140:2003. The validation was certified at the time by MicroVal as an independent certification body and further approved by the European Union Reference Laboratory (Cefas). A further validation study on the colony-count method using the original study data was carried out by the National Institute for Public Health and the Environment, the Netherlands, in 2021 (Pol-Hofstad and Jacobs-Reitsma, 2021) to reflect updated validation criteria in ISO 16140-2:2016.

The colony-count method is currently listed as one of two accepted alternative methods in the EU Community Guide to the Principles of Good Practice for the Microbiological Classification and Monitoring of Bivalve Mollusc Production and Relaying Areas (Anon, 2021), and can be considered as an equivalent to the reference MPN method as required by the Codex Standard for Live and Raw Bivalve Molluscs CXS 292-2008 (Codex Alimentarius, 2015).

2 Scope

This protocol has been produced with reference to ISO 16649-2, relevant parts of other ISO standards referenced in Section 11, and the two validation studies carried out in the Netherlands (Jacobs-Reitsma and Pol-Hofstad, 2010; Pol-Hofstad and Jacobs-Reitsma, 2021). In the context of this test detectable *E. coli* expresses β-glucuronidase activity at 44±1˚C.

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

3 Principle

The method used to enumerate *E. coli* in bivalve molluscs is a pour plate, colony-count technique. Diluted bivalve shellfish homogenate is mixed with TBGA/TBX medium in sterile Petri dishes and incubated at 37±1 °C for 4 hours (resuscitation step). The inoculated TGBA/TBX plates are then transferred to 44±1 °C for a further 18-24 hours. *E. coli* is confirmed by the presence of blue/green colonies within the medium indicative of 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. Risks of cuts and minor physical injury exist when performing this procedure, particularly when using sharp oyster knifes to open shellfish. Appropriate measures to reduce these risks should be taken. Homogenisation of shellfish should be performed in a Class II safety cabinet to reduce the risk of infection from aerosol inhalation. Laboratory procedures should conform to the recommendations given in the WHO Laboratory Biosafety Manual Third edition (WHO, 2004) or relevant national legislation or guidelines.

5 Equipment

- Autoclave (or media preparator including an autoclave component)
- Drying cabinet or ventilated oven capable of being maintained between 25±1 °C and 50±1 °C
- Balance capable of weighing to ±0.1 g and reference weights for calibration
- pH-meter, with automatic or manual temperature compensation and having a resolution of 0.01 pH units and accurate to within ±0.1 pH unit at 25 °C. Reference solutions for calibrating the pH meter
- Local Exhaust Ventilation (LEV; for weighing powders during media preparation)
- Class II safety cabinet
- Thermometer with a resolution of 0.1 °C or better at the temperature being measured
- Sterile measuring cylinder - 250 ml (a larger cylinder may be needed for some species)
- Sterile petri dishes (for solid media): diameter 90 mm; height 15-16 mm; vented
- Shucking knife, oyster cracker or other suitable equipment for opening bivalve molluscs
- Food grade plastic bags
- Weighing dish capable of holding the largest size of bivalve mollusc to be tested plus released intravalvular fluid. Must be capable of being cleaned and sterilized (for use with oyster cracker)
- Safety gloves - for example a chain mail glove (for use with the shucking knife)
- Blender and 1 litre blending jars or stomacher and stomacher bags
- Refrigerators at 3±2 °C and 5±3 °C
- Sterile container of at least 500 ml capacity (a larger capacity may be required when large bivalve molluscs are to be examined. Examples include *Panopea generosa* (geoducks), *Mya arenaria* (soft shell clams; sand gapers) and some types of razor clams)
- Safety Bunsen burner or electric micro-incinerator
- Protective gloves - single use
- Incubator or recirculating water bath at 37±1 °C and 44±1 °C

- Steamer, microwave or equivalent apparatus for melting prepared media
- Waterbath at 44-47 °C
- Pipette filler and graduated pipettes or automatic pipettor and pipette tips of a range of sizes e.g. 1 ml and 10 ml. For handling mixed shellfish homogenates (1 in 2 and 1 in 20 dilutions) open-ended pipettes may be necessary due to high concentrations of particulate matter.

6 Media and Reagents

Media stored under refrigeration should be allowed to equilibrate at room temperature before use (ISO 11133). For performance testing of media see Table 1 in section 7.

Note: Dehydrated media purchased commercially should be prepared according to the manufacturer's instructions. If any alternative products are used the laboratory must determine that they are fit for purpose.

- Ethanol
- Peptone salt solution (PSS); formula per litre - de-ionised water 1±0.01 litre, peptone 1±0.1 g, sodium chloride 8.5±0.1 g, pH 7±0.2. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121±2 °C for 15±1 minutes. Store at 5±3 °C in the dark.
- Tryptone bile glucuronide agar (TBGA; also marketed commercially as TBX); formula per litre - de-ionised water 1±0.01 litre, tryptone bile x-glucuronide medium 36.6±0.5 g, pH 7.2±0.2. Sterilize by autoclaving at 121±2 °C for 15±1 minutes. Store at 5±3 °C in the dark.

