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Centre for Environment Fisheries & Aquaculture Science

FAO Reference Centre for Bivalve Mollusc Sanitation

Third proficiency testing distribution for the detection of *Escherichia coli* in shellfish and the detection of faecal coliforms in water (PT 98)

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V7	L. Stockley	26th March 2025	Updated to included missing results

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1. Introduction

In 2019, Cefas (the Centre for Environment, Fisheries and Aquaculture Science) was designated as the Food and Agriculture Organization (FAO) Reference Centre for Bivalve Mollusc Sanitation. The aim of the FAO Reference Centre is to provide support to FAO member countries in the development of bivalve shellfish production.

Part of the FAO Reference Centre work programme is to organise a proficiency testing (PT) distribution to help support existing or new bivalve programmes and help the capability of laboratories to test for indicators of faecal contamination which may be used wherever bivalves are commercially produced and traded.

Proficiency testing (PT), also known as comparative testing, involves multiple laboratories testing identical samples and comparing results. The results of PT can help demonstrate good performance, assist in the implementation of new methods, support laboratory quality accreditations, identify opportunities for continuous improvement and help build supportive networks of laboratories with similar goals, for example the development of networks of laboratories in countries with an interest in growing safe bivalve mollusc programmes.

2. Samples

The third PT distribution comprised Lenticule™ discs originating from the UK Health Security Agency (UK HSA). The samples contained known levels of fully characterised *Escherichia coli* NCTC 9001 (*E. coli*) (supplied as certified reference material).

2.1. *E. coli* in shellfish

Participants were requested to examine the samples using their laboratory's in house method for the enumeration of *E. coli* and/or use the FAO Reference Centre generic protocol based [Generic Protocols] upon 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.

ISO 16649-3 is an internationally recognised method for the enumeration of *E. coli* in bivalve shellfish and is the stipulated European Union reference method. The level of *E. coli* in the sample should be reported in 100g of flesh.

Note: These samples are designed for laboratories testing raw bivalve molluscs from harvesting beds for classification or end product testing.

2.2. Faecal coliforms in water

Participants were requested to examine the samples using their laboratory's in house method for enumeration of faecal coliforms (FC) and/or the FAO Reference Centre generic protocol [Generic Protocols] based upon the approach set out in US FDA BAM Chapter 4 water in provided by the reference centre.

Note: These samples are designed for laboratories wishing to test water from bivalve mollusc growing areas.

2.3. Distribution

Samples were packaged according to IATA regulations, UN3373 as diagnostic specimens, division 6.2 under the packing instruction code 650 and distributed using the courier DG Global Forwarding on the 11th March 2024 to 14 participants. Relevant transport documentation, examination request forms and instructions on handling and sample reconstitution accompanied the samples. Laboratories were asked to test the *E. coli* samples in duplicate and obtain a single result for faecal coliforms, returning results on completion. Those participants that returned results have been included in this report.

2.4. Quality Control

The samples used for this PT distribution were certified according to ISO/IEC 17025 and produced under reproducible conditions compliant with ISO 17034 by the supplier (UK HSA).

2.4.1. *E. coli* in shellfish

The method used to obtain the reference results was the FAO generic protocol based on ISO 16649-3 (Anon, 2015). Three randomly selected Lenticules were examined in duplicate

under repeatability conditions by the FAO reference centre. The reference results are given in Figures 1 and 2, and Tables 1 and 3.

2.4.2. Faecal coliforms in water

The method used to obtain the reference results was the FAO generic protocol based on the approach set out in US FDA BAM Chapter 4 (Anon, 2020). Six randomly selected Lenticules were examined under repeatability conditions by the FAO reference centre. The reference results are given in Figures 3 and 4, and Tables 5 and 7.

3. Analysis of participants results

3.1. *E. coli* in shellfish - Sample 1 and 2

Each participant's *E. coli* Most Probable Number (MPN) value (MPN/100g) reported were compared against the median of all participants' results, reference results were omitted from the calculation. The median is used rather than the mean as it is less affected by outlying results. The acceptability limits for participants were calculated as the participants' median ±2.68 theoretical standard deviations (SD_T) and ±4 SD_T following the recommended approach described by the UKHSA for PT performance assessments of MPN tests and Lenticule discs (FEPTU562.13.pdf (publishing.service.gov.uk). Reported MPN values were log_{10} transformed before being compiled into charts as shown in Figures 1 and 2.

