FAO Reference Centre for Bivalve Mollusc Sanitation Proficiency Testing Scheme

Enumeration of *Escherichia coli* and the detection of *Salmonella* spp. in bivalve molluscan shellfish (PT 100)

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Centre for Environment Fisheries & Aquaculture Science

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Version	Author	Date	Comment
Draft V1	L. Stockley	06.01.25	First internal review to JL
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FINAL V1	L. Stockley	23.01.25	Quality control completed
FINAL V2	L. Stockley	07.03.25	Participant review - minor change to ID number and arrival time

Contents

This scheme is intended to provide proficiency testing (PT) samples for laboratories undertaking examination of live bivalve molluscs for bacteriological determinands.

The scheme is organised by Cefas, the FAO Reference Centre (FAO RC) for Bivalve Mollusc Sanitation. The scheme is intended to complement the Cefas/UK HSA Shellfish External Quality Assessment (EQA) Scheme through assessing elements of the procedure (initial sample preparation and preparation of initial dilutions) not covered by the Shellfish Scheme Proficiency testing for food, water and environmental microbiology - GOV.UK (www.gov.uk) and to provide additional data to laboratories for ISO 17025 (Anon, 2017a) accreditation purposes.

A scoring system is used to help assess participants' performance. Details of this system are included as Appendix 2 of this report. The purpose of scoring is to help identify incorrect or outlying results. Further information on the use of scoring in PT and on recommended procedures for following up on poor performance can be accessed via the Cefas website (FAO Reference Centre for Bivalve Mollusc Sanitation - Cefas (Centre for Environment, Fisheries and Aquaculture Science)).

If you are experiencing problems with any aspects of these distributions, please contact Cefas (contact details below), or alternately refer to the troubleshooting guide included as Appendix 3 of this report.

Further advice on microbiological testing of bivalve shellfish can be obtained via the Cefas website (FAO Reference Centre for Bivalve Mollusc Sanitation - Cefas (Centre for Environment, Fisheries and Aquaculture Science)).

Due to the nature of this scheme repeat samples are not available.

1. Sample preparation

1.1. Sample 1 - Pacific oysters

A single batch of 640 Pacific oysters (*Magallana gigas*) was collected from a UK commercial harvesting area on the 14th November 2024 and were evenly spread across 6 trays and immersed in a small-scale depuration unit that had been partially filled with 500 litres of filtered (50 micron filter) seawater and maintained at a temperature of 16 °C. Seawater was re-circulated at 25 litres per min (with UV) for 3 days to allow the shellfish to acclimatise and remove any bacterial content. The oyster trays were then removed from the tank on the 17th November 2024 and 100 ml of inoculum containing known levels of *E. coli* (≈2.1 x 10^6 cfu/100 ml), *Klebsiella pneumoniae* (≈5.5 x 10^5 cfu/100 ml) and *Salmonella* Typhimurium (≈1.0 x 10^6 cfu/100 ml) were added to the tank and thoroughly mixed. The oysters were then re-immersed in the tank and were left for approximately 4 hours with constant re-circulation (without UV). After 4 hours of exposure the oysters were removed from the tank and samples of 22 oysters were randomly selected and placed into individual bags. The sample bags were then placed in the fridge at 3±2 °C prior to dispatch on the 18th November 2024.

1.2. Sample 2 – Pacific oysters

A single batch of 640 Pacific oysters (*M. gigas*) was collected from a UK commercial harvesting area on the 18th November 2024. Prior to packing the shellfish were placed in a large sterile container and thoroughly mixed. Samples of 22 oysters were randomly selected and placed into individual bags.

2. Sample distribution and examination

Each sample was packed in accordance with the Cefas protocol for packaging shellfish for transportation. Samples were dispatched at 10:00 on the 18th November 2024 to 24 participating laboratories using DG Global Forwarding. Participants were requested to analyse the samples immediately on receipt using their routine methods. The deliveries for laboratories 96, 203 and 207 experienced issues at customs causing the material to be returned back to the sender (Cefas). Laboratory 527 received their material a week after the dispatch date and recorded an internal sample temperature of 12°C. The results reported for this laboratory have not been included in the assessment of participants' results.

2.1. Sample temperature

Participants were requested to record the internal sample temperature on arrival. Temperatures recorded by participants are shown in Appendix 1.