7 Microbiological reference cultures

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

Table 1: Microbiological reference strains

Media type	Control strain	WDCM [a]	Criteria	Characteristic reaction	Strain choice [c]
Tryptone bile glucuronide agar (TBGA/TBX)	*Escherichia coli*	00202	Blue to blue-green colonies	β-glucuronidase positive (weak)	2
	Escherichia coli	00012 or 00013	Blue to blue-green colonies	β-glucuronidase positive	1
	Enterococcus faecalis	00009 or 00087	Total inhibition	no growth	1
	Pseudomonas aeruginosa	00025	White to beige colonies	β-glucuronidase negative	1
	Citrobacter freundii [b]	00006			

[a] Follow the link WDCM Reference Strain Catalogue to obtain NCTC number for WDCM reference strains.
[b] Following the sub-culturing of *C. freundii* onto TBGA/TBX plates, growth is not always present. We therefore recommend the use of *P. aeruginosa* over *C. freundii.*
[c] Reference strains given in ISO 16649-2 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 Procedure

8.1 Sample transport and receipt

Samples must be placed in an intact food-grade plastic bag, or equivalent, and properly packed in a cool box with ice packs – packed in this manner they should reach a temperature between 0 °C and 10 °C within 4 hours and then maintain this for at least 24 hours[1]. The specific cool box, ice pack and transport condition combination should be validated to ensure that this can be achieved (https://www.cefas.co.uk/nrl/information-centre/nrl-laboratory-protocols/). For samples where less than 4 hours have elapsed between collection from the growing area and receipt at the laboratory, internal air temperature (or between-shellfish sample temperature) should be less than the temperature recorded at the time of sampling. Samples from harvesting areas should be rinsed, but not immersed, and drained at 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 samples being taken in support of a growing area sanitation programme, it is recommended that verification studies supporting the use of sample transport criteria outside of the ranges given in ISO 6887-3 are approved by the responsible authority. (See Section 4.3.6 and Annex 13 of the FAO/WHO technical guidance (https://www.fao.org/3/cb5072en/cb5072en.pdf).

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

8.2 Sample storage

Upon receipt in the laboratory the temperature of the samples should be recorded. Samples should be examined immediately - if storage in the laboratory is necessary, then samples should be stored at 3±2 °C and should be processed within 24 hours of collection. If, due to logistical problems, microbiological analysis of samples taken in support of a growing area programme cannot be started within 24 hours of sample collection, a verification study should be undertaken to show that extended storage does not affect the microbiological content of the sample.

[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

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 bivalve molluscs 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.

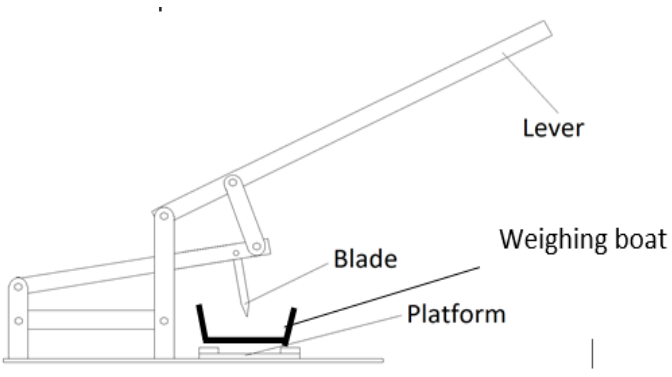


Figure 1: Oyster cracker apparatus



Figure 2: Opening an oyster using an oyster cracker

8.4.2 Preparation of bivalve molluscs 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

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

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.

8.5 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.

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

8.5.1 Homogenisation in a blender

Measure 1 ml of sterile PSS per 1 g of shellfish (±2 ml) using a measuring cylinder. Place contents of sterilised container into a 1 litre blender jar[3,4], with the PSS 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 (1 in 2 dilution) back into the sterilised container.

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 50 g of homogenised shellfish into another set of 3 stomacher bags, add 50 ml of PSS and homogenise for a further 2-3 minutes. After homogenisation decant the contents (1 in 2 dilution) into a new sterilised container.

8.5.3 Preparation of 1 in 20 dilution

Make a further decimal dilution (1 in 20) using the 1 in 2 dilution as prepared in 8.5.1 or 8.5.2

[3] If shellfish are particularly small, it may be necessary to use a smaller blender jar to achieve a consistent homogenate.
[4] If shellfish are particularly large, it may be necessary to split the sample across multiple blender jars and then pool the homogenates together in the sterilised container after blending.

by adding 1 ml of the 1 in 2 dilution to 9 ml of PSS.

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

Note: Inclusion of the 1 in 20 dilution is dependent on the purpose of testing. It is required where it is necessary to determine values of >15000 *E. coli* per 100 g, for example, under the EU classification system the upper limit for Class C samples is 46000 *E. coli* per 100 g (using the 1 in 20 dilution allows values up to 150000 *E. coli* per 100 g to be determined). In other circumstances it may only be necessary to test using the 1 in 2 dilution only.

8.6 Inoculation and incubation

Melt TBGA/TBX medium using a steamer, microwave or equivalent, then leave to cool in a waterbath at 44-47 °C.

Note: If bottles are extremely hot after melting, e.g. using a microwave, allow to cool down on the bench for a short time before adding to the waterbath to prevent cracking.

Note: Melted medium must not be used until it has cooled to the target temperature.

Inoculate 5 sterile Petri dishes (90 mm diameter) with 2 ml of the 1 in 2 dilution. Inoculate 2 sterile Petri dishes (90 mm diameter) with 2 ml of the 1 in 20 dilution (if used). Use a new sterile pipette for each dilution.

Pour approximately 15-18 ml of the melted and cooled TBGA/TBX medium into each Petri dish. Carefully mix the inoculum with the medium by gentle swirling and allow the mixture to solidify, with the Petri dishes standing on a cool horizontal surface.

Incubate the TGBA/TBX plates inverted for 4 hours at 37±1 °C, followed by 18–24 hours at 44±1 °C.

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

Note: The time which elapses between the distribution of the inoculum in a dish and pouring of the medium shall not exceed 15 minutes.