Note: The median and upper and lower limits (±2.68SD_T and ±4SD_T) were calculated from participants' results. The value for SD_T used in these calculations is based on the inherent variability of a 5 x 3 MPN method (0.24 log_{10}). Reference values were excluded from the calculation of the participants' median.

Note: Values reported as >16,000 MPN/100g and zero were assigned a value of 16,001 and 17 respectively for statical purposes.

3.1.1. *E. coli* in shellfish - Sample 1

- **Sample contents**

Escherichia coli (certified reference material) 5.0 x 10^2 – 5.0 x 10^4 per Lenticule™ disc.

- **Sample results**

Table 1: Participants' and reference results median, median ±2.68 and ±4 SD_T

Results	Range	Median	GM	Median ±2.68*SD_T	Median ±4*SD_T
Participants'	0 – 9.2 x 10^4	1.3 x 10^4	2.9 x 10^3	3.0 x 10^3 – 5.7 x 10^4	1.4 x 10^3 – 1.2 x 10^5
Reference	4.9 x 10^3 – 1.3 x 10^4	7.9 x 10^3	7.8 x 10^3	-	-

Results are in *E. coli* per 100g. GM - geometric mean, SD_T - theoretical standard deviation (0.24 log_{10})

Table 2: Participants' results returned

Lab ID.	*E. coli* (per 100g)	
	Replicate 1	Replicate 2
40 [a]	0	
60	7,900	13,000
118	92,000	54,000
136	700	700
195	24,000	13,000
276	3,300	2,300
290	17,000	22,000
299	13,000	13,000
307 [a]	>16,000	>16,000
325	13,000	11,000
530 – M [b]	33	33
530 – S [b]	40	48

Key:

a; results reported as zero and >16,000, treated as 17 and 16,001 respectively for statistical analysis.

b; laboratory 530 participated using 2 different methods.

3.1.2. *E. coli* in shellfish - Sample 2

- **Sample contents**

Escherichia coli (certified reference material) 5.0 x 10^2 – 5.0 x 10^4 per Lenticule™ disc.

- **Sample results**

Table 3: Participants' and reference results median, median ±2.68 and ±4 SD_T

Results	Range	Median	GM	Median ±2.68*SD_T	Median ±4*SD_T
Participants'	4.6 x 10^2 – 2.4 x 10^4	7.0 x 10^3	3.7 x 10^3	1.6 x 10^3 – 3.1 x 10^4	7.7 x 10^2 – 6.4 x 10^4
Reference	2.3 x 10^3 – 1.3 x 10^4	6.4 x 10^3	6.5 x 10^3	-	-

Results are in *E. coli* per 100g. GM - geometric mean, SD_T - theoretical standard deviation (0.24 log_{10})

Table 4: Participants' results reported

Lab ID.	*E. coli* (per 100g)	
	Replicate 1	Replicate 2
40 [a]	0	
60	4,900	13,000
118	3,300	24,000
136	3,500	3,500
195	7,900	7,000
276	11,000	7,900
290	7,000	7,900
299	4,900	4,900
307 [a]	>16,000	>16,000
325	7,900	7,900
530 – M [b]	510	510
530 – S [b]	760	460

Key:

a; results reported as zero and >16,000, treated as 17 and 16,001 respectively for statistical analysis.

b; laboratory 530 participated using 2 different methods.