3. Results

3.1. Reference results – *E. coli*

Six randomly selected samples were analysed for *E. coli* in duplicate on the 19th November 2024 under repeatability conditions using Cefas SOP No. 1175 (Table 1) based on ISO 16649-3 (Anon 2015). Sample homogeneity was assessed following the procedure described in ISO 22117 (Anon, 2019). Where no *E. coli* was detected (<18 MPN/100g) results were scored at 17 MPN/100g for statistical evaluation. The sample material as distributed was considered sufficiently homogenous.

Table 1 - *E. coli* MPN/100g reference results

Sample No.	Range	Median	Geomean	Median ±3*SD
Sample 1	$1.3 \times 10^3 – 1.3 \times 10^4$	6.0×10^3	5.0×10^3	$1.1 \times 10^3 – 3.1 \times 10^4$
Sample 2	$<1.8 \times 10^1 – 4.5 \times 10^1$	$<1.8 \times 10^1$	1.9×10^1	n/a – 8.9×10^1

3.2. Reference results – *Salmonella* spp.

Six randomly selected samples were analysed for *Salmonella* spp. on the 19th November 2024 under repeatability conditions using Cefas SOP No. 1176 (Table 2) based on ISO 6579-1 (Anon 2017b).

Table 2 – *Salmonella* spp. reference results

Sample No.	*Salmonella* spp.	No. of replicates giving the expected results
Sample 1	Present in 25g	6
Sample 2	Absent in 25g	6

3.3. Participants' results

Performance assessment was carried out according to the procedures described in the Cefas/UK HSA shellfish EQA scheme for a single distribution, with minor modifications (Appendix 2). Reference values were excluded from the calculation of the participants' median. Participants' results and scores allocated for PT 100 are shown in Tables 3, 4, 5, 6, Figure 1 and Appendix 3.

3.3.1. *E. coli* results

Table 3 – *E. coli* MPN/100g Participants' results

Sample No.	Range	Median	Geomean	Median±3*SD	Median±5*SD
Sample 1	$4.5 \times 10^2 – 1.3 \times 10^4$	3.1×10^3	2.7×10^3	$5.9 \times 10^2 – 1.6 \times 10^4$	$1.9 \times 10^2 – 4.9 \times 10^4$
Sample 2	$<1.8 \times 10^1 – 2.0 \times 10^1$	$<1.8 \times 10^1$	1.7×10^1	n/a – 8.9×10^1	n/a – 2.7×10^2

Note: The median and upper and lower limits (±3 SD and ±5 SD) were calculated from participants' results. SD calculations were based on the inherent variability of the 5 x 3 MPN method (0.24 $\log_{10}$). Reference values

were excluded from the calculation of participants' median.

Table 4 – Participants' results and allocated scores (MPN/100g)

Lab ID.	Sample 1 – Pacific oysters			Sample 2 – Pacific oysters		
	Rep 1	Rep 2	Score	Rep 1	Rep 2	Score
3	7900	13000	12	20	<18	12
10	2300	3300	12	<18	<18	12
12 [a]	VOID	3300	7	<18	<18	12
31	2400	3500	12	<18	<18	12
41	1300	1700	12	<18	<18	12
70	2300	2300	12	20	<18	12
72	4600	3100	12	<18	<18	12
120	1300	3300	12	<18	<18	12
125	3300	2300	12	20	20	12
129	3100	3300	12	<18	<18	12
131	1300	2200	12	<18	<18	12
142	3300	4600	12	<18	<18	12
189	1300	2300	10	<18	<18	12
195	4900	4900	12	<18	20	12
235	1300	450	5	<20	<20	8
286	1300	780	12	<18	<18	12
290	1700	2300	12	<18	<18	12
311	7900	4900	12	<18	<18	12
315	4900	7900	12	<18	<18	12
366	3300	1300	12	<18	<18	12
527 [b]	130	80	-	490	330	-

Key: a = A void result was reported for one replicate for Sample 1, total score for this sample is out of 7; b = Due to the extended duration of the sample transport these results have been omitted from the assessment but are included in the graph.