8.7 Counting the colony forming units

After incubation examine all inoculated TBGA/TBX plates for the presence of typical i.e. blue to blue-green colonies. Record the number of typical colonies on each of the 5 plates inoculated with the 1 in 2 dilution and each of the 2 plates inoculated with the 1 in 20 dilution (if used). Atypical colonies should not be included in the count.

Note: In this protocol the maximum countable number of typical colonies for a set of plates at a single dilution is set at a level that corresponds to an average of 150 typical colonies per plate i.e. 750 colonies in total for a set of 5 plates or 300 colonies in total for a set of 2 plates (8.8.1). It is not possible for a set of 5 plates to average ≤150 typical colonies and to meet the requirements on the reliability of colony counts of parallel plates (8.8.2) where a single plate within the set contains ≥192 typical colonies. Likewise, it is not possible for a set of 2 plates to average ≤150 typical colonies and to meet the requirements on the reliability of colony counts of parallel plates where one of the plates contains ≥173 typical colonies.

Therefore, where a count of ≥192 typical colonies is reached for any single plate in the set of 5 at the 1 in 2 dilution, or a count of ≥173 typical colonies is reached for either of the plates in the set of 2 at the 1 in 20 dilution (if used), the entire set at the affected dilution can be considered to be above the maximum countable number, and the remaining plates in the set do not need to be counted.

8.8 Test results

Test results are generated from the colony counts for all plates (8.7). The counts may be checked against the maximum countable number and for acceptable reliability, and results calculated and reported as described in sections 8.8.1 to 8.8.4. Alternatively, the Shellfish colony-count calculator can be used (8.8.5). This calculator simultaneously takes into account the maximum countable number, carries out checks for acceptable reliability, calculates the result and shows how the result should be expressed when reporting.

8.8.1 Maximum countable number of colonies

In this protocol the maximum countable number of typical colonies for a set of plates at a single dilution is set at a level that corresponds to an average of 150 typical colonies per plate i.e. 750 colonies in total for a set of 5 plates or 300 colonies in total for a set of 2 plates.

Where only the 1 in 2 dilution is tested, and the maximum countable number of colonies is exceeded, then results should be reported as **>15000 *E. coli* per 100 g**.

Where both 1 in 2 and 1 in 20 dilutions are tested, and the maximum countable number of colonies is exceeded for the set of plates using the 1 in 2 dilution, calculation of results should be carried out using the colony count at the 1 in 20 dilution.

Note: If the maximum countable number of colonies is exceeded for the set of plates using the 1 in 2 dilution, and the 2 plates at the 1 in 20 dilution contain fewer than 17 colonies in total, the ratio between the total colony counts at the two dilutions is not acceptable (8.8.3) and the test result should be considered as **Void**.

Note: If the maximum countable number of colonies is exceeded for the set of plates using the 1 in 2 dilution, and the results at the 1 in 20 dilution are rejected due to unacceptable variation between the counts on the two plates (8.8.2), then results should be reported as **>15000 *E. coli* per 100 g.**

Where the maximum countable number of colonies is exceeded for the set of plates using the 1 in 20 dilution, then results should be reported as **>150000 *E. coli* per 100 g**.

8.8.2 Determination of the reliability of colony counts on parallel plates

To ensure reliable results, the pattern of individual colony counts for all parallel plates within a set at a single dilution should be assessed for acceptable levels of variability using a *G*-test approach (e.g. Sokal and Rohlf, 2012) as recommended in ISO 7218 and detailed (for 2 parallel plates) in ISO 14461-2.

For **5 parallel plates** (1 in 2 dilution), determine the χ^2 statistic for the set using the following equation:-

$$\chi^2 = \left\{2\left[C_{\mathrm{a}} \cdot \ln\left(\frac{C_{\mathrm{a}}}{(\sum C)/5}\right) + C_{\mathrm{b}} \cdot \ln\left(\frac{C_{\mathrm{b}}}{(\sum C)/5}\right) + C_{\mathrm{c}} \cdot \ln\left(\frac{C_{\mathrm{c}}}{(\sum C)/5}\right) + C_{\mathrm{d}} \cdot \ln\left(\frac{C_{\mathrm{d}}}{(\sum C)/5}\right) + C_{\mathrm{e}} \cdot \ln\left(\frac{C_{\mathrm{e}}}{(\sum C)/5}\right)\right]\right\}$$

where

C_{a} is the count on the first plate, C_{b} is the count on the second plate,….. C_{e} is the count on the fifth plate.

$\sum C$ is the sum of the counts on all 5 plates.

The variability between the parallel counts is considered acceptable where χ^2 is less than or equal to 13.277 (the critical value of χ^2 for p=0.01 and 4 degrees of freedom).

For **2 parallel plates** (1 in 20 dilution if used), determine the χ2 statistic for the set using the following equation:-

$$\chi^2 = \left\{2\left[C_{\mathrm{a}} \cdot \ln\left(\frac{C_{\mathrm{a}}}{(\sum C)/2}\right) + C_{\mathrm{b}} \cdot \ln\left(\frac{C_{\mathrm{b}}}{(\sum C)/2}\right)\right]\right\}$$

where

C_{a} is the count on the first plate and C_{b} is the count on the second plate.

$\sum C$ is the sum of the counts on both plates.

The variability between the parallel counts is considered acceptable where χ^2 is less than or equal to 6.635 (the critical value of χ^2 for p=0.01 and 1 degree of freedom).

Alternatively, for 2 parallel plates the acceptability of the counts can be determined using the table in Appendix 2.

