Centre for Environment Fisheries & Aquaculture Science

Cefas

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 95)

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Draft V1	L. Stockley	11.12.23	First internal review to JL
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Final V1	L. Stockley	10.01.24	No changes required. Issued as final copy

Contents

This scheme is intended to provide proficiency testing (PT) samples for laboratories undertaking examination of live bivalve molluscs from production areas in accordance with Regulation (EC) No. 2017/625 and from throughout the production chain in accordance with Regulation (EC) No. 2073/2005.

The scheme is organised by Cefas, the FAO Reference Centre for Bivalve Mollusc Sanitation. The scheme is intended to compliment the Cefas/UK HSA Shellfish 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 accreditation purposes.

A scoring system is used to help assess participants' performance. Details of this system are included as Appendix II 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 (https://www.cefas.co.uk/international-centres-of-excellence/seafood-safety/).

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 III of this report.

Further advice on microbiological testing of bivalve shellfish can be obtained via the Cefas website (https://www.cefas.co.uk/international-centres-of-excellence/seafood-safety/).

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 550 Pacific oysters (*Magallana gigas*) were collected from a UK commercial harvesting area on the 23rd October 2023. Prior to packing the shellfish were placed in a large sterile container and thoroughly mixed. Sample 1 comprised of approximately 22 randomly selected oysters per participant.

1.2. Sample 2 – Homogenate

Approximately 200 Pacific oysters (*Magallana gigas*) were collected from a UK commercial harvesting area on the 9th October 2023 and a portion was tested to confirm the absence of *E. coli* and *Salmonella* spp.. before storing at <20 °C for approximately 2 weeks. The shellfish were then defrosted, shucked and homogenised. Homogenised shellfish were pooled together and mixed before being aliquoted into sterile bottles in 100 ml volumes and frozen again until sample preparation took place. On the 22nd October 2023 the aliquoted homogenate was removed from the freezer to defrost overnight prior to spiking and dispatch on the 23rd October 2023.

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 23rd October 2023 to 20 participating laboratories using DG Global Forwarding. Participants were requested to analyse the samples immediately on receipt using their routine methods.

2.1. Sample temperature

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

3. Results

3.1. Reference results – *E. coli*

Six randomly selected samples were analysed for *E. coli* in duplicate on the 24th October 2023 under repeatability conditions using Cefas SOP No. 1175 (Table 1). Sample homogeneity was assessed following the procedure described in ISO 22117. 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. and type	Range	Median	GM	Median ±3*SD_T
Sample 1 – Pacific oysters	<1.8 x 10^1 – 2.0 x 10^1	<1.8 x 10^1	1.7 x 10^1	3.2 x 10^0 – 8.9 x 10^1
Sample 2 – Homogenate	1.3 x 10^3 – 7.9 x 10^3	3.0 x 10^3	3.0 x 10^3	5.7 x 10^2 – 1.6 x 10^4

3.2. Reference results – *Salmonella* spp.

Six randomly selected samples were analysed for *Salmonella spp.* on the 24th October 2023 under repeatability conditions using Cefas SOP No. 1176 (Table 2).

Table 2 – *Salmonella* spp. reference results

Sample No. and type	*Salmonella* spp.	No. of replicates giving the expected results
Sample 1 – Pacific oysters	Absent in 25g	6
Sample 2 – Homogenate	Present 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 II). Reference values were excluded from the calculation of the participants' median. Participants' results and scores allocated for PT 95 are shown in Tables 3, 4, 5, 6 and Figures 1[1] and 2.

3.3.1. *E. coli* results

Table 3 – Participants' results

Sample No. and type	*E. coli* MPN/100g *Range*	Median	GM	Median±3*SD_T
Sample 1 – Pacific oysters	<1.8 x 10^1 – 4.9 x 10^2	<1.8 x 10^1	2.5 x 10^1	3.2 x 10^0 – 8.9 x 10^1
Sample 2 – Homogenate	1.8 x 10^1 – 1.1 x 10^4	3.3 x 10^3	2.6 x 10^3	6.3 x 10^2 – 1.7 x 10^4

Note: The median and upper and lower limits (±3 SD and ±5 SD) were calculated from participants' results. SD_T 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.

[1] Samples reported as <18 MPN /100g were assigned a value of 17 to enable a full assessment to be made.

