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 83)

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Cefas Document Control

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Version	Author	Date	Comment
Draft 1	L. Stockley	11.01.22	Released for first internal review to CBA.
Draft 2	L. Stockley	17.01.22	Amended following CBA comments
Final V1	L. Stockley	21.01.22	Final for issue to FSA and Participants

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/UKHSA Shellfish Scheme through examination of aspects of the methods not covered under the Shellfish Scheme Proficiency testing for food, water and environmental microbiology - GOV.UK (www.gov.uk) (initial sample preparation and preparation of initial dilutions) and to provide additional data for 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 – Common mussels

A single batch of 800 Common mussels (*Mytilus edulis*) were collected from a UK commercial harvesting area on the 5th December 2021. Prior to packing the shellfish were placed in a large sterile container and thoroughly mixed. Sample 1 comprised of approximately 35 randomly selected mussels from this bulk material.

1.2. Sample 2 and 3 - Homogenate

Approximately 300 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area on the 9th November 2021 and a portion was tested to confirm the absence of *E. coli* and *Salmonella* spp.. before storing at <20 °C for approximately 1 week. 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 5th December 2021 the aliquoted homogenate was removed from the freezer to defrost overnight prior to spiking and dispatch on the 6th December 2021.

1.2.1. Sample 2 – Homogenate

For Sample 2, 100ml of homogenate was spiked with *E. coli* (≈ 5.3×10^4 cfu/sample) and *Salmonella* spp. (1.2×10^4 cfu/sample) on the day of dispatch.

1.2.2. Sample 3 - Homogenate

For Sample 3, 100ml of homogenate was spiked with *E. coli* (≈ 4.7×10^2 cfu/sample) and *Salmonella* spp. (≈ 2.9×10^4 cfu/sample) on the day of dispatch.

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 6th December 2021 to 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 7th December 2021 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 *	$2.3 \times 10^2 – 7.0 \times 10^3$	1.1×10^3	1.1×10^3	$2.1 \times 10^2 – 5.8 \times 10^3$
Sample 2 – Homogenate	$3.3 \times 10^3 – 3.5 \times 10^4$	7.5×10^3	7.7×10^3	$1.4 \times 10^3 – 3.9 \times 10^4$
Sample 3 - Homogenate	$1.3 \times 10^2 – 4.9 \times 10^2$	2.8×10^2	2.6×10^2	$5.3 \times 10^1 – 1.5 \times 10^3$

* Results obtained from one set of replicate results were removed from the analysis due to problems with testing an individual sample.

3.2. Reference results – *Salmonella* spp.

Six randomly selected samples were analysed on the 7th December 2021 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 - Homogenate	Present in 25g	6
Sample 3 - Homogenate	Present in 25g	6

3.3. Participants' results

Performance assessment was carried out according to the procedures described in the Cefas/UKHSA EQA shellfish scheme for a single distribution, with minor modifications (Appendix II) by calculating the participants median and ±3 and ±5 standard deviations (δ) (upper and lower limits) from the participants' reported MPN 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 the participants' median. Participants' results and scores allocated for PT 83 are shown in Tables 3, 4, 5, 6 and Figures 1, 2 and 3.

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	$3.3 \times 10^2 – 4.9 \times 10^3$	1.2×10^3	1.3×10^3	$2.3 \times 10^2 – 6.3 \times 10^3$
Sample 2 - Homogenate	$1.7 \times 10^3 – 1.6 \times 10^5$	2.4×10^4	1.6×10^4	$4.6 \times 10^3 – 1.2 \times 10^5$
Sample 3 - Homogenate	$2.0 \times 10^1 – 2.3 \times 10^3$	3.3×10^2	3.3×10^2	$6.3 \times 10^1 – 1.7 \times 10^3$

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 - Homogenate			Sample 2 - Homogenate			Sample 3 - Homogenate		
	Rep 1	Rep 2	Score	Rep 1	Rep 2	Score	Rep 1	Rep 2	Score
3	1100	2300	12	24000	22000	12	330	780	12
10	690	1700	12	3300	1700	6	230	130	12
12	2300	780	12	54000	24000	12	220	230	12
31	4600	4900	12	160000	28000	9	230	230	12
70	3300	1700	12	92000	92000	12	490	690	12
72	690	1300	12	54000	4900	12	230	230	12
120	330	330	12	14000	7900	12	230	130	12
125	2300	2300	12	35000	54000	12	1100	1700	12
129	2300	2300	12	2300	3300	6	230	170	12
131	1100	780	12	2300	2300	6	490	330	12
142	2200	1700	12	13000	14000	12	20	78	7
189	690	780	12	24000	35000	12	780	330	12
286	1300	780	12	35000	54000	12	330	330	12
311	690	780	12	2300	2300	6	330	330	12
366	780	780	12	92000	54000	12	2200	2300	6