Where colony counts on a set of parallel plates at one dilution are not acceptable according to the above criteria, then calculation of results should be carried out using the colony counts at the other dilution. Where parallel plate counts at both dilutions are not acceptable, or where only one dilution is used and the parallel plate counts are not acceptable, then the test result should be considered as **Void**. This should occur in <1% of cases.

Note: If using the colony-count calculator (8.8.5), all steps described in this section are carried out automatically.

8.8.3 Determination of the reliability of colony counts at successive dilutions

Where plates are prepared using both 1 in 2 and 1 in 20 dilutions, to ensure reliable results, the ratio between the total counts (the sum of all plates) at the 1 in 2 and 1 in 20 dilutions should be assessed for acceptability using a *G*-test approach (e.g. Sokal and Rohlf, 2012) as recommended in ISO 7218 and detailed (for successive dilutions where the same numbers of plates are used) in ISO 14461-2.

Determine the χ^2 statistic for the ratio of the total counts at the two dilutions using the following equation:-

$$\chi^2 = \left\{2\left[\sum upper \cdot \ln\left(\frac{\sum upper}{10.0 \cdot (\sum upper + \sum lower)/10.4}\right) + \sum lower \cdot \ln\left(\frac{\sum lower}{0.4 \cdot (\sum upper + \sum lower)/10.4}\right)\right]\right\}$$

where

$\sum upper$ is the total count of the 5 plates at the 1 in 2 dilution.

$\sum lower$ is the total count of the 2 plates at the 1 in 20 dilution.

The ratio between the total counts at the two dilutions is considered acceptable where χ^2 is less than or equal to 6.635 (the critical value of χ^2 for p=0.01 and 1 degree of freedom).

Alternatively, the acceptability of the ratio of the total counts at the two dilutions can be determined using the table in Appendix 3.

Where the ratio between the total colony counts at the two dilutions is not acceptable according to the above criteria, then the test result should be considered as **Void**. This should occur in 1% of cases.

Note: If using the colony-count calculator (8.8.5), all steps described in this section are carried out automatically.

8.8.4 Calculation and reporting of results

Results are calculated using colony counts for all plates retained after the checks described in 8.8.1, 8.8.2 and 8.8.3. Where plates are prepared using both 1 in 2 and 1 in 20 dilutions and there are no issues with exceedance of the maximum countable number (8.8.1) or reliability of the counts at either dilution according to 8.8.2 and 8.8.3, then counts from all plates must be used in the calculation even if e.g. no colonies are present at the 1 in 20 dilution.

Where the sum of colony counts, $\sum C$,of all retained plates is ≥10, calculate the result N (in *E. coli* per 100 g) as a weighted mean using the following equations depending on which dilutions are retained:-

If plates at 1 in 2 and 1 in 20 dilutions are retained:

$$N = 100 \cdot \left(\frac{\sum C}{5.2}\right)$$

If plates at 1 in 2 dilution only are retained:

$$N = 100 \cdot \left(\frac{\sum C}{5}\right)$$

If plates at 1 in 20 dilution only are retained:

$$N = 100 \cdot \left(\frac{\sum C}{0.2}\right)$$

Round off the results to two significant figures and report as ***N E. coli* per 100 g**.

Example:

Where individual plate counts at the 1 in 2 and 1 in 20 dilutions are (10,15,8,11,14) and (0,1) respectively:-

$$N = 100 \cdot \left(\frac{(10 + 15 + 8 + 11 + 14 + 0 + 1)}{5.2}\right)$$

$$N = 100 \cdot \left(\frac{59}{5.2}\right) = 1134.62$$

Results are rounded off to two significant figures and reported as **1100 *E. coli* per 100 g**.

Where the sum of colony counts, $\sum C$,of all retained plates is between 1 and 9, calculate the result *N* (in *E. coli* per 100 g) as a weighted mean using the above equations.

Round off the results to two significant figures and report as ***N E. coli* per 100 g estimated**.

Example:

Where individual plate counts at the 1 in 2 and 1 in 20 dilutions are (2,1,2,2,1) and (0,0) respectively:-

$$N = 100 \cdot \left(\frac{(2+1+2+2+1+0+0)}{5.2}\right)$$

$$N = 100 \cdot \left(\frac{8}{5.2}\right) = 153.85$$

Results are rounded off to two significant figures and reported as **150 *E. coli* per 100 g estimated**.

Where no colonies are present on the retained plates, the result should be reported as ***<N E. coli* per 100 g,** where N is calculated using the above equations, substituting 1 for $\sum C$ as follows:-

If plates at both dilutions are retained report the results as **<19 *E. coli* per 100 g**

If plates at 1 in 2 dilution only are retained report the results as **<20 E. coli per 100 g**

If plates at 1 in 20 dilution only are retained report the results as **<500 *E. coli* per 100 g**

Note: If using the colony-count calculator (8.8.5), all steps described in this section are carried out automatically.

8.8.5 Use of the Shellfish colony-count calculator

Two copies of the calculator are available from the FAORC website (Method Guidance and Calculation Spreadsheets); one is designed for tests carried out using both 1 in 2 and 1 in 20 dilutions (5 and 2 plates respectively, “two dilutions”), and one designed for tests carried out using only the 1 in 2 dilution (5 plates, “one dilution”).

Note: Where both dilutions are used in the test, the calculator for two dilutions must be used, even if e.g. no colonies are present at the 1 in 20 dilution.

Shellfish colony-count calculator – two dilutions:-

- Download and open a copy of the two dilution colony-count calculator from the website.

  Note: Only cream-coloured cells can be overwritten, all other cells are locked.