Figure 1: *E. coli* in shellfish - Sample 1

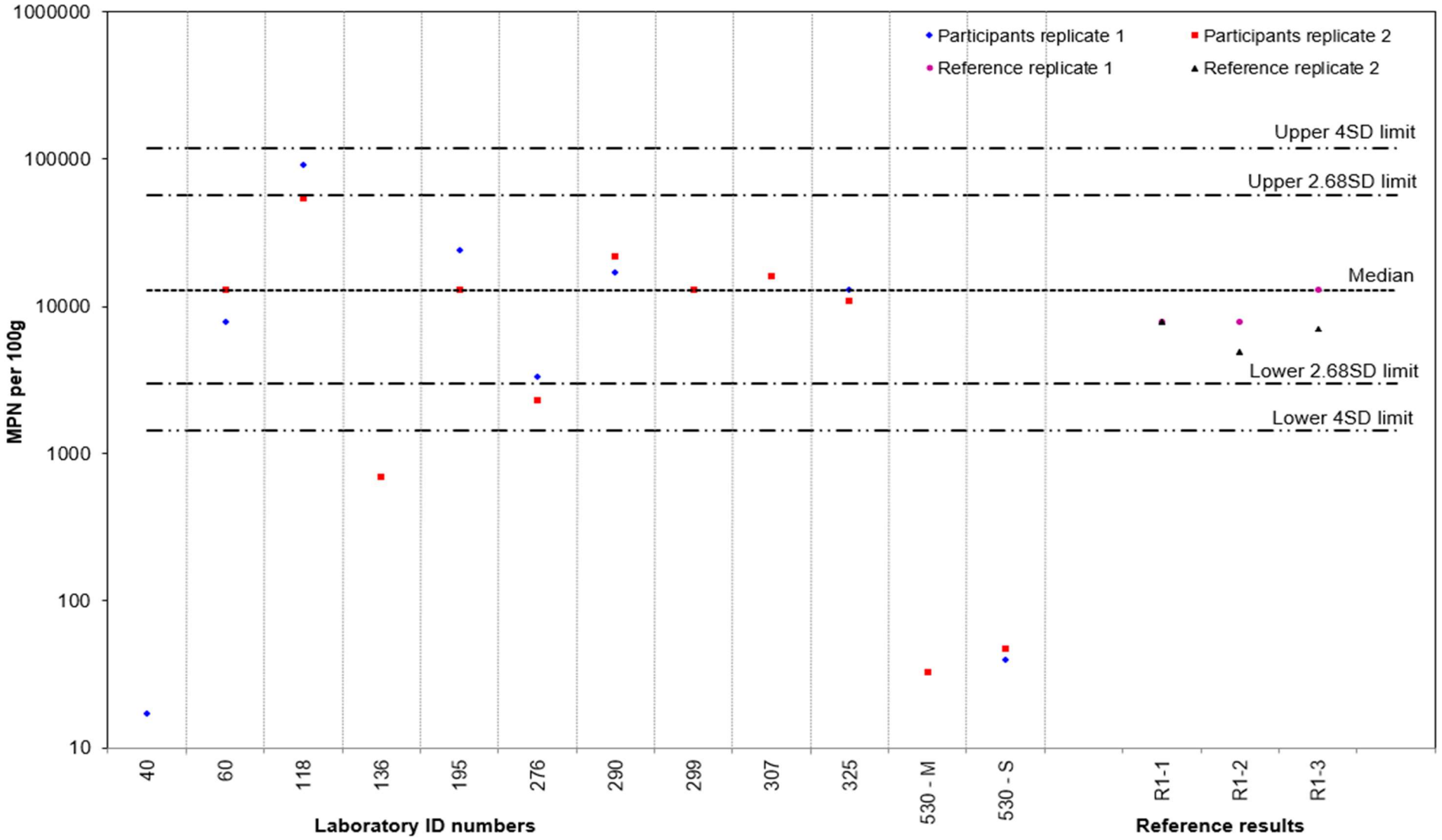
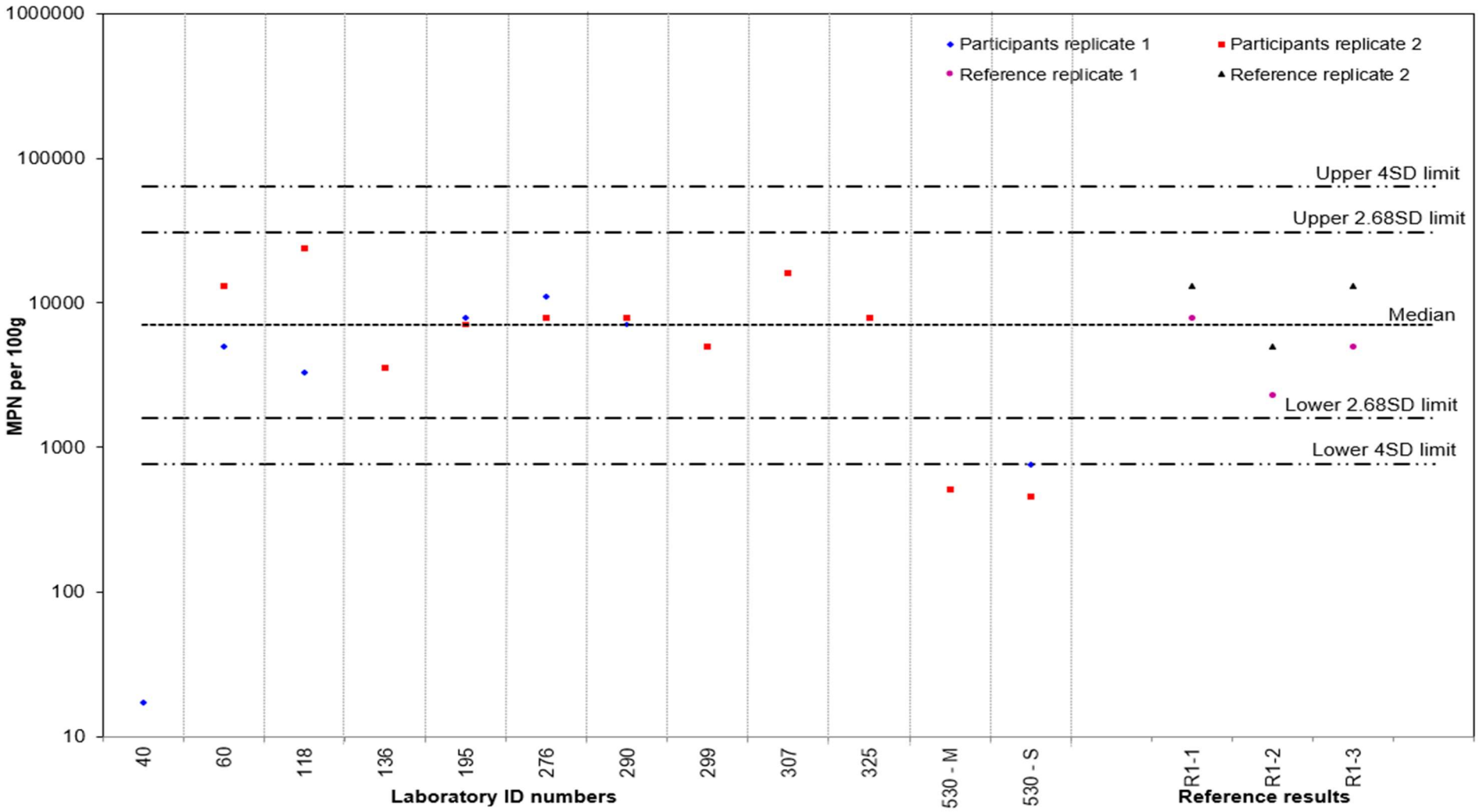


