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 90)

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Version	Author	Date	Comment
Draft V1	L. Stockley	03.01.2023	First internal review to CBA.
Draft V2	L. Stockley	03.01.2023	Internal review from CK (project manager).
Draft V3	L. Stockley	09.01.2023	Minor changes made before distribution to all participants for review.
Final V1	L. Stockley	27.01.2023	Minor changes following participants comments

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 600 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area on the 21st November 2022. Prior to packing the shellfish were placed in a large sterile container and thoroughly mixed. Sample 1 comprised of approximately 22 randomly selected oysters.

1.2. Sample 2 – Pacific oysters

Approximately 600 Pacific oysters (*C. gigas*) 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 5 days to allow the shellfish to acclimatize and remove any bacterial content. The oyster trays were removed from the tank and 1 litre of inoculum containing known levels of *E. coli* (≈3.8 x 10^4 cfu/100ml), *Klebsiella pneumoniae* (≈2.3 x 10^4 cfu/100ml) and *Salmonella typhimurium* (≈9.4 x 10^4 cfu/100ml) originating from shellfish was added to the tank and thoroughly mixed. The oysters were re-immersed in the tank and were left for 3.5 hours with constant re-circulation (without UV). After 3.5 hours of exposure the oysters were removed from the tank and transferred to a large container before 22 randomly selected oysters were 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 21st November 2022 to 23 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 in duplicate on the 22nd November 2022 under repeatability conditions for *E. coli* using SOP No. 1175 (Table 1). Sample homogeneity was assessed following the procedure described in ISO 22117. The sample material 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	4.5 x 10^1 – 7.8 x 10^2	2.3 x 10^2	2.2 x 10^2	4.4 x 10^1 – 1.2 x 10^3
Sample 2 – Pacific oysters	2.3 x 10^3 – 5.4 x 10^4	7.9 x 10^3	8.8 x 10^3	1.5 x 10^3 – 4.1 x 10^4

3.2. Reference results – *Salmonella* spp.

Six randomly selected samples were analysed on the 22nd November 2022 under repeatability conditions for *Salmonella spp.* using 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 – Pacific oysters	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 90 are shown in Tables 3, 4, 5, 6 and Figures 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 – 2.3 x 10^3	2.2 x 10^3	1.7 x 10^2	4.1 x 10^1 – 1.1 x 10^3
Sample 2 – Pacific oysters	1.7 x 10^3 – 5.4 x 10^4	8.6 x 10^3	8.9 x 10^3	1.6 x 10^3 – 4.5 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.

Table 4 – Participants' 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	690	170	12	11000	7900	12
10	330	490	12	54000	13000	9
12	170	310	12	4900	7900	12
31	230	330	12	11000	17000	12
41	330	2300	9	13000	4900	12
69	220	450	12	11000	11000	12
70	330	490	12	24000	35000	12
72	330	490	12	11000	24000	12
120	330	330	12	7900	13000	12
125	78	130	12	35000	24000	12
129	780	330	12	24000	4900	12
131 *	NE	NE	-	NE	NE	-
142	170	230	12	3300	7900	12
189	110	140	12	4900	4900	12
195	<18	<18	6	4900	4900	12
212	210	600	12	1700	2300	12
235	<18	45	9	1800	5400	10
273 **	<10	<10	12	100	80	12
286	20	78	9	11000	4600	12
290	<18	<18	6	7000	7000	12
357	45	68	8	5400	9200	8
366	210	210	10	7900	11000	12
513	130	45	12	13000	13000	12

* Samples sent but not able to be tested due to laboratory training.
** Results reported per g rather than per 100g. The results were multiplied by 100 to include on graph.

Table 5 – Summary statistics of participants' results

E. coli	Sample 1	Sample 2
Participants reporting duplicate results for *E. coli* MPN	22	22
Participants reporting the absence of *E. coli*	3	0
Participants reporting both replicate MPN results within expected range [1]	17	21
Participants reporting a single MPN result within expected range [1]	0	0
Participants reporting one replicate MPN result outside expected range [1]	3	1
Participants reporting both replicate MPN results outside expected range [1]	2	0
Participants reporting one replicate MPN results as censored results	0	0
Participants reporting both replicate MPN results as censored results	0	0
Participants reporting tube combination and / or MPN results inconsistent with ISO 7218 [2]	3	1

[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' allocated scores (*Salmonella* spp. in 25g)