- Each row of the calculator will generate results for a single sample (there are enough rows for 200 samples – example details have been added to row 5 but can be overwritten).
- Add the sample name or unique identifier to column B.
- Add the colony counts for the 5 plates at the 1 in 2 dilution to columns D to H.
- Add the colony counts for the 2 plates at the 1 in 20 dilution to columns J and K.

  Note: Where the maximum countable number for either set was exceeded and the full count not completed (8.7), the set can be “blocked out” on the calculator by adding any number ≥151 to all relevant columns.

- Where the variability between counts on parallel plates within a set is unacceptable, “!!!!!” will appear in column Y (1 in 2 dilution) and/or column AG (1 in 20 dilution).
- Where the ratio between total counts at the two dilutions is unacceptable, “!!!!!” will appear in column AP.
- Raw and rounded results calculated using the 1 in 2 dilution only, the 1 in 20 dilution only and both dilutions are shown in columns AW to BJ.
- Results (to be reported in ***E. coli* per 100 g**), taking into account exceedance of maximum countable numbers at one or both dilutions, all checks for acceptability, expression of results at low colony counts (<10) and rounding of results to two significant figures, are shown in column CD.
- Lower and upper 95% confidence limits (section 9) are provided in columns CF and CG for information.

Shellfish colony-count calculator – one dilution:-

- Download and open a copy of the one dilution colony-count calculator from the website.

  Note: Only cream-coloured cells can be overwritten, all other cells are locked.

- Each row of the calculator will generate results for a single sample (there are enough rows for 200 samples – example details have been added to row 3 but can be overwritten).
- Add the sample name or unique identifier to column B.
- Add the colony counts for the 5 plates at the 1 in 2 dilution to columns D to H.

  Note: Where the maximum countable number was exceeded and the full count not completed (8.7), the set can be "blocked out" on the calculator by adding any number ≥151 to all relevant columns.
- Where the variability between counts on parallel plates within the set is unacceptable, "!!!!!" will appear in column V.
- Raw and rounded results are shown in columns AB and AC.
- Results (to be reported in ***E. coli* per 100 g**), taking into account exceedance of maximum countable numbers, all checks for acceptability, expression of results at low colony counts (<10) and rounding of results to two significant figures are shown in column AO.
- Lower and upper 95% confidence limits (section 9) are provided in columns AQ and AR for information.

On both versions of the calculator, final result cells are conditionally colour-coded according to the EU system classification bracket in which the results sit (based on EU Regulation 2019/627; European Union, 2019):-

- ≤230 – shaded green
- 240 - 700 – shaded yellow
- 710 – 4600 – shaded peach
- 4700 – 46000 – shaded pink
- >46000 – shaded red

Results that are ambiguous in terms of classification brackets (>15000, <500) are shaded grey and void results are shaded black with yellow text.

Note: Where final results are shown in the form "***N* estimated**", these should be reported as "***N E. coli* per 100 g estimated**".

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 for the result is not required when reporting 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.

ISO 7218 includes a formula for determination of theoretical confidence intervals for colony-count methods; the confidence interval becomes narrower in relative terms as the colony count increases. According to this formula, the confidence interval for a result of 230 E. coli per 100 g obtained using results at both 1 in 2 and 1 in 20 dilutions and derived from a colony count of 12 is 100 – 360. The confidence interval for a result of 4600 E. coli per 100g derived from a colony count of 239 is 4000 – 5200. These intervals are included as an output on the calculation spreadsheet (8.8.5).

ISO/TS 19036 gives guidance on the estimation of MU 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

It is recommended that laboratories undertaking microbiological examination of samples as part of a bivalve mollusc sanitation programme should take part in comparative laboratory testing relevant to the sample type(s) and method(s). Participation may be required under the sanitation programme (by national legislation or official procedures) or specified by importing countries. Comparative testing provides an indication of performance relative to other participating laboratories and can help improve the performance of the laboratory. Examples of comparative testing for bivalve molluscs are the FAORC PT scheme (see https://www.cefas.co.uk/icoe/seafood-safety/services/proficiency-testing-and-quality-assurance/) and the UK HSA/Cefas Shellfish EQA scheme (for further information contact foodeqa@ukhsa.gov.uk).

10.2 Internal quality controls

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

Positive and negative controls for TBGA/TBX medium should be included with each set of examinations (that is, those processed at approximately the same time). The control strains listed in Table 1 (Section 7) may be used for this purpose. An uninoculated plate of TBGA/TBX should also be incubated with each set as a sterility control. Where more than one batch of medium has been used for the sample examinations, positive, negative and sterility controls should be included for each batch.

10.3 Internal quality assurance (IQA)

It is recommended that regular (e.g. monthly) monitoring using known levels of target organism are examined to ensure that routine *E. coli* procedures continue to be effective. Certified reference materials containing a target number of microorganisms (for example Lenticule™ discs) are available from commercial sources for use in internal quality assurance procedures. The target

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

11 References

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

Anon, 2021. Community Guide to the Principles of Good Practice for the Microbiological Classification and Monitoring of Bivalve Mollusc Production and Relaying Areas with regard to Implementing Regulation 2019/627, Issue 4. Available online at (aesan.gob.es).

Codex Alimentarius, 2015. Standard for Live and Raw Bivalve Molluscs. CXS 292-2008, 2015 revision.

European Union, 2005. Regulation (EC) No 2073/2005 of the European Parliament and the Council, 15 November 2005 on microbiological criteria for foodstuffs.

European Union, 2017. Regulation (EU) 2017/625 of the European Parliament and of the Council on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products.

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

ISO 6887-3. Microbiology of the food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 3: Specific rules for the preparation of fish and fishery products (as amended). International Organization for Standardization, Geneva.