Table 5 – Summary statistics of participants' results

E. coli	Sample 1 – Pacific oysters	Sample 2 - Homogenate	Sample 3 - Homogenate
Participants reporting duplicate results for *E. coli* MPN	15	15	15
Participants reporting the absence of *E. coli*	0	0	0
Participants reporting both replicate MPN results within expected range [1]	15	10	13
Participants reporting a single MPN result within expected range [1]	0	0	0
Participants reporting one replicate MPN result outside expected range [1]	0	1	1
Participants reporting both replicate MPN results outside expected range [1]	0	4	1
Participants reporting one replicate MPN results as censored results	0	0	0
Participants reporting both replicate MPN results as censored results	0	0	0
Participants reporting tube combination and / or MPN results inconsistent with ISO 7218 [2]	0	0	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		Sample 2		Sample 3	
	Rep 1	Score	Rep 1	Score	Rep 1	Score
3	Not Detected	2	Detected	2	Not Detected	0
10	NE	-	Detected	2	Detected	2
12	Not Detected	2	Not Detected	0	Detected	2
31	Not Detected	2	Detected	2	Detected	2
70	Not Detected	2	Detected	2	Detected	2
72	Not Detected	2	Detected	2	Detected	2
120	Not Detected	2	Detected	2	Detected	2
125	Not Detected	2	Detected	2	Detected	2
129	NE	-	NE	-	NE	-
131	Not Detected	2	Detected	2	Detected	2
142	Not Detected	2	Detected	2	Detected	2
189	Not Detected	2	Detected	2	Detected	2
286	Not Detected	2	Detected	2	Detected	2
311	NE	-	Detected	2	Detected	2
366	Not Detected	2	Detected	2	Detected	2

NE – Not examined

4. Comments

4.1. General comments

- All laboratories except Laboratory 3 received the samples within 24 hours of dispatch as recommended by the FAO RC.
- All 15 laboratories analysed the samples on the day of arrival.
- Information provided by laboratories on the arrival temperature of the samples showed the maximum temperature recorded by participants did not exceed the recommended transport temperature of <10°C set out in the FAO RC generic protocol.
- Laboratory 129 did not examine the sample materials for *Salmonella* spp. as they do not undertake this test in their laboratory.

4.2. Result comments

4.2.1. Sample 1

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

***Salmonella spp.* –** Twelve 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 311 did not examine for *Salmonella* spp..

4.2.2. Sample 2

***E. coli* –** Eleven laboratories returned duplicate *E. coli* MPN/100g results between ±3 SD of the participants' median for Sample 2 (Figure 2) and obtained a maximum score of 12. Laboratory 31 reported 1 replicate result and laboratories 10, 129 and 311 reported both replicate results between ±3 and ±5 SD of the participants' median and scored 9 and 6 respectively.

***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. Sample 3

***E. coli* –** Nine laboratories returned duplicate *E. coli* MPN/100g results between ±3 SD of the participants' median for Sample 3 (Figure 3) and obtained a maximum score of 12. Laboratory 145 reported both replicate results ±3 and ±5 SD of the participants' median and scored 6 and laboratory 578 reported 1 replicate result outside ±5 SD of the participants' median and scored 7.

***Salmonella* spp. –** Thirteen laboratories returned results for *Salmonella* spp. with all correctly reporting the presence of *Salmonella* spp. in Sample 3 and received a score of 2. Laboratory 3 incorrectly reporting the absence of *Salmonella* spp. and received a score of 0.

