Food and Agriculture Organization of the United Nations

Cefas INTERNATIONAL CENTRES OF EXCELLENCE

Centre for Environment Fisheries & Aquaculture Science

FAO Reference Centre for Bivalve Mollusc Sanitation

Second Proficiency Testing distribution for the detection of *Escherichia coli* in shellfish and the detection of Faecal Coliforms in water (PT 93)

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Cefas Document Control

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Contents

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

This second PT distribution comprised of Lenticule ™ discs. The samples originated from the UK Health Security Agency (UK HSA) External Quality Assessment (EQA) scheme and contained a mixture of fully characterised bacterial isolates. The proportions and types of organisms in the reconstituted samples are designed to mirror those that may be found in real bivalve molluscs or bathing beach (marine) waters, bathing pool waters and river, lake, or stream waters.

2.1. *E. coli* in shellfish

Participants were requested to examine the samples using their laboratory's in house method for the enumeration of *Escherichia coli (E. coli*) and/or use the FAO Reference Centre generic protocol [Generic Protocols] based 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 (2015).

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 for water.

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 12th September 2022 to 21 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

Sample quality control (homogeneity and stability testing) for all samples was assessed following procedures described in ISO 22117 by the supplier (UK HSA). The sample material distributed was considered sufficiently homogenous.

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).

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.

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

Twenty laboratories were sent a set of lenticules to analyse for *E. coli* with 13 returning results. Each participant's *E. coli* Most Probable Number (MPN) value (MPN/100g) reported was compared against the calculated median MPN from all participants' results, with reference results being omitted from the calculation. The expected range was calculated using the participants' median ±2.68 theoretical standard deviations (SD_T) (satisfactory values). Upper and lower limits (±4 SD_T above and below the participants' median) were also calculated for the same sample. Reported MPN values were log_{10} transformed before being compiled into charts as shown in Figures 1 and 2.

Following the assessment of participants' results, the FAO reference centre recommends those participants reporting outlying MPN results (falling outside ±4 SD_T of the participants' medium) carry out an investigation into the possible cause.

Note: The median is used instead of the mean as it is affected less by outlying results. The median and upper and lower limits (±2.68 SD_T and ±4 SD_T) were calculated from participants' results. The value for SD_T used in these calculations is based on the inherent variability of the 5 x 3 MPN method (0.26 log_{10}). Reference values were excluded from the calculation of the participants' median.

Note: Values reported as >16,000 MPN/100g and <18 or zero MPN/100g were assigned a value of 16001 and 17 respectively for calculations and displaying on the graphs.

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

- **Sample contents**

Escherichia coli ($2.3x10^2 – 7.8x10^2$) (wild strain), *Salmonella* Indiana 1,4,12: z:1,7 (32 per disc) (wild strain), *Serratia fonticola* ($4.5x10^4$) (wild strain)

- **Sample results**

Reference results were obtained by testing six randomly selected Lenticules under repeatability conditions and are included in Table 1 and Figure 1.

Table 1: Participants' and reference results median, median ±2.68$_T$ and ±4 SD$_T$

Results	Range	Median	GM	Median ±2.68*SD$_T$	Median ±4*SD$_T$
Participants'	$0 – 2.2 \times 10^3$	2.3×10^2	1.4×10^2	$4.6 \times 10^1 – 1.1 \times 10^3$	$2.1 \times 10^1 – 2.5 \times 10^3$
Reference	$2.3 \times 10^2 – 7.8 \times 10^2$	3.4×10^2	3.9×10^2	$6.8 \times 10^1 – 1.7 \times 10^3$	$3.1 \times 10^1 – 3.7 \times 10^3$