ISO 7218. Microbiology of the food chain – General requirements and guidance for microbiological examinations. International Organization for Standardization, Geneva.

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

ISO 14461-2. Milk and milk products - Quality control in microbiological laboratories - Part 2: Determination of the reliability of colony counts of parallel plates and subsequent dilution steps.

ISO 16140:2003. Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods. International Organization for Standardization, Geneva.

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ISO 16649-2. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* – Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. International Organization for Standardization, Geneva.

ISO 16649-3:2015. Microbiology of the food chain - Horizontal method for the enumeration of β-

glucuronidase-positive *Escherichia coli* - Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. International Organization for Standardization, Geneva.

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

Jacobs-Reitsma W. F and Pol-Hofstad I. E., 2010. Expert lab report on the MicroVal ISO 16140:2003 validation of the TBX pour plate method (ISO 16649-2) for enumeration of *Escherichia coli* in bivalve molluscs, RIKILT report 2007LR07.

Pol-Hofstad I. E. and Jacobs-Reitsma W. F., 2021. Validation of the TBX pour plate method (ISO 16649-2) for the enumeration of *Escherichia coli* in Live Bivalve Molluscs: renewal study for alignment with EN ISO 16140-2:2016. RIVM report 2021-0127.

Sokal, R. R. and Rohlf, F. J., 2012. Biometry: The Principles and Practice of Statistics in Biological Research (Fourth ed.). New York: Freeman. ISBN-13: 978-0-7167-8604-4.

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

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

ISO 6887-3 specifies that for any bivalve species, a test sample shall contain a minimum of 10 animals, and the combined weight of flesh and intravalvular liquid shall be at least 50 g (for very small species such as Donax spp. a minimum amount of 25 g is permitted). To ensure sufficient material for the test it is recommended to use the following sample numbers 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 [5]	Scientific name [6]	Sample size [7]
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

[5] Species of relevance provided by European NRLs.
[6] 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.
[7] Sample sizes given in this table have been provided by European NRLs. For species not given in the table, sufficient shellfish should be opened to obtain 50 g of flesh and liquor, with the provision that a minimum of ten animals should be used in all cases. 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.

	Soft shell clam (Sand gaper)	*Mya arenaria*	10 - 12
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

Appendix 2: Limits of acceptable variability for colony counts of two parallel Petri dishes

For a set of two parallel Petri dishes at a single dilution, variability between the plates is acceptable provided that for a given upper plate count as shown in the “Upper colony count” column in the table below, the lower plate count in the set of 2 plates is no lower than the number shown in the “Lower colony count” column e.g. where the upper count in the set of 2 plates is 100, the other plate must contain at least 67 colonies for variability to be acceptable.

Colony count	
Upper	Lower
1	0
2	0
3	0
4	0
5	1
6	1
7	1
8	1
9	2
10	2
11	3
12	3
13	4
14	4
15	5
16	5
17	6
18	6
19	7
20	7
21	8
22	9
23	9
24	10
25	11
26	11
27	12
28	12
29	13
30	14
31	14
32	15
33	16
34	16
35	17
36	18
37	19
38	19
39	20
40	21
41	21
42	22
43	23

Colony count	
Upper	Lower
44	24
45	24
46	25
47	26
48	27
49	27
50	28
51	29
52	29
53	30
54	31
55	32
56	32
57	33
58	34
59	35
60	36
61	36
62	37
63	38
64	39
65	39
66	40
67	41
68	42
69	42
70	43
71	44
72	45
73	46
74	46
75	47
76	48
77	49
78	50
79	50
80	51
81	52
82	53
83	54
84	54
85	55
86	56

Colony count	
Upper	Lower
87	57
88	58
89	58
90	59
91	60
92	61
93	62
94	62
95	63
96	64
97	65
98	66
99	67
100	67
101	68
102	69
103	70
104	71
105	71
106	72
107	73
108	74
109	75
110	76
111	76
112	77
113	78
114	79
115	80
116	81
117	81
118	82
119	83
120	84
121	85
122	86
123	86
124	87
125	88
126	89
127	90
128	91
129	91

Colony count	
Upper	Lower
130	92
131	93
132	94
133	95
134	96
135	96
136	97
137	98
138	99
139	100
140	101
141	102
142	102
143	103
144	104
145	105
146	106
147	107
148	107
149	108
150	109
151	110
152	111
153	112
154	113
155	113
156	114
157	115
158	116
159	117
160	118
161	119
162	120
163	120
164	121
165	122
166	123
167	124
168	125
169	125
170	126
171	127
172	128

Appendix 3: Limits of acceptable ratios for colony counts at successive dilutions

For a set of five parallel Petri dishes at the 1 in 2 dilution and two parallel dishes at the 1 in 20 dilution, the ratio between the total counts at the two dilutions is acceptable provided that for a given total count at the 1 in 2 dilution as shown in the below table, the total count at the 1 in 20 dilution falls within the lower and upper limits in the table e.g. where the total count at the 1 in 2 dilution is 100, the total count at the 1 in 20 dilution must be at least 1 and no more than 10 for the ratio to be acceptable. Where the total count at the 1 in 2 dilution is above the maximum countable number i.e. >750, then there is no upper limit for acceptable counts at the 1 in 20 dilution.

