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FAO Reference Centre for Bivalve Mollusc Sanitation

Enumeration *of Escherichia coli* and the detection of *Salmonella* spp. In bivalve molluscan shellfish (PT 80)

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Draft V1	L. Stockley	07/02/20	Released for internal review
Draft V2	L. Stockley	10/02/20	Version updated following RH comments
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Final V2	L. Stockley	09/03/20	Updated following transcription error identified by participant

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/PHE Shellfish Scheme through examination of aspects of the methods not covered under the Shellfish Scheme (http://www.hpa.org.uk/ProductsServices/MicrobiologyPathology/ExternalQualityAssessmentProficiencyTesting/EQAPTForFoodWaterAndEnvironmentalMicrobiology/ShellfishScheme/ (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.

Sample preparation

Sample 1

A single batch of 600 Pacific Oysters (*Crassostrea gigas*) were collected from a UK commercial harvesting area on the 25th November 2019. Prior to packing the shellfish were placed in a large disinfected container and thoroughly mixed. Sample 1 comprised of approximately 24 randomly selected oysters.

Preparation of shellfish homogenate

Approximately 500 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area on the 20th November 2019 and were tested to confirm the absence of *E. coli* and *Salmonella* spp.. On arrival, the oysters were shucked and homogenised before being split into two homogenates.

Sample 2

For Sample 2, 100 ml volumes of homogenate were dispensed into sterile bottles on the 21st November 2019 and stored at 3±2 °C until distribution. Sample 2 was spiked with *E. coli* (≈1.4 x 10^4 cfu/sample) on the day of dispatch.

Sample 3

For Sample 3, 100 ml volumes of homogenate were dispensed into sterile bottles on the 21st November and stored at 3±2 °C. Sample 3 was spiked with *E. coli* (2.7 x 10^3 cfu/sample) and *Salmonella* spp. (*S. typhimurium* at ≈1.8 x 10^2 cfu/sample) on the day of dispatch.

Sample distribution and examination

Each sample was packed in accordance with the Cefas protocol for packaging shellfish for transportation. Samples were despatched at 10:00 on the 25th November 2019 to 15 participating laboratories. Participants were requested to analyse the samples immediately on receipt using their routine methods.

Sample temperature

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

Results

Reference results - *E. coli*

Six randomly selected samples were analysed in duplicate on Tuesday 26th November 2019 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 - Oysters	<18	-	-	-
Sample 2 - Homogenate	2.3 x 10^2 – 2.3 x 10^3	2.3 x 10^2	3.4 x 10^2	4.4 x 10^1 – 1.2 x 10^3
Sample 3 - Homogenate	2.3 x 10^2 – 1.3 x 10^3	3.3 x 10^2	3.5 x 10^2	6.3 x 10^1 – 1.7 x 10^3

GM - geometric mean, SD_T - theoretical standard deviation (0.24 $\log_{10}$)

Reference results – *Salmonella* spp.

Six randomly selected samples were analysed conditions for *Salmonella spp.* using SOP No. 1176 on Tuesday 26th November 2019 under repeatability (Table 2).

Table 2: Reference results

Sample No.		
Sample 1 – Oysters	Absent in 25g	6
Sample 2 - Homogenate	Absent in 25g	6
Sample 3 - Homogenate	Present in 25g	6

Participants' results

Performance assessment was carried out 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 80 are shown in Tables 3, 4, 5, 6 and Figures 1 and 2.

Table 3: Summary statistics of participants' results

Participants reporting duplicate results for *E. coli* MPN	14	12	12
Participants reporting correctly the absence of *E. coli*	14	0	0
Participants reporting both replicate MPN results within expected range[1]	-	5	9
Participants reporting a single MPN result within expected range[1]	-	0	0
Participants reporting one replicate MPN result outside expected range	-	4	1
Participants reporting both replicate MPN results outside expected range	-	3	2
Participants reporting one replicate MPN results as censored results	-	0	0
Participants reporting both replicate MPN results as censored results	-	0	0