Table 5 – Summary statistics of participants' results

E. coli	Sample 1	Sample 2
Participants reporting duplicate results for *E. coli* MPN	20	21
Participants reporting a single result for *E. coli* MPN	1	0
Participants reporting both replicate MPN results within expected range [1]	18	20
Participants reporting a single MPN result within expected range [1]	1	0
Participants reporting one replicate MPN result outside expected range [1]	1	0
Participants reporting both replicate MPN results outside expected range [1]	1	1
Participants reporting tube combination and / or MPN results inconsistent with ISO 7218 [2]	2	0
Participant reported an error when transcribing results on the report form	1	0

[1] expected range = participants' median ± theoretical 3SD,

[2] points deducted from participants returning results inconsistent with ISO 7218

3.3.2. *Salmonella* spp. reference results

Table 6 - Participants' results and allocated scores (*Salmonella* spp. in 25g)

Lab ID.	Sample 1 Rep 1	Score	Sample 2 Rep 1	Score
3	Not Detected	0	Not Detected	2
10	Detected	2	Not Detected	2
12	Detected	2	Not Detected	2
31	Detected	2	Not Detected	2
41	Detected	2	Not Detected	2
70	Detected	2	Not Detected	2
72	Detected	2	Not Detected	2
120	Detected	2	Not Detected	2
125	Detected	2	Not Detected	2
129	NE	-	NE	-
131	Detected	2	Not Detected	2
142	Detected	2	Not Detected	2
189	Detected	2	Not Detected	2
195	Detected	2	Not Detected	2
235	Detected	2	Not Detected	2
286	Detected	2	Not Detected	2
290	Detected	2	Not Detected	2
311	Detected	2	Not Detected	2
315	Detected	2	Not Detected	2
366	Detected	2	Not Detected	2
527 [a]	Detected	-	Not Detected	-

Key: NE – Not examined; a = Due to the extended duration of the sample transport these results have been omitted from the assessment.

4. Comments

4.1. General comments

- Twenty-one out of 24 participating laboratories received their samples with 18 laboratories receiving the material within 48 hours of dispatch as recommended by the FAO RC. Fifteen laboratories analysed the samples on the day of arrival.
- Nineteen laboratories recorded the sample arrival temperature between 0°C and 10°C as stated in the FAO RC generic protocol. For laboratory 235 the arrival temperature was recorded as -1°C. The laboratory did not record any shellfish mortalities following this reduction in temperature and continued to test the samples.
- Laboratory 527 experienced significant delays in receiving PT 100 material (sample arrived after 7 days in transit). Due to the extended length of transit time and elevated arrival temperature (>10°C), the results reported from this laboratory were omitted from the performance assessment but have been included in the graph.
- Laboratories 96, 203 and 207 also experienced issues at customs, these samples were returned

to the FAO RC.

4.2. Result comments

4.2.1. Sample 1

***E. coli* –** Eighteen laboratories returned duplicate *E. coli* MPN/100g results between ±3 SD of the participants' median for Sample 1 (Figure 1) of which 17 laboratories obtained a maximum score of 12. Laboratory 12 reported a single replicate result between ±3 SD of the participants' median and one void result due to a category 3 tube combination and scored 7 out of 7. Laboratory 235 reported one replicate result between ±3 SD of the participants' median and one replicate result between ±3 and ±5 SD of the participants' median.

Two laboratories (laboratories 189 and 235) had points deducted for reporting tube combinations inconsistent with the guidance given in ISO 7218 (Anon, 2024) for interpretation of MPN tables or for the reporting of an incorrect MPN value for the reported tube combination. Participants are reminded that an MPN calculator referenced in ISO 7218:2024 may be used to calculate results.

***Salmonella spp.* –** Nineteen laboratories returned results for *Salmonella* spp. with 18 correctly reporting the presence of *Salmonella* spp. and received a maximum score of 2. Laboratory 3 did not detect the presence of *Salmonella* spp. and received a score of 0.

4.2.2. Sample 2

***E. coli* –** Twenty laboratories returned duplicate *E. coli* MPN/100g results between ±3 SD of the participants' median for Sample 2. Nineteen laboratories received the maximum score of 12 however laboratory 235 had points deducted for reporting tube combinations inconsistent with the guidance given in ISO 7218 for interpretation of MPN tables or for the reporting of an incorrect MPN value for the reported tube combination. This laboratory is reminded that for official control testing of live bivalve molluscs in the EU, the MPN calculator referenced in ISO 7218:2024 should be used.

Note: Due to the large number of <18 results for this sample a figure showing participant results is not included.

***Salmonella* spp. –** Nineteen laboratories returned results for *Salmonella* spp. with all correctly reporting the absence of *Salmonella* spp. and received a maximum score of 2.