1 in 2 Total count	1 in 20 Lower limit	1 in 20 Upper limit
0	0	1
1	0	1
2	0	1
3	0	2
4	0	2
5	0	2
6	0	2
7	0	2
8	0	2
9	0	2
10	0	3
11	0	3
12	0	3
13	0	3
14	0	3
15	0	3
16	0	3
17	0	3
18	0	4
19	0	4
20	0	4
21	0	4
22	0	4
23	0	4
24	0	4
25	0	4
26	0	4
27	0	4
28	0	4
29	0	5
30	0	5
31	0	5
32	0	5
33	0	5
34	0	5
35	0	5
36	0	5
37	0	5
38	0	5
39	0	5
40	0	6
41	0	6
42	0	6
43	0	6
44	0	6
45	0	6
46	0	6
47	0	6
48	0	6
49	0	6
50	0	6
51	0	6
52	0	6
53	0	7
54	0	7
55	0	7
56	0	7
57	0	7
58	0	7
59	0	7
60	0	7
61	0	7
62	0	7
63	0	7
64	0	7
65	0	7
66	0	8
67	0	8
68	0	8
69	0	8
70	0	8
71	0	8
72	0	8
73	0	8
74	0	8
75	0	8
76	0	8
77	0	8
78	0	8
79	0	8
80	0	9
81	0	9
82	0	9
83	0	9
84	0	9
85	1	9
86	1	9
87	1	9
88	1	9
89	1	9
90	1	9
91	1	9
92	1	9
93	1	9
94	1	9
95	1	10
96	1	10
97	1	10
98	1	10
99	1	10
100	1	10
101	1	10
102	1	10
103	1	10
104	1	10
105	1	10
106	1	10
107	1	10
108	1	10
109	1	10
110	1	11
111	1	11
112	1	11
113	1	11
114	1	11
115	1	11
116	1	11
117	1	11
118	1	11
119	1	11
120	1	11
121	1	11
122	1	11
123	1	11
124	1	11
125	1	11
126	1	12
127	1	12
128	1	12
129	1	12
130	1	12
131	1	12
132	1	12
133	1	12
134	1	12

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
135	1	12
136	1	12
137	1	12
138	1	12
139	1	12
140	1	12
141	1	13
142	1	13
143	1	13
144	1	13
145	1	13
146	1	13
147	1	13
148	1	13
149	1	13
150	1	13
151	1	13
152	1	13
153	1	13
154	1	13
155	1	13
156	2	13
157	2	13
158	2	14
159	2	14
160	2	14
161	2	14
162	2	14
163	2	14
164	2	14
165	2	14
166	2	14
167	2	14
168	2	14
169	2	14
170	2	14
171	2	14
172	2	14
173	2	14
174	2	15
175	2	15
176	2	15
177	2	15
178	2	15
179	2	15

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
180	2	15
181	2	15
182	2	15
183	2	15
184	2	15
185	2	15
186	2	15
187	2	15
188	2	15
189	2	15
190	2	15
191	2	16
192	2	16
193	2	16
194	2	16
195	2	16
196	2	16
197	2	16
198	2	16
199	2	16
200	2	16
201	2	16
202	2	16
203	2	16
204	2	16
205	2	16
206	2	16
207	3	16
208	3	17
209	3	17
210	3	17
211	3	17
212	3	17
213	3	17
214	3	17
215	3	17
216	3	17
217	3	17
218	3	17
219	3	17
220	3	17
221	3	17
222	3	17
223	3	17
224	3	17

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
225	3	18
226	3	18
227	3	18
228	3	18
229	3	18
230	3	18
231	3	18
232	3	18
233	3	18
234	3	18
235	3	18
236	3	18
237	3	18
238	3	18
239	3	18
240	3	18
241	3	18
242	3	18
243	3	19
244	3	19
245	3	19
246	3	19
247	3	19
248	3	19
249	3	19
250	3	19
251	4	19
252	4	19
253	4	19
254	4	19
255	4	19
256	4	19
257	4	19
258	4	19
259	4	19
260	4	20
261	4	20
262	4	20
263	4	20
264	4	20
265	4	20
266	4	20
267	4	20
268	4	20
269	4	20

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
270	4	20
271	4	20
272	4	20
273	4	20
274	4	20
275	4	20
276	4	20
277	4	20
278	4	21
279	4	21
280	4	21
281	4	21
282	4	21
283	4	21
284	4	21
285	4	21
286	4	21
287	4	21
288	4	21
289	4	21
290	4	21
291	4	21
292	4	21
293	5	21
294	5	21
295	5	21
296	5	22
297	5	22
298	5	22
299	5	22
300	5	22
301	5	22
302	5	22
303	5	22
304	5	22
305	5	22
306	5	22
307	5	22
308	5	22
309	5	22
310	5	22
311	5	22
312	5	22
313	5	22
314	5	23

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
315	5	23
316	5	23
317	5	23
318	5	23
319	5	23
320	5	23
321	5	23
322	5	23
323	5	23
324	5	23
325	5	23
326	5	23
327	5	23
328	5	23
329	5	23
330	5	23
331	5	23
332	5	23
333	6	24
334	6	24
335	6	24
336	6	24
337	6	24
338	6	24
339	6	24
340	6	24
341	6	24
342	6	24
343	6	24
344	6	24
345	6	24
346	6	24
347	6	24
348	6	24
349	6	24
350	6	24
351	6	25
352	6	25
353	6	25
354	6	25
355	6	25
356	6	25
357	6	25
358	6	25
359	6	25

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
360	6	25
361	6	25
362	6	25
363	6	25
364	6	25
365	6	25
366	6	25
367	6	25
368	6	25
369	6	25
370	6	26
371	6	26
372	7	26
373	7	26
374	7	26
375	7	26
376	7	26
377	7	26
378	7	26
379	7	26
380	7	26
381	7	26
382	7	26
383	7	26
384	7	26
385	7	26
386	7	26
387	7	26
388	7	27
389	7	27
390	7	27
391	7	27
392	7	27
393	7	27
394	7	27
395	7	27
396	7	27
397	7	27
398	7	27
399	7	27
400	7	27
401	7	27
402	7	27
403	7	27
404	7	27