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

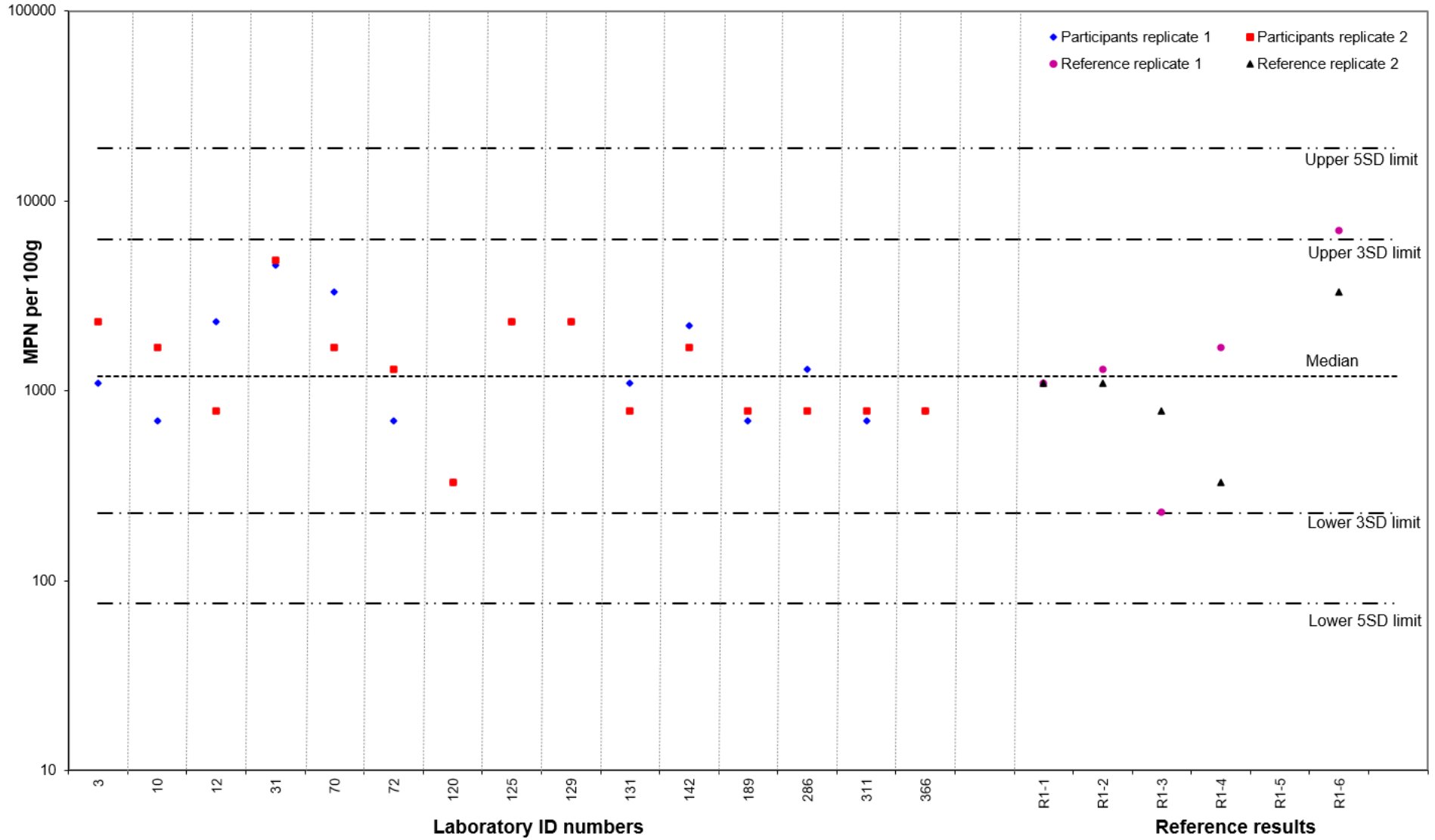


Figure 2 - Sample 2 – Shellfish homogenate - Participants' and FAO reference *E. coli* MPN results plotted against the participants' median