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

Table 4: Participants' results

Sample and type	*E. coli* MPN/100g			
	Range	Median	GM	Median±3*SD_T
Sample 1 - Oysters	0 - <67	-	-	-
Sample 2 - Homogenate	$1.6 \times 10^2 – 3.5 \times 10^4$	7.7×10^3	4.9×10^3	$1.4 \times 10^3 – 4.0 \times 10^4$
Sample 3 - Homogenate	$<67 – 7.9 \times 10^3$	2.3×10^3	1.3×10^3	$4.4 \times 10^2 – 1.2 \times 10^4$

GM - geometric mean, SD_T – theoretical standard deviation (0.24)

Table 5: Participants' results and allocated scores for Sample 1, 2 and 3 *E. coli*

Lab ID	Sample 1			Sample 2		Sample 3	
	E. coli MPN/100g			*E. coli* MPN/100g		*E. coli* MPN/100g	
	Rep 1	Rep 2	Score [b]	Rep 1	Rep 2	Rep 1	Rep 2
3	<18	<18	12	24000	24000	4900	4600
10	<18	<18	12	330	330	230	230
41	<18	<18	12	2300	2300	2300	1300
47	0	0	12	24000	35000	2300	2300
48	<18	<18	12	13000	24000	3300	3300
54	<18	<18	12	17000	35000	1300	1700
96 [c]	<18	<18	12	13000	35000	1700	3300
98	NR	NR	0	NR	NR	NR	NR
168	<20	<20	12	NE	NE	NE	NE
189	<18	<18	12	2300	2300	2300	2300
203	<18	<18	12	2300	2300	2300	2300
212 [a]	<67	<67	8	160	170	<67	<67
223	<18	<18	12	35000	35000	7900	1300
235	<20	<20	12	NE	NE	NE	NE
271	<20	<20	12	790	2400	790	330

NE – Not examined.

NR – Not returned.

[a] MPN tube combination is not required for this method, the maximum overall score is reduced to reflect this (8).

[b] Participants undertaking PT to satisfy the requirement of EU Food and Feed regulations should follow stipulated reference methods. An assessment of methods used in this PT have not been included when scoring MPN values.

Table 6: Participants' results and allocated scores for Sample 1, 2 and 3 *Salmonella spp.*

Lab ID	Sample 1		Sample 2		Sample 3	
	Sal. spp. in 25g		*Sal.* spp. in 25g		*Sal.* spp. in 25g	
	Rep 1	Score	Rep 1	Score	Rep 1	Score
3	Not Detected	2	Not Detected	2	Detected	2
10	Not Detected	2	Not Detected	2	Detected	2
41	Not Detected	2	Not Detected	2	Detected	2
47	Not Detected	2	Not Detected	2	Detected	2
48	Not Detected	2	Not Detected	2	Detected	2
54	Not Detected	2	Not Detected	2	Detected	2
96	Not Detected	2	Not Detected	2	Detected	2
98	NR	0	NR	0	NR	0
168	Not Detected	2	NE	-	NE	-
189	Not Detected	2	Not Detected	2	Detected	2
203	Not Detected	2	Not Detected	2	Detected	2
212	NE	-	NE	-	NE	-
223	Not Detected	2	Not Detected	2	Detected	2
235	Not Detected	2	NE	-	Detected	2
271	Not Detected	2	Not Detected	2	Detected	2

NE – Not examined.

NR – Not returned.

General comments

Fifteen laboratories were sent material, 14 laboratories returned results. Information provided by laboratories on arrival times showed 12 (86%) laboratories received the material the day after dispatch (27th November 2019), with 4 (33%) laboratories analysing the material immediately on arrival. Two laboratories received material

within 48 hours of dispatch. Arrival temperatures were recorded in the range of 0 – 8°C. All temperature data, arrival and analysis dates and times recorded by participants are shown in Appendix I.

Sample analyses

Fourteen laboratories returned a completed report form within the specified timeframe. Laboratory 98 did not return the report form or provide a reason why the samples were not examined, a final score of 0 was given for all samples. Laboratory 168 did not analyse sample 2 and 3 as the containers were damaged in transit. Laboratory 235 gave no reason why sample 2 and 3 were not examined. Laboratory 212 does not routinely examine bivalve shellfish samples for Salmonella and therefore did not analyse the samples for *Salmonella* spp..