4.2.3. Results summary

Those laboratories who have lost marks for the enumeration of *E. coli* and/or *Salmonella* spp. detection should in the first instance refer to the troubleshooting guide included as Appendix 4. Laboratories are reminded that the MPN calculator from ISO 7218 or the MPN tables provided by the FAO RC should be used for MPN determination.

5. References

Anon, 2015. ISO 16649-3. 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.

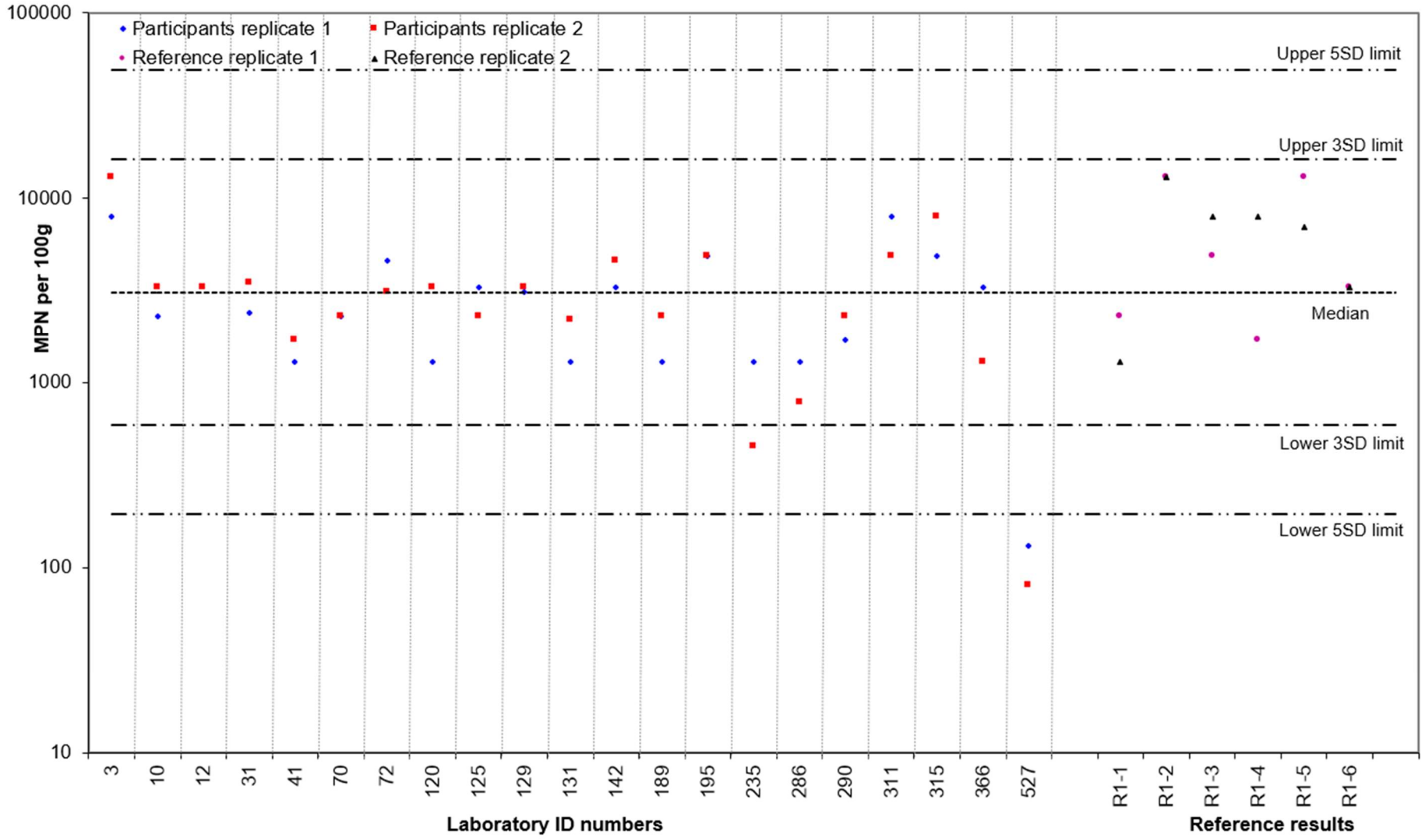
Anon, 2017a. ISO/IEC 17025. General requirements for the competence of testing and calibration laboratories.