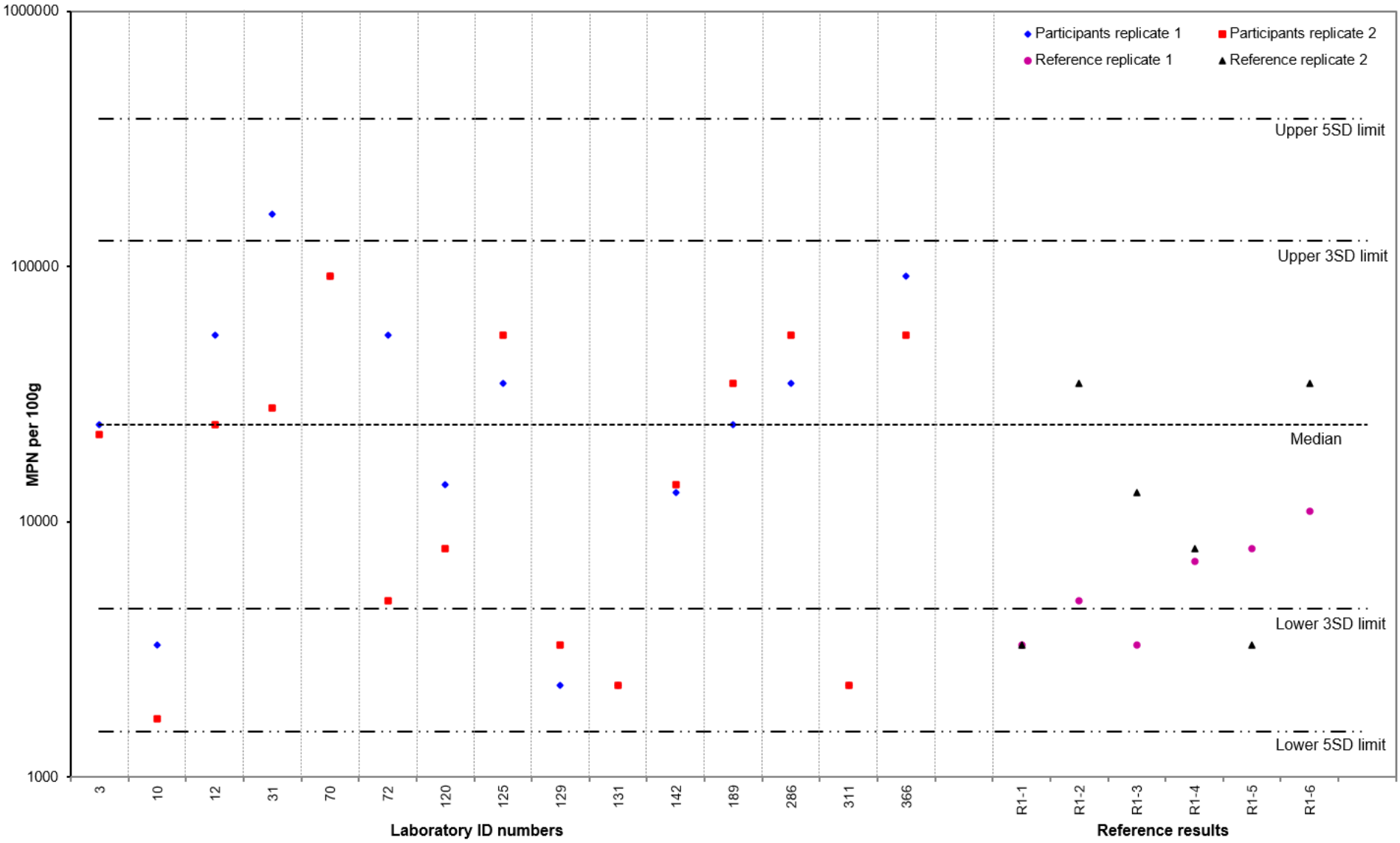
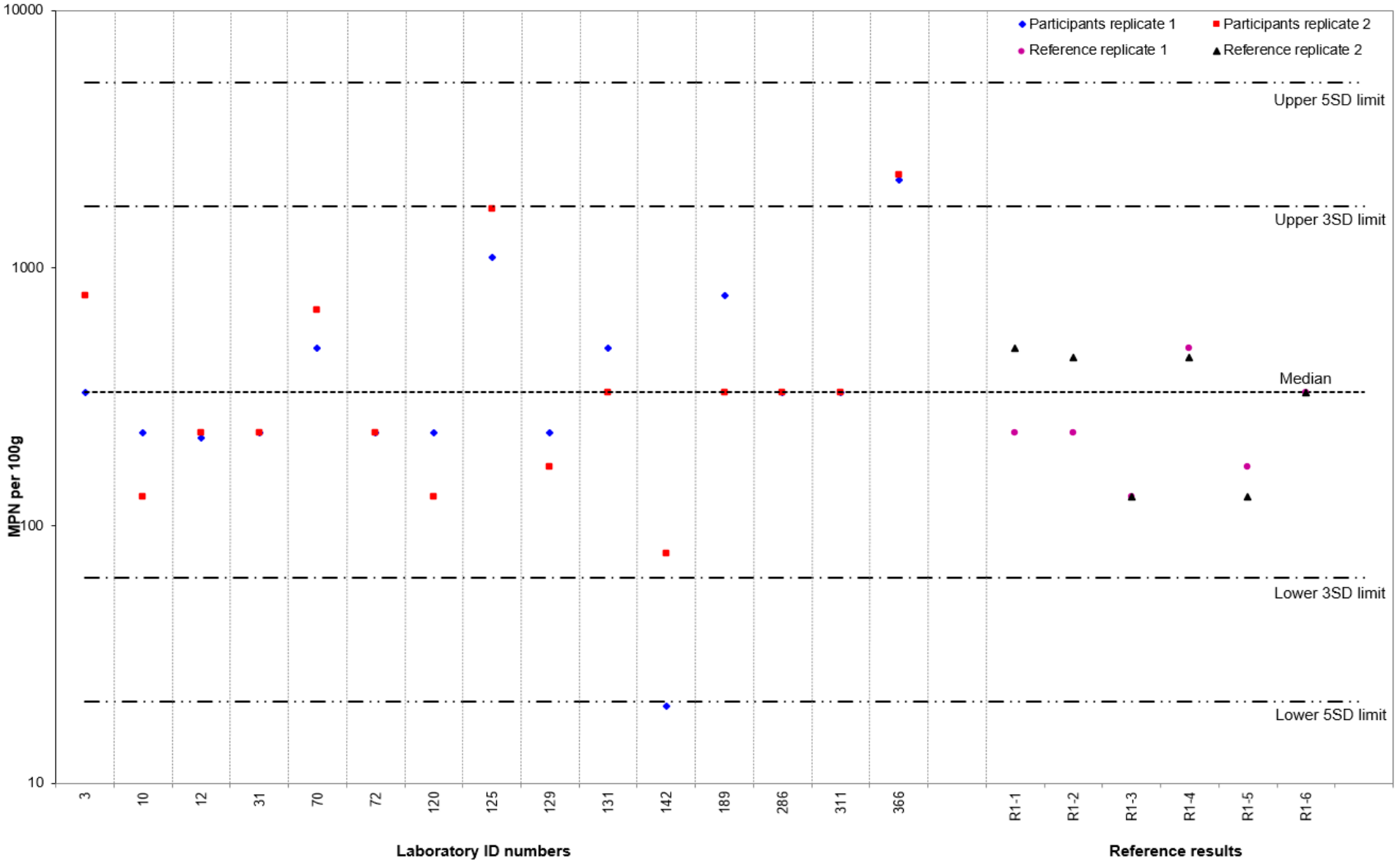