Lab ID.	Sample 1 Rep 1	Score	Sample 2 Rep 1	Score
3	Not Detected	2	Detected	2
10	Detected	0	Detected	2
12	Not Detected	2	Detected	2
31	Not Detected	2	Detected	2
41	Detected	0	Detected	2
69	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	-	Detected	2
131	NE	-	NE	-
142	Not Detected	2	Detected	2
189	Not Detected	2	Detected	2
195	Not Detected	2	Detected	2
212	NE	-	NE	-
235	Not Detected	2	Detected	2
273	Not Detected	2	Detected	2
286	Not Detected	2	Detected	2
290	Not Detected	2	Detected	2
357	Not Detected	2	Detected	2
366	Not Detected	2	Detected	2
513	Not Detected	2	Detected	2

NE – Not examined

4. Comments

4.1. General comments

- Eighteen laboratories received their samples within 48 hours of dispatch as recommended by the FAO Reference Centre with 15 laboratories analysing the samples on the day of arrival.
- 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. Laboratories 142, 195 and 290 recorded an internal arrival temperature exceeding 10°C. The reason for the increases in temperature were due to an extended transport time (more than 10 days in transit) and the samples being transferred to an alternative transport container with no cool packs included.
- Laboratory 131 did not analyse PT 90 material due to staff training being carried out at the time of receiving the PT material.

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 15 obtaining a maximum score of 12. Laboratories 41, 235 and 286 reported 1 replicate result between ±3 and ±5 SD of the participants' median and scored 9. Laboratories 195 and 290 reported both replicate results between ±3 and ±5 SD of the participants' median and scored 6. Laboratories 357 and 366 had points deducted for reporting a tube combination inconsistent with MPN reported and / or tube combination selected not consistent with the rules given in ISO 7218, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

***Salmonella spp.* –** Eighteen laboratories returned results for *Salmonella* spp. with all correctly reporting the absence of *Salmonella* spp. in Sample 1 and received a score of 2. Laboratories 10 and 41 incorrectly reporting the presence of *Salmonella* spp. and received a score of 0.

4.2.2. Sample 2

***E. coli* –** Twenty-one laboratories returned duplicate *E. coli* MPN/100g results between ±3 SD of the participants' median for Sample 2 (Figure 2) with 19 obtaining a maximum score of 12. Laboratory 10 reported 1 replicate result between ±3 and ±5 SD of the participants' median and scored 9. Laboratories 235 and 357 had points deducted for reporting a tube combination selected not consistent with the rules given in ISO 7218, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.

***Salmonella* spp. –** Thirteen laboratories returned results for *Salmonella* spp. with all correctly reporting the presence of *Salmonella* spp. in Sample 2 and received a score of 2. Laboratory 12 incorrectly reporting the absence of *Salmonella* spp. and received a score of 0.

4.2.3. Results summary

For those laboratories who have lost marks for the enumeration *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 2010. ISO TS 22117:2010. Microbiology of food and animal feeding stuffs – Specific

requirements and guidance for proficiency testing by interlaboratory comparison.

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.