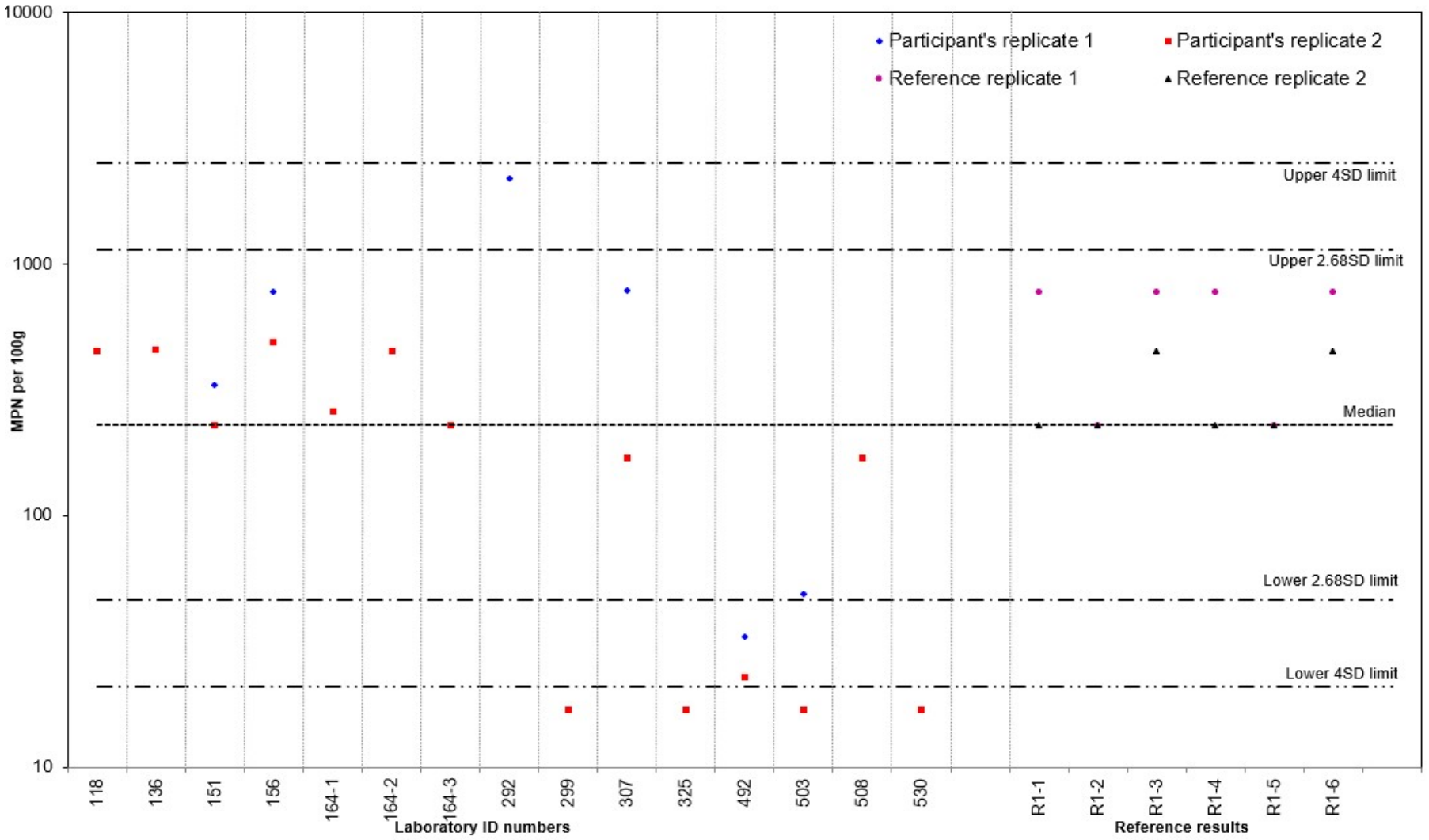
GM - geometric mean, SD$_T$ - theoretical standard deviation (0.26 $\log_{10}$)

Table 2: Participants' results

Lab ID.	*E. coli* (per 100g)	
	Replicate 1	Replicate 2
118	450	450
136	460	460
151	330	230
156	780	490
164-1	260	260
164-2	450	450
164-3	230	230
292	2200	NR
299	0	0
307	790	170
325	0	0
492	33	23
503	49	17
508	170	170
530	0	0

NR – Not reported

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



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

- **Sample contents**

Escherichia coli ($1.7x10^4$ - $5.4x10^4$) (wild strain), *Bacillus circulans* ($3.0x10^3$) (wild strain), *Klebsiella oxytoca* ($5.0x10^4$) (wild strain), *Providencia rettgeri* ($5.0x10^3$) (wild strain)

- **Sample results**

Reference results were obtained by testing six randomly selected Lenticules under repeatability conditions and are included in Table 3 and Figure 2.

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

Results	Range	Median	GM	Median ±2.68*SD_T	Median ±4*SD_T
Participants'	$5.4 \times 10^1 – 3.3 \times 10^5$	7.0×10^3	3.7×10^3	$1.4 \times 10^3 – 3.5 \times 10^4$	$6.4 \times 10^2 – 6.7 \times 10^4$
Reference	$1.7 \times 10^4 – 5.4 \times 10^4$	$2.9x 10^4$	3.2×10^4	$5.9 \times 10^3 – 1.5 \times 10^5$	$2.7 \times 10^3 – 3.2 \times 10^5$