Sample 1 – Oysters

***E. coli* –** Fourteen laboratories returned duplicate *E. coli* MPN/100g and reported the absence of *E. coli* in the sample with all obtaining full marks.

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

Additional statistical analyses of participants result for *E. coli* for Sample 2 and 3 only

The organisers observed a range of MPN values amongst participants returned results (See Table 4) in excess of 2 $\log_{10}$. A review of historic datasets from PT distributions from 2008 to 2019 demonstrated that this was within a normal range. However, both reference results generated by the organising laboratory and several participants results were approximately 2 $\log_{10}$ lower than expected for sample 2 and 1 $\log_{10}$ lower for sample 3 (spiking level was estimated at $\approx 1.4 \times 10^4$ ml^{-1} for sample 2 and $\approx 2.7 \times 10^3$ ml^{-1} for sample 3), so despite demonstrating sufficient homogeneity (ISO 22117), this anomaly triggered an investigation at the organising laboratory (see troubleshooting guide). The investigation did not identify clear causal factors, although suggested a number of avenues for further investigation, including the effect of refrigerated storage of homogenates prior to spiking. Therefore, the organisers made the decision not to score participants for *E. coli* for samples 2 and 3. The potential for storage to impact *E. coli* results will be considered further within the work programme of the FAO Reference Centre.

Sample 2 – Homogenate

***E. coli* –** Five laboratories (3, 41, 48, 189 and 203) returned duplicate *E. coli* MPN/100g results falling between ±3 SD of the participants' median (Figure 3). Laboratories 47, 54 96 and 271 reported 1 replicate result between ±3 SD of the participants' median and the second replicate result between ±3 SD and ±5 SD of the participants' median. Laboratories 10 and 212 returned both replicate results outside ±5 SD of the participants' median.

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

Sample 3 – Homogenate

***E. coli* –** Nine laboratories (3, 41, 47, 48, 54, 96, 189, 203 and 223) returned duplicate *E. coli* MPN/100g results falling between ±3 SD of the participants' median (Figure 4). Laboratory 271 reported 1 replicate result between ±3 SD of the participants' median and the second replicate result between ±3 SD and ±5 SD of the participants' median. Laboratory 10 reported both between ±3 SD and ±5 SD of the participants' median. Laboratory 212 returned both replicate results outside ±5 SD of the participants' median.

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

References

Anon 2001. 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˚C using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

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: Results chart Sample 2 – Shellfish homogenate