Anon, 2017b. ISO 6579-1. Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp.

Anon, 2019. ISO 22117. Microbiology of the food chain – Specific requirements and guidance for proficiency testing by interlaboratory comparison.

Anon, 2024. ISO 7218. Microbiology of the food chain – General requirements and guidance for microbiology examinations.

Figure 1 - Sample 1 – Pacific oysters - Participants' and FAO reference *E. coli* MPN results plotted against the participants' median



6. Appendices

6.1. Appendix 1 – Participants sample information

Table 7 – Sample arrival and temperature

Lab ID.	Participants' records		Internal temp. (°C)	Storage (°C)	Date analysed
	Date	Time			
3	20.11.24	14:30	2.8	4	20.11.24
10	19.11.24	10:30	2.8	-	19.11.24
12	21.11.24	11:20	2.5	3 to 5	21.11.24
31	19.11.24	11:30	4.7	5	19.11.24
41	19.11.24	13:00	2.1	3 ± 2	19.11.24
70	19.11.24	08:30	2.3	-	19.11.24
72	19.11.24	09:00	3.6	3.8	19.11.24
120	19.11.24	11:10	2.2	3	19.11.24
125	19.11.24	11:14	4.2	4	19.11.24
129	21.11.24	14:45	4	-	21.11.24
131	19.11.24	09:15	2.6	3.1	19.11.24
142	20.11.24	09:10	4.51	-	20.11.24
189	20.11.24	12:00	1.6	4	20.11.24
195	20.11.24	09:45	4.8	4	20.11.24
235	20.11.24	15:45	-1	5	21.11.24
286	19.11.24	11:12	1.1	refrigerated	20.11.24
290	20.11.24	16:20	4.2	5.2	21.11.24
311	19.11.24	10:30	2.8	-	19.11.24
315	19.11.24	13:00	5.4	5 ± 3	19.11.24
366	19.11.24	17:30	6	4.5	20.11.24
527	25.11.24	10:00	12	2 to 4	26.11.24

6.2. Appendix 2 – Proficiency Testing scoring

Table 8 - *E. coli* MPN scores allocated to participants returning 2 replicate results

Result	Returning of results	Score allocated		Total score
		Rep. 1	Rep. 2	
Both replicate MPN results are within the expected range.	2	5	5	12
One replicate MPN result is outside the expected range and falls between the median ±3SD and median ±5SD values.	2	5	2	9
Both replicate MPN results are outside the expected range and fall between the median ±3SD and median ±5SD values.	2	2	2	6
One replicate MPN result is outside the median ±5SD value.	2	5	0	7
Both replicate MPN results are outside the expected range. The first falls between the median ±3SD and median ±5SD value and the second falls outside the median ±5SD values.	2	2	0	4
Both replicate MPN results reported are outside the median ±5SD value.	2	0	0	2

Table 9 – *E. coli* MPN scores allocated to participants returning 1 single replicate result

Result	Returning of results	Score allocated	Total score
Single replicate MPN result is within the expected range.	2	5	7
Single replicate MPN result is outside the expected range and falls between the median ±3SD and median ±5SD values.	2	2	4
Single replicate MPN result reported is outside the median ±5SD value.	2	0	2

Table 10 – *E. coli* score deductions

Result	Scores deducted	
	Rep. 1	Rep. 2
Tube combination inconsistent with MPN reported and / or tube combination selected not consistent with rules given in ISO 7218 or MPN tables provided by the FAO RC.	2	2
High censored result (e.g. MPN = >18000 per 100g)	2	2
Sample not examined or results returned late - no explanation received	12	

Table 11 – *Salmonella* spp. scoring

Result	Scores allocated
Fully correct results	2
Misleading result, e.g. failure to isolate Salmonella	0