Figure 2: *E. coli* in shellfish - Sample 2



3.2. Faecal coliforms in water – Samples 1 and 2

Each participant's reported results for faecal coliforms were compared against the median of all participants' results, reference results were omitted from the calculation. The median is used rather than the mean as it is less affected by outlying results. The acceptable limits were calculated as the participants' median ±2.68 SD_T and ±4SD_T above and below the participants' median for the same sample. The acceptability limits for participants were calculated as the participants' median ±2.68 SD_T and ±4 SD_T following the recommended approach described by the United Kingdom Health Protection Agency for PT performance assessments of MPN tests and Lenticule™ discs (FEPTU562.13.pdf (publishing.service.gov.uk). Reported results were log_{10} transformed before being compiled into charts as shown in Figures 3 and 4.

Note: The median and upper and lower limits (±2.68SD_T and ±4SD_T) were calculated from participants' results. The value for SD_T used in these calculations is based on the inherent variability of a 5 x 3 MPN method (0.24 log_{10}). Reference values were excluded from the calculation of the participants' median.

Note: Values reported as zero were assigned a value of 0.5 for statistical purposes.

3.2.1. Faecal coliforms in water – Sample 1

- **Sample content**

Escherichia coli (certified reference material) 3.0 x 10^1 – 1.2 x 10^2 per Lenticule™ disc.

- **Sample results**

Table 5: Participants' and reference results median, median ±2.68 and ±4 SD_T

Results	Range	Median	GM	Median ±2.68*SD_T	Median ±4*SD_T
Participants'	0 – 4.5 x 10^2	1.4 x 10^1	8.0 x 10^0	3.2 x 10^0 – 6.2 x 10^1	1.5 x 10^0 – 1.3 x 10^2
Reference	2.3 x 10^1 – 1.3 x 10^2	7.9 x 10^1	6.8 x 10^1	-	-

Results are in faecal coliforms per 100ml. GM - geometric mean, SD_T - theoretical standard deviation (0.24 log_{10})

Table 6: Participants' results reported

Lab ID	Faecal coliforms per 100ml	
	Replicate 1	Replicate 2
40	0	
60 - F [a]	20	
60 - M [a]	17	
118	450	
136	92	
195	0	
276 [b]	6.8	4
299	14	
307	1	
325 - F [a]	21	
325 - M [a]	22	
530	0	

Key

a; laboratory 60 and 325 participated using 2 different methods.

b; laboratory 276 reported duplicate results.