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	<18	<18	12	2300	2300	12
10	<18	<18	12	1400	NE	7
12	<18	<18	12	3300	2700	12
31	<18	20	12	NE	NE	-
70	<18	<18	12	3100	1400	12
72	<18	20	12	3300	2300	12
120	20	<18	12	4900	4900	12
125	<18	<18	12	3300	3300	12
129	20	20	12	7900	2300	12
131	20	<18	12	11000	11000	12
142	<18	45	12	20	18	2
189	<18	20	12	3300	3300	12
195	490	330	2	3300	3300	12
235	<18	<18	12	2200	1800	12
286	<18	<18	12	1700	3300	12
290	330	450	2	3300	4600	12
315	<18	<18	12	7900	4900	12
366	<18	<18	12	4900	4600	12
477	<18	<18	12	7900	3300	12

Table 5 – Summary statistics of participants' results

E. coli	Sample 1	Sample 2
Participants reporting duplicate results for *E. coli* MPN	19	17
Participants reporting both replicate MPN results within expected range [1]	17	16
Participants reporting a single MPN result within expected range [1]	0	1
Participants reporting one replicate MPN result outside expected range [1]	0	0
Participants reporting both replicate MPN results outside expected range [1]	2	1
Participants reporting tube combination and / or MPN results inconsistent with ISO 7218 [2]	0	0

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

[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	NE	-	NE	-
10	Not Detected	2	Detected	2
12	Not Detected	2	Detected	2
31	Not Detected	2	Detected	2
70	Not Detected	2	Detected	2
72	Not Detected	2	Detected	2
120	Not Detected	2	Detected	2
125	Not Detected	2	Detected	2
129	NE	-	NE	-
131	Not Detected	2	Detected	2
142	Not Detected	2	Detected	2
189	Not Detected	2	Detected	2
195	Not Detected	2	Detected	2
235	Not Detected	2	Detected	2
286	Not Detected	2	Detected	2
290	Not Detected	2	Detected	2
315	Not Detected	2	Detected	2
366	Not Detected	2	Detected	2
477	Not Detected	2	Detected	2

NE – Not examined

4. Comments

4.1. General comments

- Nineteen out of 20 participating laboratories received their samples with 17 laboratories receiving the material within 48 hours of dispatch as recommended by the FAO Reference Centre. Fifteen laboratories analysed the samples on the day of arrival. Laboratory 96 did not receive PT 95 material due issues experienced at customs.
- The sample arrival temperature provided by 19 laboratories showed the maximum temperature recorded did not exceed the recommended transport temperature of <10°C set out in the FAO RC generic protocol.

4.2. Result comments

4.2.1. Sample 1

***E. coli* –** Seventeen laboratories returned duplicate *E. coli* MPN/100g results between ±3 SD of the participants' median for Sample 1 (Figure 1) with all obtaining a maximum score of 12.

Laboratories 195 and 290 reported both replicate results outside ±5 SD of the participants' median and scored 2.

***Salmonella spp.* –** Seventeen laboratories returned results for *Salmonella* spp. with all correctly reporting the absence of *Salmonella* spp. in Sample 1 and received a score of 2.

4.2.2. Sample 2

***E. coli* –** Sixteen laboratories returned duplicate *E. coli* MPN/100g results between ±3 SD of the participants' median for Sample 2 (Figure 2) with 15 obtaining a maximum score of 12. Laboratory 10 reported a single replicate result which was between ±3 SD of the participants' median and scored 7. Laboratory 142 reported both replicate results outside ±5 SD of the participants' median and scored 2.

***Salmonella* spp. –** Seventeen laboratories returned results for *Salmonella* spp. with all correctly reporting the presence of *Salmonella* spp. in Sample 2 and received a 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 (<1 out of the maximum 2 score) should in the first instance refer to the troubleshooting guide included as Appendix III. Laboratories are reminded that the 5 x 3 MPN tables from ISO 7218 or those provided by the FAO Reference Centre should be used for MPN determination.

5. References

Anon 2007. ISO 7218. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiology examinations.

Anon 2013. ISO 7218:2007/FDAM 1:2013. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - Amendment 1.

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 2017. 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 TS 22117:2019. Microbiology of food and animal feeding stuffs – Specific requirements and guidance for proficiency testing by interlaboratory comparison.