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

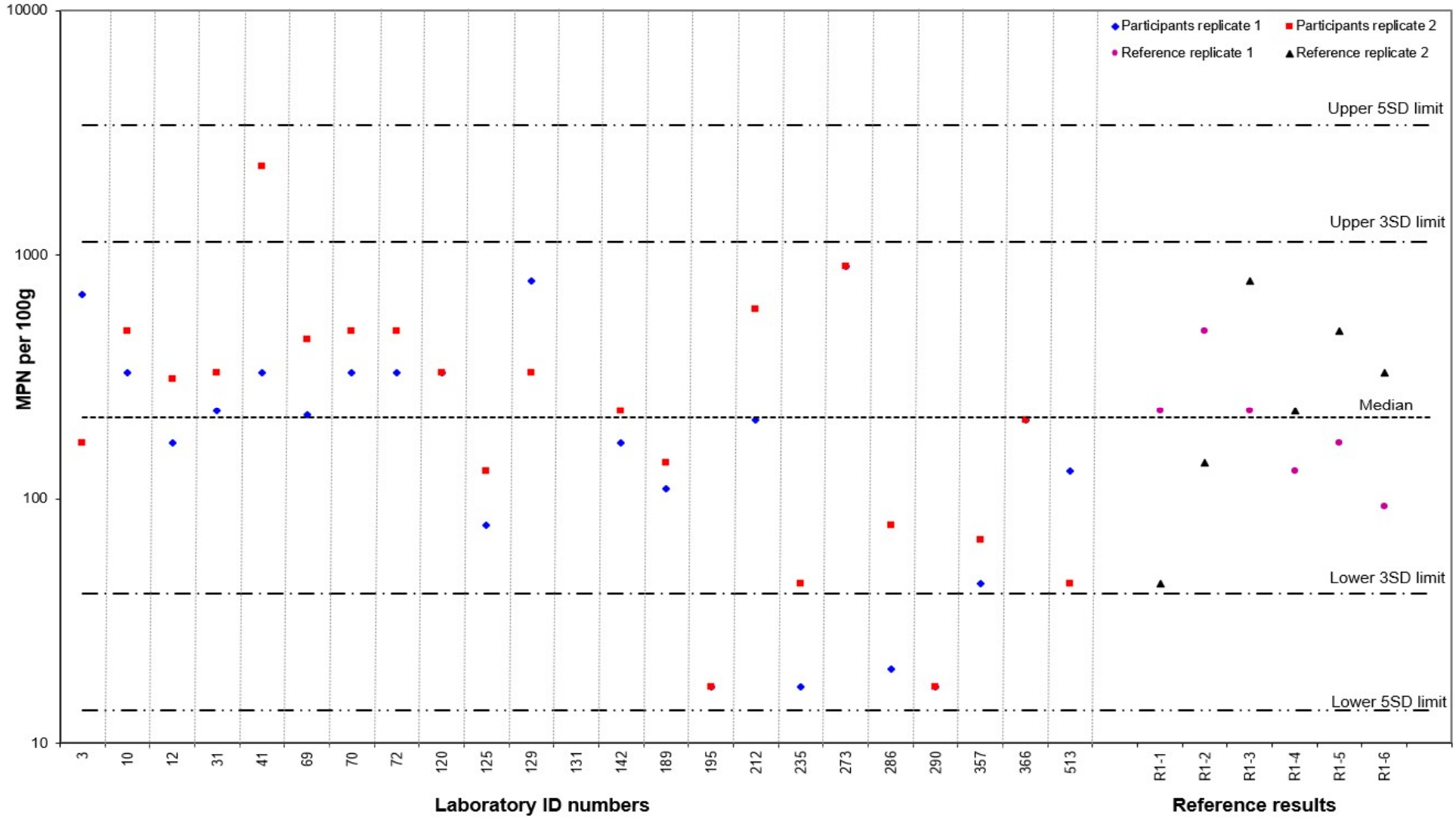
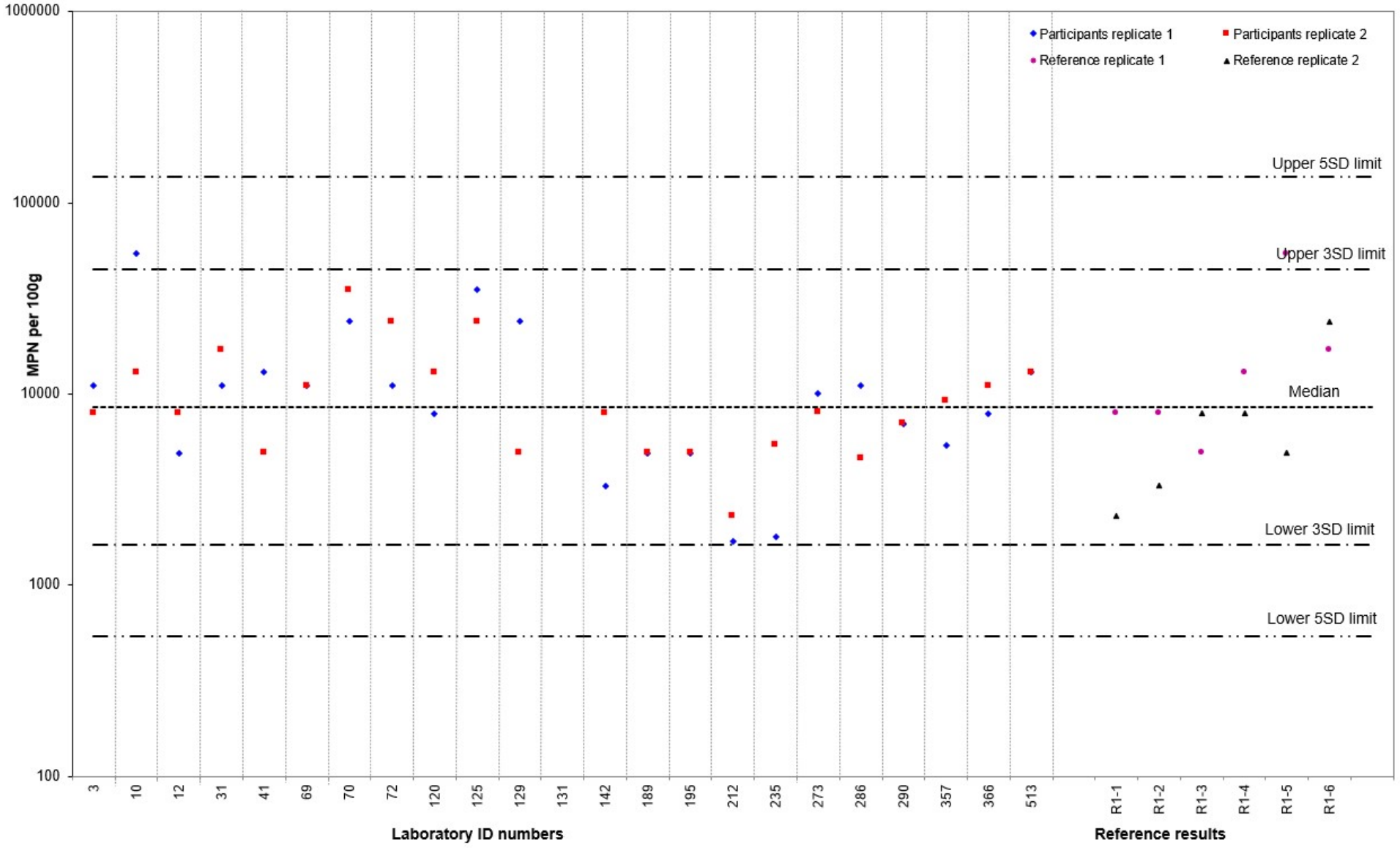