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
405	7	27
406	7	27
407	7	28
408	7	28
409	7	28
410	8	28
411	8	28
412	8	28
413	8	28
414	8	28
415	8	28
416	8	28
417	8	28
418	8	28
419	8	28
420	8	28
421	8	28
422	8	28
423	8	28
424	8	28
425	8	28
426	8	29
427	8	29
428	8	29
429	8	29
430	8	29
431	8	29
432	8	29
433	8	29
434	8	29
435	8	29
436	8	29
437	8	29
438	8	29
439	8	29
440	8	29
441	8	29
442	8	29
443	8	29
444	8	29
445	8	30
446	9	30
447	9	30
448	9	30
449	9	30

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
450	9	30
451	9	30
452	9	30
453	9	30
454	9	30
455	9	30
456	9	30
457	9	30
458	9	30
459	9	30
460	9	30
461	9	30
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463	9	30
464	9	31
465	9	31
466	9	31
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468	9	31
469	9	31
470	9	31
471	9	31
472	9	31
473	9	31
474	9	31
475	9	31
476	9	31
477	9	31
478	9	31
479	9	31
480	9	31
481	9	31
482	9	31
483	10	32
484	10	32
485	10	32
486	10	32
487	10	32
488	10	32
489	10	32
490	10	32
491	10	32
492	10	32
493	10	32
494	10	32

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
495	10	32
496	10	32
497	10	32
498	10	32
499	10	32
500	10	32
501	10	32
502	10	33
503	10	33
504	10	33
505	10	33
506	10	33
507	10	33
508	10	33
509	10	33
510	10	33
511	10	33
512	10	33
513	10	33
514	10	33
515	10	33
516	10	33
517	10	33
518	11	33
519	11	33
520	11	33
521	11	33
522	11	34
523	11	34
524	11	34
525	11	34
526	11	34
527	11	34
528	11	34
529	11	34
530	11	34
531	11	34
532	11	34
533	11	34
534	11	34
535	11	34
536	11	34
537	11	34
538	11	34
539	11	34

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
540	11	34
541	11	35
542	11	35
543	11	35
544	11	35
545	11	35
546	11	35
547	11	35
548	11	35
549	11	35
550	11	35
551	11	35
552	11	35
553	12	35
554	12	35
555	12	35
556	12	35
557	12	35
558	12	35
559	12	35
560	12	35
561	12	36
562	12	36
563	12	36
564	12	36
565	12	36
566	12	36
567	12	36
568	12	36
569	12	36
570	12	36
571	12	36
572	12	36
573	12	36
574	12	36
575	12	36
576	12	36
577	12	36
578	12	36
579	12	36
580	12	37
581	12	37
582	12	37
583	12	37
584	12	37

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
585	12	37
586	12	37
587	12	37
588	13	37
589	13	37
590	13	37
591	13	37
592	13	37
593	13	37
594	13	37
595	13	37
596	13	37
597	13	37
598	13	37
599	13	37
600	13	38
601	13	38
602	13	38
603	13	38
604	13	38
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612	13	38
613	13	38
614	13	38
615	13	38
616	13	38
617	13	38
618	13	38
619	13	38
620	13	39
621	13	39
622	14	39
623	14	39
624	14	39
625	14	39
626	14	39
627	14	39
628	14	39
629	14	39

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
630	14	39
631	14	39
632	14	39
633	14	39
634	14	39
635	14	39
636	14	39
637	14	39
638	14	39
639	14	39
640	14	40
641	14	40
642	14	40
643	14	40
644	14	40
645	14	40
646	14	40
647	14	40
648	14	40
649	14	40
650	14	40
651	14	40
652	14	40
653	14	40
654	14	40
655	14	40
656	15	40
657	15	40
658	15	40
659	15	41
660	15	41
661	15	41
662	15	41
663	15	41
664	15	41
665	15	41
666	15	41
667	15	41
668	15	41
669	15	41
670	15	41
671	15	41
672	15	41
673	15	41
674	15	41

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
675	15	41
676	15	41
677	15	41
678	15	41
679	15	42
680	15	42
681	15	42
682	15	42
683	15	42
684	15	42
685	15	42
686	15	42
687	15	42
688	15	42
689	16	42
690	16	42
691	16	42
692	16	42
693	16	42
694	16	42
695	16	42
696	16	42
697	16	42
698	16	42
699	16	43
700	16	43
701	16	43
702	16	43
703	16	43
704	16	43
705	16	43
706	16	43
707	16	43
708	16	43
709	16	43
710	16	43
711	16	43
712	16	43
713	16	43
714	16	43
715	16	43
716	16	43
717	16	43
718	16	43
719	16	44

1 in 2	1 in 20	
Total count	Lower limit	Upper limit
720	16	44
721	16	44
722	17	44
723	17	44
724	17	44
725	17	44
726	17	44
727	17	44
728	17	44
729	17	44
730	17	44
731	17	44
732	17	44
733	17	44
734	17	44
735	17	44
736	17	44
737	17	44
738	17	44
739	17	45
740	17	45
741	17	45
742	17	45
743	17	45
744	17	45
745	17	45
746	17	45
747	17	45
748	17	45
749	17	45
750	17	45
>750	17	n/a

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Centre for Environment, Fisheries & Aquaculture Science

Cefas

Pakefield Road, Lowestoft, Suffolk, NR33 0HT, UK

The Nothe, Barrack Road, Weymouth, DT4 8UB, UK

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

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