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

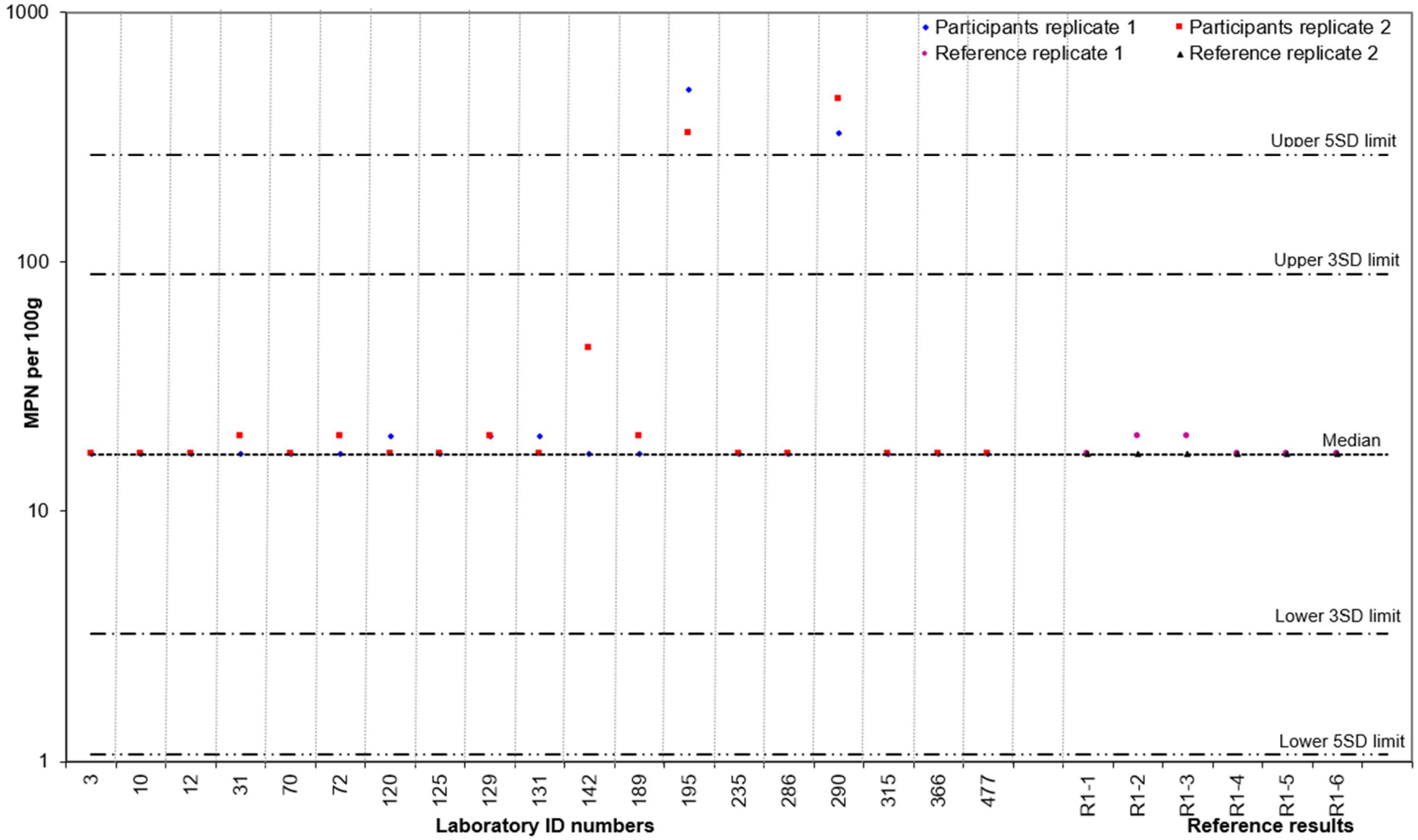
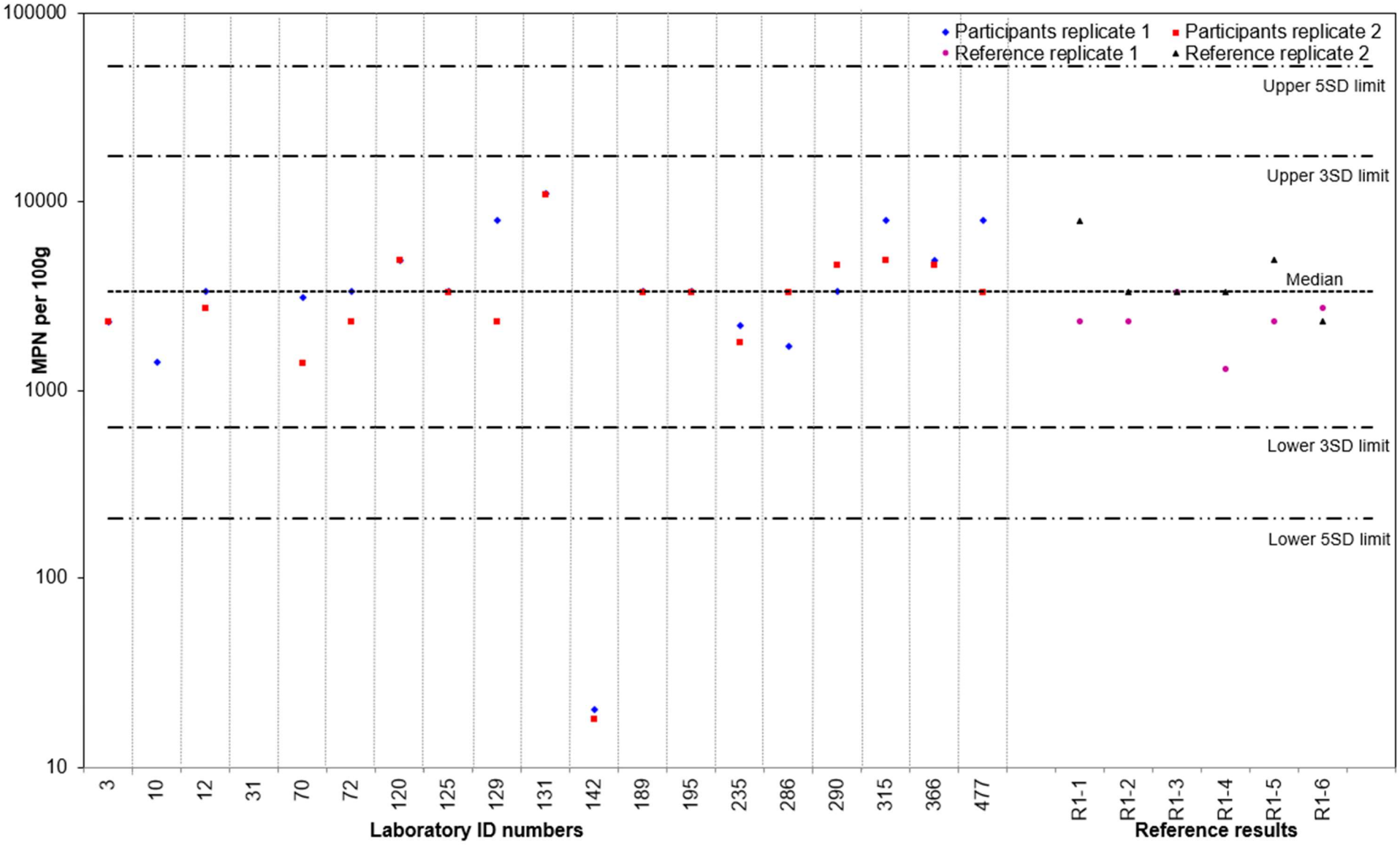