Figure 2 - Sample 2 – Shellfish homogenate - Participants' and FAO reference *E. coli* MPN results plotted against the participants' median



6. Appendix

6.1. Appendix 1 – Participants sample information

Table 7 – Sample arrival and temperature

Lab ID.	Participants' records		Internal temp. (°C)	Storage (°C)	Date analysed
	Date	Time			
3	23/11/2022	14:00	4.2	4.00	23/11/2022
10	22/11/2022	14:00	4.5	-	22/11/2022
12	22/11/2022	11:22	1.5	-	22/11/2022
31	22/11/2022	10:42	2	5.00	22/11/2022
41	23/11/2022	12:00	4.7	-	23/11/2022
69	23/11/2022	13:00	5.7	-	23/11/2022
70	22/11/2022	19:12	3	3.50	22/11/2022
72 *	22/11/2022	09:00	3	3.00	23/11/2022
120	22/11/2022	11:40	2.73	3.00	22/11/2022
125	22/11/2022	13:00	2	4.00	22/11/2022
129	23/11/2022	09:00	4.1	4.90	23/11/2022
131 **	-	-	-	-	-
142 ***	22/11/2022	09:05	18.03	-	22/11/2022
189	22/11/2022	11:30	0.2	3 to 5	22/11/2022
195 ******	30/11/2022	13:30	13.9	-	30/11/2022
212	23/11/2022	12:00	4.7	-	23/11/2022
235	23/11/2022	Am	3	3.00	24/11/2022
273	24/11/2022	10:00	4	4.00	25/11/2022
286	22/11/2022	09:40	1.4	-	22/11/2022
290 ******	30/11/2022	10:00	19	-	30/11/2022
357	23/11/2022	17:00	1.3	4	24/11/2022
366	22/11/2022	10:00	4.2	4.8	22/11/2022
513	24/11/2022	09:30	2.4 - 4	-	24/11/2022

* Wrong sample included in box. A new sample was sent the following day.

** Samples sent but not able to be tested due to laboratory training.

*** Following dispatch, the samples were transferred to an alternative container without cool packs causing the sample temperature to exceed the recommended temperature.

**** The transit time of these samples exceeded the FAO Reference Centre recommendations due to the incorrect import permit paperwork was not in place on arrival at customs.

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 NRL.	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.

Centre for Environment Fisheries & Aquaculture Science

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