Figure 3 - Sample 3 – Shellfish homogenate - Participants' and FAO reference *E. coli* MPN results plotted against the participants' median



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	08/12/2021	11:45	6.9	4.4	08/12/2021
10	07/11/2021	11:00	2.2	-	07/12/2021
12	07/12/2021	12:27	3.5	-	07/12/2021
31	07/12/2021	09:55	4.6	4.2	08/12/2021
70	07/12/2021	09:30	3.3	-	-
72	07/12/2021	8	9.1	5	07/12/2021
120	07/12/2021	10:00	5.5	3	07/12/2021
125	07/12/2021	09:05	1.1	3	07/12/2021
129	07/12/2021	11:30	3.2	5	07/12/2021
131	07/12/2021	10:00	5	3.5	07/12/2021
142	07/12/2021	08:50	8.6	-	07/12/2021
189	07/12/2021	16:00	1.4	-	-
286	07/12/2021	08:10	2.2	4.48	07/12/2021
311	07/12/2021	11:00	2.2	-	07/12/2021
366	07/12/2021	12:30	6.9	-	07/12/2021

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
Single replicate MPN result is within the expected range.	2	2
Single replicate MPN result is outside the expected range and falls between the median ±3SD and median ±5SD values.	2	2
Single replicate MPN result reported is outside the median ±5SD value.	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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We are the government's marine and freshwater science experts. We help keep our seas, oceans and rivers healthy and productive and our seafood safe and sustainable by providing data and advice to the UK Government and our overseas partners. We are passionate about what we do because our work helps tackle the serious global problems of climate change, marine litter, over-fishing and pollution in support of the UK's commitments to a better future (for example the UN Sustainable Development Goals and Defra's 25 year Environment Plan).

We work in partnership with our colleagues in Defra and across UK government, and with international governments, business, maritime and fishing industry, non-governmental organisations, research institutes, universities, civil society and schools to collate and share knowledge. Together we can understand and value our seas to secure a sustainable blue future for us all, and help create a greater place for living.

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