3.2.2. Faecal coliforms in water – Sample 2

- **Sample content**

Escherichia coli (certified reference material) $3.0 \times 10^1 – 1.2 \times 10^2$ per Lenticule™ disc.

- **Sample results**

Table 7: Participants' and reference results median, median ±2.68 and ±4 SD_T

Results	Range	Median	GM	Median ±2.68*SD_T	Median ±4*SD_T
Participants'	$0 – 4.0 \times 10^2$	9.7×10^0	7.2×10^0	$2.2 \times 10^0 – 4.2 \times 10^1$	$1.1 \times 10^0 – 8.8 \times 10^1$
Reference	$2.3 \times 10^1 – 1.3 \times 10^2$	7.9×10^1	6.8×10^1	-	-

Results are in faecal coliforms per 100ml. GM - geometric mean, SD_T - theoretical standard deviation ($0.24 \log_{10}$)

Table 8: Participants' results reported

Lab ID	Faecal coliforms per 100ml
40	0
60 - F [a]	40
60 - M [a]	22
118	0
136	24
195	11
276	4
299	21
307	7
325 - F [a]	10
325 - M [a]	9.3
530	6.3

Key

a; laboratory 60 and 325 participated using 2 different methods.

Figure 3: Faecal coliforms in water - Sample 1

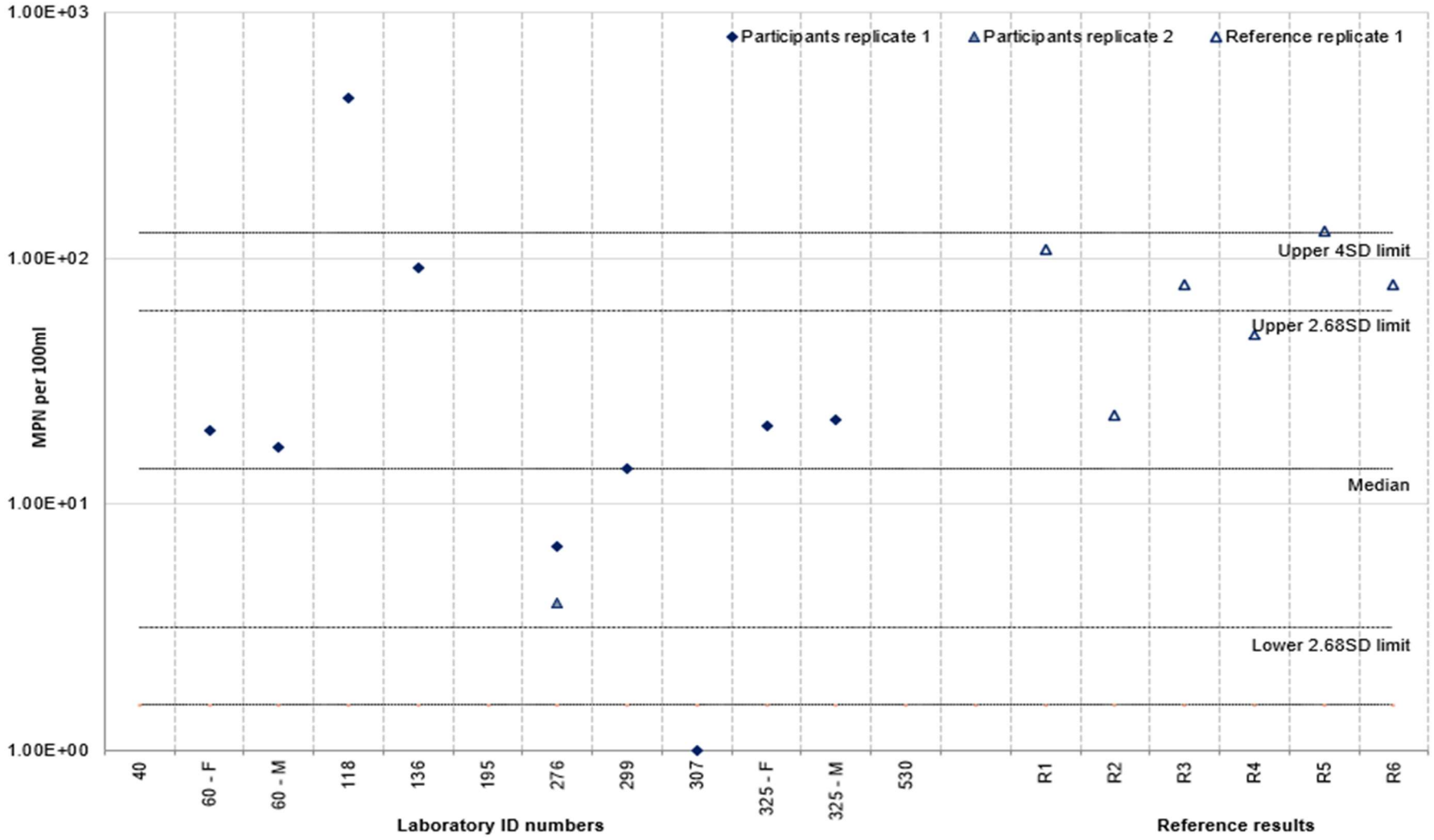
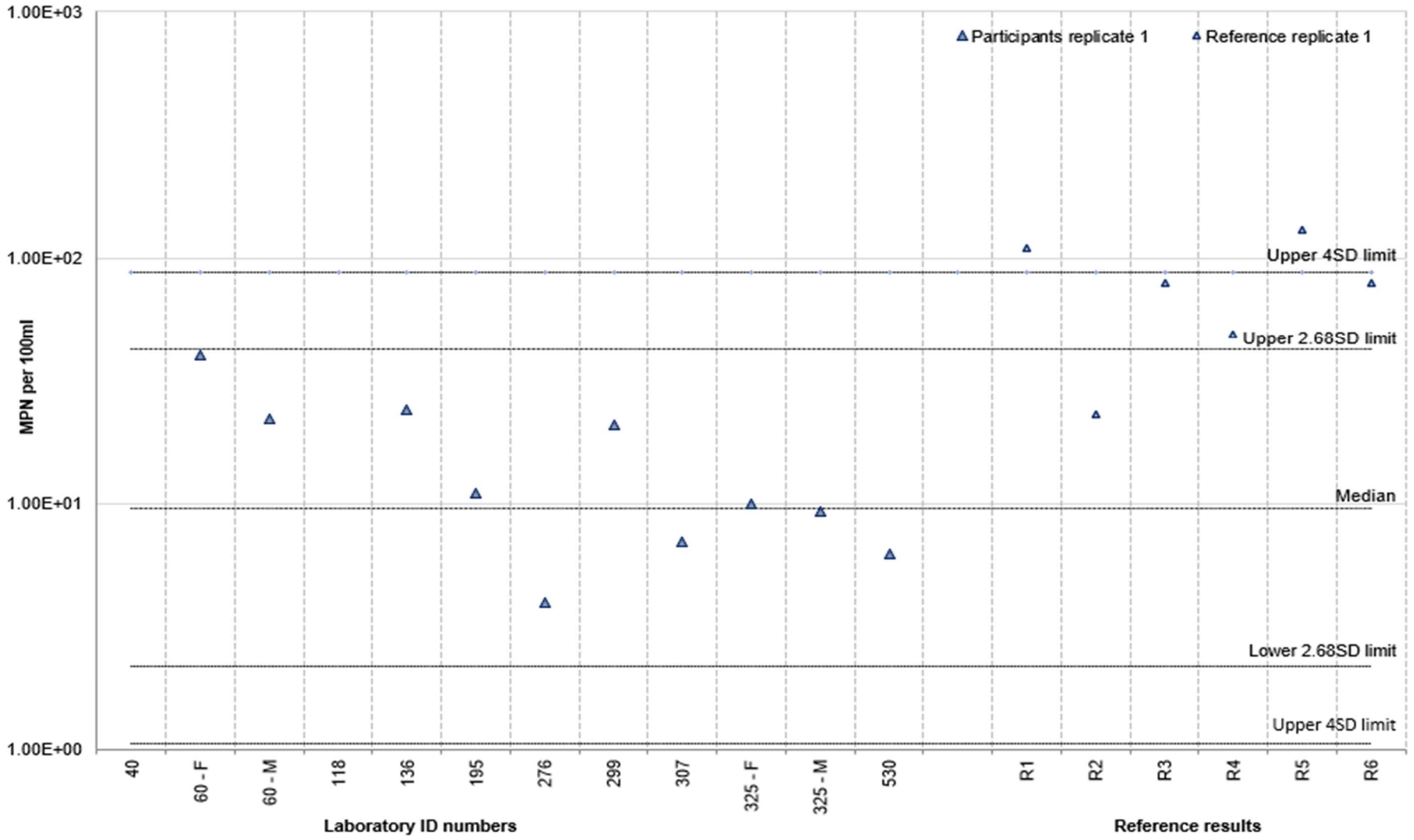