6.3. Appendix 3 – Participants results reported

Lab ID.	Sample 1 – Pacific oysters						Sample 2 – Pacific oysters					
	Replicate 1			Replicate 2			Replicate 1			Replicate 2		
	Raw data	Tube combo used	MPN/ 100g	Raw data	Tube combo used	MPN/ 100g	Raw data	Tube combo used	MPN/ 100g	Raw data	Tube combo used	MPN/ 100g
3	5530	5530	7900	5540	5540	13000	1000	1000	20	0000	0000	<18
10	5500	5500	2300	5510	5510	3300	0000	0000	<18	0000	0000	<18
12 [a]	5442	5442	void	5510	5510	3300	0000	0000	<18	0000	0000	<18
31	550	550	2400	551	551	3500	0000	0000	<18	0000	0000	<18
41	5400	5400	1300	5410	5410	1700	0000	0000	<18	0000	0000	<18
70	5500	5500	2300	5500	5500	2300	1000	1000	20	0000	0000	<18
72	5511	5511	4600	5501	5501	3100	0000	0000	<18	0000	0000	<18
120	5400	5400	1300	5510	5510	3300	0000	0000	<18	0000	0000	<18
125	5510	5510	3300	5500	5500	2300	1000	1000	20	1000	1000	20
129	5501	5501	3100	5510	5510	3300	0000	0000	<18	0000	0000	<18
131	5400	5400	1300	5420	5420	2200	0000	0000	<18	0000	0000	<18
142	5510	5510	3300	5511	5511	4600	0000	0000	<18	0000	0000	<18
189	5400	5400	1300	5500	5400	2300	0000	0000	<18	0000	0000	<18
195	5520	5520	4900	5520	5520	4900	0000	0000	<18	1000	1000	20
235	5400	540	1300	5200	520	450	0000	0000	<20	0000	0000	<20
286	5400	5400	1300	5300	5300	780	0000	0000	<18	0000	0000	<18
290	5410	5410	1700	5500	5500	2300	0000	0000	<18	0000	0000	<18
311	5530	5530	7900	5520	5520	4900	0000	0000	<18	0000	0000	<18
315	5520	5520	4900	5530	5530	7900	0000	0000	<18	0000	0000	<18
366	5510	5510	3300	5400	5400	1300	0000	0000	<18	0000	0000	<18
527 [b]	4000	4000	130	3000	3000	80	5200	5200	490	5100	5100	330

Key: a = A void result was reported for one replicate for Sample 1, total score for this sample is out of 7; b = Due to the extended duration of the sample transport these results have been omitted from the assessment but are included in the graph. Yellow shading denoted score deductions.

6.4. Trouble shooting advice

1. **Methods –** Ensure that the method used is appropriate for the examination of the sample.
    a. Ensure that any dilutions have been calculated correctly.
    b. Ensure that the dilutions analysed are as specified on the report form.
    c. Ensure that MPN tables (if used) are interpreted correctly.

2. **Interpretation of MPN value**

    Record the number of TBX positives for each dilution to give a three- or four-figure tube combination number. Use the MPN calculator for 100g test portions referenced in ISO 7218 (Anon, 2024) (ISO Standards Maintenance Portal), one of the MPN calculators available from the Cefas FAO Reference Centre website (Technical Guidance and Calculation Spreadsheets - Cefas (Centre for Environment, Fisheries and Aquaculture Science)) or the MPN tables in the FAO Reference Centre *E. coli* generic protocol (Generic Protocols - Cefas (Centre for Environment, Fisheries and Aquaculture Science)) to calculate results expressed as MPN per 100g.

    Note: In all cases the MPN must be calculated using the number of positive tubes counted at ALL tested dilutions, even if lower dilutions are completely negative. For example, if a four-dilution combination of 5,3,0,0 is obtained the result should be reported as 780 (MPN result for a combination of 5,3,0,0) rather than 790 (MPN result for a combination of 5,3,0).

    Note: Only category 1 or 2 tube combinations should be reported – category 3 combinations should be recorded/reported as 'void'. The MPN tables in the FAO Reference Centre generic *E. coli* protocol only include category 1 or 2 tube combinations: any tube combination that does not appear in the MPN table is an unacceptable (category 3) combination.

3. **Culture media** - Check the quality control data for media to ensure that they are within specifications and performing adequately.

4. **Equipment** - Check that the equipment used for the procedures (incubators, refrigerators, measuring instruments) are calibrated and performing adequately.

5. **Staff training** - Check that the staff performing the tests are fully trained and familiar with all the procedural steps.

6. **Clerical procedures** - Check that the sample labeling, laboratory numbering and clerical procedures are adequate as well as procedures for ensuring test results are reported accurately and on time.

7. **Accreditation**- Check that quality procedures are documented and always adhered to.

8. **Internal quality assessment (IQA)** – Ensure adequate controls are in place and follow-up procedures are in place to deal with IQA failures.

Further advice can be obtained from the FAO RC on request.

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