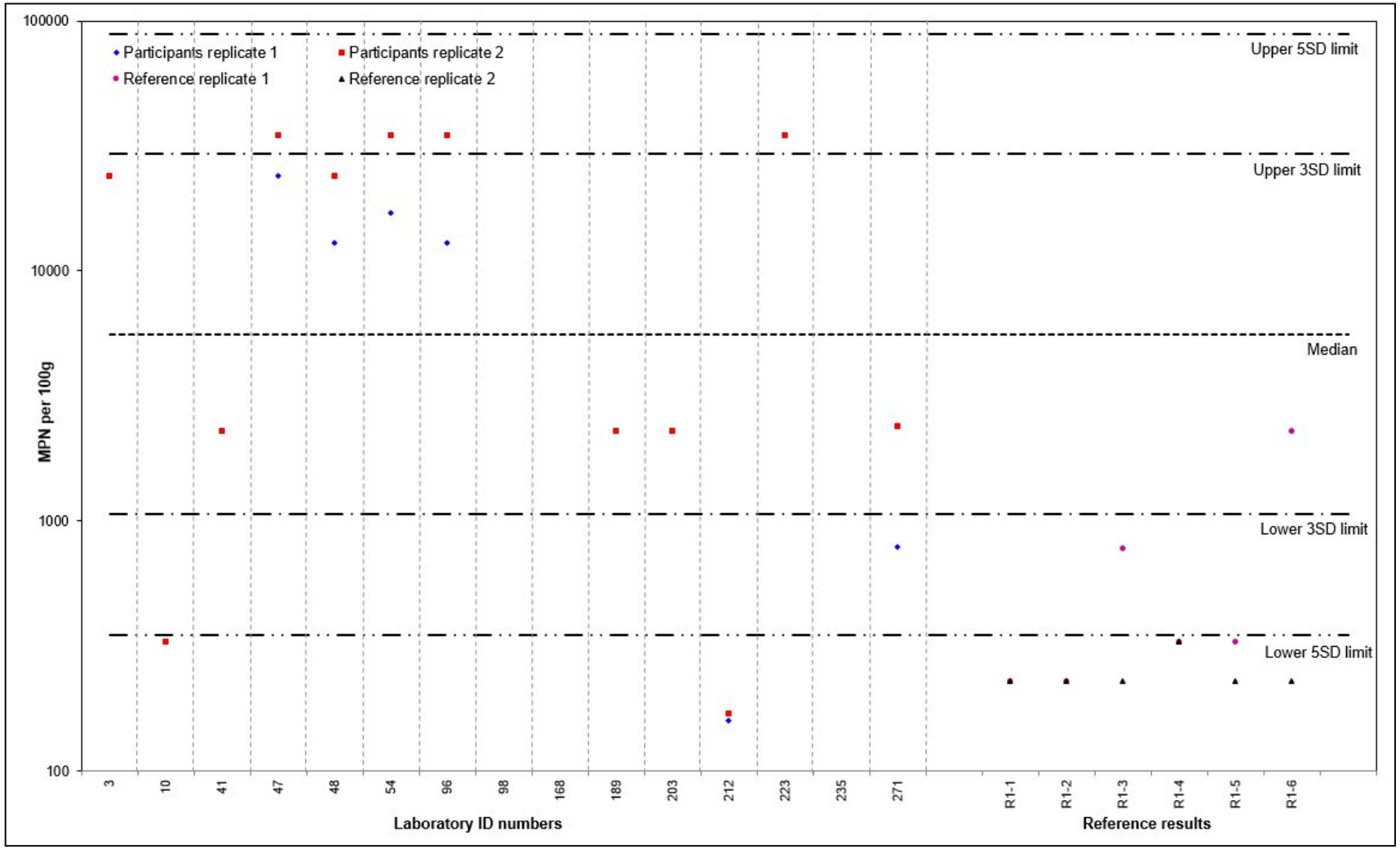
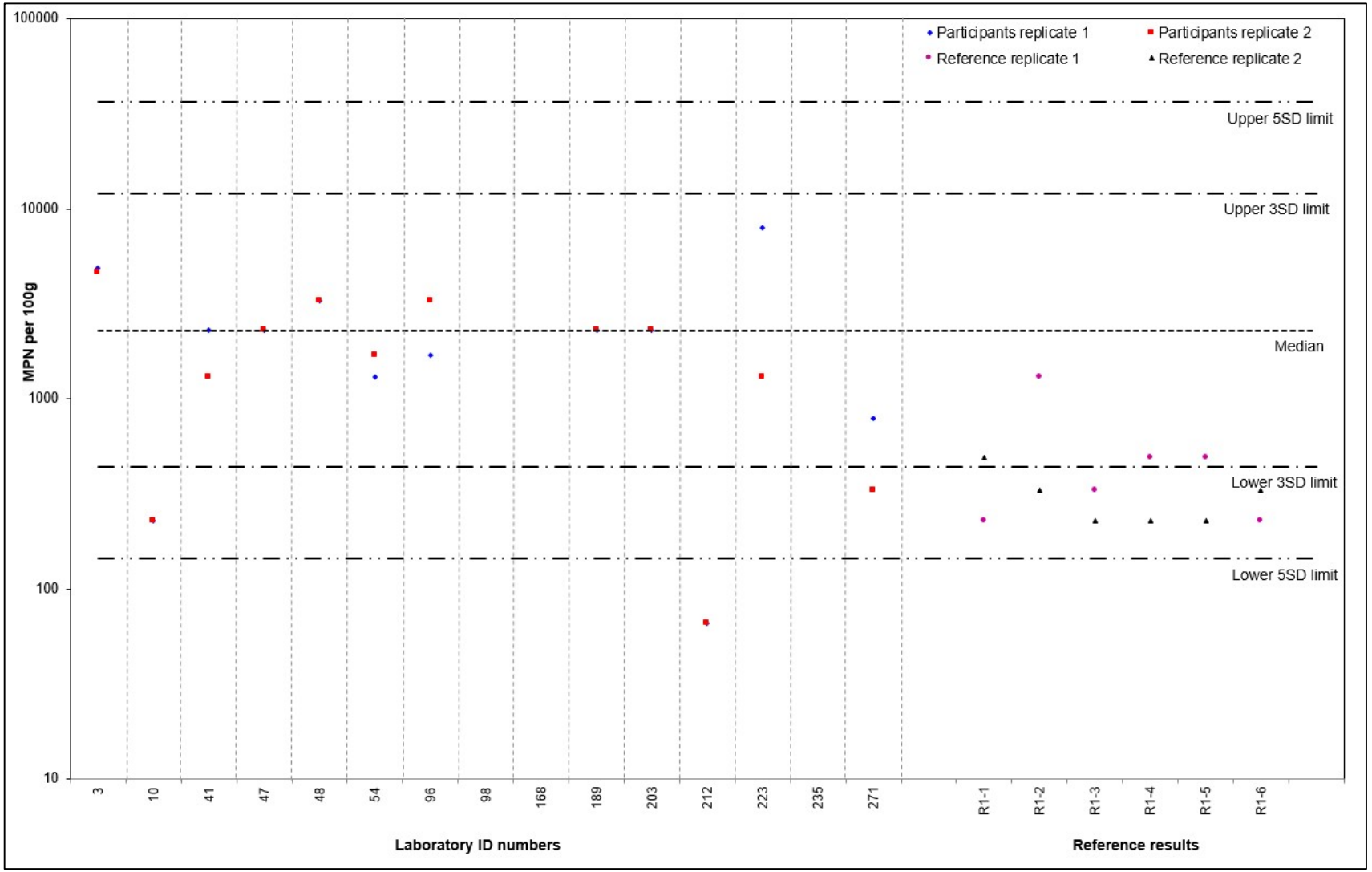