Figure 4: Faecal coliforms in water - Sample 2



4. Analysis of results

4.1. General comments

Fourteen laboratories were sent material, with 11 laboratories returning results (11 returned results for *E. coli* in shellfish, 10 returned results for faecal coliforms in water). The methods used to test the samples are shown in Tables 9 and 10 with the number of laboratories citing each method. Due to a capacity issue, one laboratory was unable to perform the MPN method according to the FAO generic protocols.

Table 9: Method used for the enumeration of *E. coli* in shellfish

Method reference	No. of Labs
ISO 16649 - 3 (MPN) (FAO Reference Centre Generic protocol)	7
APHA (1992). Compendium of Methods for the Microbiological Foods. 2nd Edition, American Public Health Association, Washington, DC. (MPN - 4 dilutions, 3 tubes)	1
NCh3056:2007	1
MPN and Simplate	1

Table 10: Method used for the enumeration of faecal coliforms in water

Method reference	No. of labs
Determination of faecal coliform bacteria in seawater by the most probable number (MPN) technique (Based on Bacteriological Analytical Manual – BAM) (FAO Reference Centre Generic Protocol)	5
ISO 9308-1	2
COLILERT-18 Test Kit (MPN Method)	1
Standard Methods for the examination of water and wastewater 23rd Edition 2017, Capitulo 9221 E.1	1
Membrane filtration; SANS 5221, 4.5:2018	1
Membrane filtration methods (mFC, Sartorius PADS)	1

4.2. Sample analyses

Eleven laboratories returned results for the third FAORC Lenticule^{TM} PT distribution.

Note: For those laboratories experiencing problems please contact us (FAO Reference Centre) for assistance.

4.2.1. *E. coli* in shellfish

Ten laboratories reported duplicate results for both samples. Laboratory 40 reported a single result for both samples. Laboratories 118, 136 and 530 appeared to report incorrect MPN values for the tube combinations reported, comparing with the expected MPN values for those combinations as detailed in the FAO Reference Centre generic protocol. Laboratory 530 reported replicate results for both samples using 2 separate methods.

4.2.1.1. Sample 1

Six laboratories returned duplicate *E. coli* MPN/100g results falling within ±2.68 SD_T of the participants' median (Figure 1). Laboratories 118 and 276 each reported one replicate within ±2.68 SD_T of the participants' median and one replicate >2.68 SD_T but <4 SD_T different from the participants' median, Laboratories 40, 136 and 530 (for both methods used) reported all replicates outside ±4 SD_T of the participants' median.

4.2.1.2. Sample 2

Nine laboratories returned duplicate *E. coli* MPN/100g results falling within ±2.68 SD_T of the participants' median (Figure 2). Laboratories 40 and 530 (for both methods used) reported results outside ±4 SD_T of the participants' median.

4.2.2. Faecal coliforms in water

Ten laboratories reported results for both samples. Laboratory 60 and 325 reported results for both samples using 2 separate methods. Laboratory 276 reported duplicate results for one sample.

4.2.2.1. Sample 1

Four laboratories returned results (faecal coliforms per 100ml) that fell between ±2.68 SD_T of the participants' median (Figure 3). Laboratory 136 reported a result >2.68 SD_T but <4

SD_T different from the participants' median and laboratories 118 and 307 reported results that fell outside ±4 SD_T of the participants' median. Laboratories 40, 195 and 530 did not detect faecal coliforms in this sample.

4.2.2.2. Sample 2

Eight laboratories returned results (faecal coliforms per 100ml) that fell between ±2.68 SD_T of the participants' median (Figure 4). Laboratories 40 and 118 did not detect faecal coliforms in this sample.

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 2020 - US FDA BAM, Chapter 4 Determination of faecal coliform bacteria in seawater by the most probable number (MPN) technique

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