Figure 2 - Sample 2 – Shellfish homogenate - Participants' and FAO reference *E. coli* MPN results plotted against the participants' median



6. Appendix

6.1. Appendix 1 – Participants sample information

Table 7 – Sample arrival and temperature

Lab ID.	Participants' records Date	Time	Internal temp. (°C)	Storage (°C)	Date analysed
3	24/10/2023	11:35	4	4	25/10/2023
10	24/10/2023	10:00	2.8	-	24/10/2023
12	24/10/2023	11:10	2.4	2.9	24/10/2023
31	24/10/2023	10:09	2.4	5	24/10/2023
70	24/10/2023	09:00	3	4	25/10/2023
72	24/10/2023	08:30	3.7	4	24/10/2023
120	24/10/2023	11:30	2.23	3	24/10/2023
125	24/10/2023	11:08	5.6	4	24/10/2023
129	24/10/2023	09:10	1.5	4.9	24/10/2023
131	24/10/2023	09:32	3.2	3.6	24/10/2023
142	24/10/2023	09:20	5.31	-	24/10/2023
189	24/10/2023	11:20	1	4	24/10/2023
195	26/10/2023	13:40	8	-	26/10/2023
235	24/10/2023	11:00		3	25/10/2023
286	24/10/2023	10:10	2.5	-	24/10/2023
290	27/10/2023	10:00	10	-	27/10/2023
315	24/10/2023	10:30	3	3	24/10/2023
366	24/10/2023	07:50	4	4	24/10/2023
477	24/10/2023	12:55	4.5	-	24/10/2023

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 replicates 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 replicates 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 replicates MPN results reported is 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 7218:2007/Amd 1:2013 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 – 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.

   Interpretation of MPN tables

   Record the number of TBX positives for each dilution to give a three figure tube combination number. Use the MPN tables included in ISO 7218 and the FAO RC generic *E. coli* protocol. Only category 1 or 2 tube combinations are included in the tables and should be reported.

   Where more than three dilutions have been tested for a sample, use the Excel spreadsheet MPN calculator (http://standards.iso.org/iso/7218/) to determine the MPN from all the dilutions tested. Combinations that do not appear in the tables or obtained from the Excel calculator as category 3 are not acceptable and should not be used.

   If the tube combination result is an unacceptable combination, the result is reported as 'void'.

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

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

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

5. **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.

6. **Accreditation**- Check that quality procedures are documented and adhered to at all times.

7. **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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