Figure 2: Results chart Sample 3 – Shellfish homogenate



Appendix I

Sample arrival and temperature

Lab ID	Date arrived	Time of arrival	Sample (°C)	Storage (°C)	Date analysed
3	26/11/2019	16:30	3.2	4.3	27/11/2019
10	26/11/2019	10:00	1.2	-	26/11/2019
41	26/11/2019	11:35	5.8	3.0	26/11/2019
47	26/11/2019	10:00	2.6	-	26/11/2019
48	26/11/2019	15:00	4.3	4.0	27/11/2019
54	27/11/2019	10:35	6	-	27/11/2019
96	26/11/2019	12:15	3.1	4.0	27/11/2019
98	NR	NR	NR	NR	NR
168	28/11/2019	11:00	8	3 ± 2	
189	26/11/2019	13:00	Freeze	+5	27/11/2019
203	26/11/2019	14:00	4.8	3 ±2	27/11/2019
212	26/11/2019	-	-	-	26/11/2019
223	26/11/2019	14:00	2.8	3-5	27/11/2019
235	26/11/2019	11:00	-	4.0	26/11/2019
271	26/11/2019	13:45	3.5	2.0	27/11/2019

NR – Not returned

Appendix II:

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

Result	Returning of results	Score allocated		Total score
		Replicate 1	Replicate 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

***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

***E. coli* score deductions**

Result	Score deducted	
	Replicate 1	Replicate 2
High censored result (e.g. MPN = >18000 per 100g).	2	2
Sample not examined or results returned late - no explanation received.	12	

***Salmonella* spp. scoring**

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

Appendix III:

Troubleshooting 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

    Where three dilutions have been tested for a sample, record the number of TBGA/TBX positives for each dilution to give a three figure tube combination number. Use the MPN tables included in ISO 7218 and the EURL 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 and that you have procedures for ensuring that 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 controls (IQC)** – Ensure adequate controls are in place and follow-up procedures are in place to deal with IQC failures.

Further advice can be obtained from the EURL on request.

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