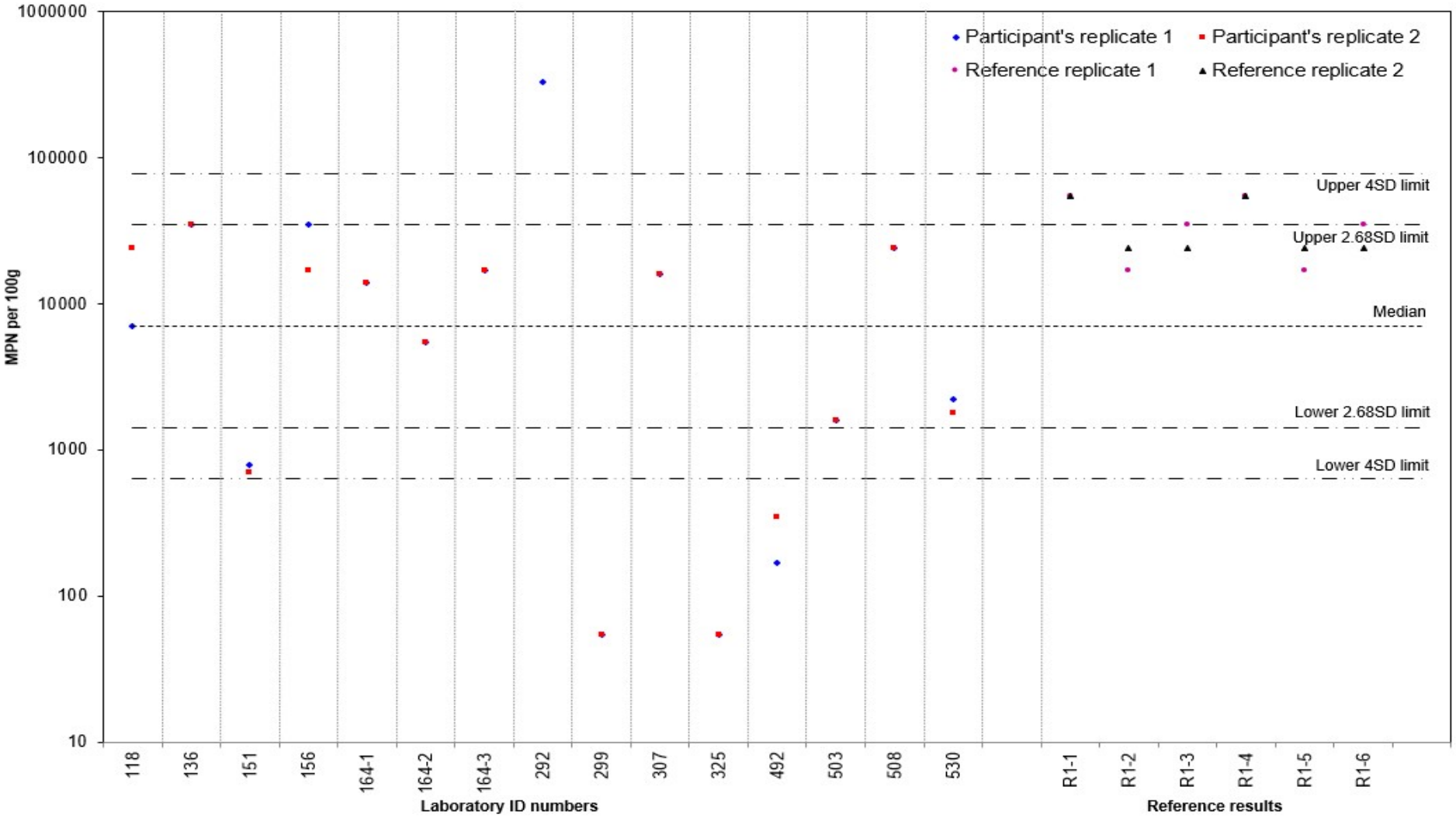
GM - geometric mean, SD_T - theoretical standard deviation (0.26 log_{10})

Table 4: Participants results reported

Lab ID.	*E. coli* (per 100g)	
	Replicate 1	Replicate 2 *
118	7000	24000
136	35000	35000
151	790	700
156	35000	17000
164-1	14000	14000
164-2	5400	5400
164-3	17000	17000
292	330000	NR
299	54	54
307	>16000	>16000
325	54	54
492	170	350
503	1600	1600
508	24000	24000
530	2200	1800

NR – Not reported

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



2.6. Faecal coliforms in water – Samples 1 and 2

Twenty laboratories were sent a set of lenticules to analyse for FC with 13 returning results. From the results received a large portion were record as zero for both samples. Due to these low numbers, statistical analysis was not possible.

Following assessment of the participants results, the FAO reference centre recommends those participants reporting values below the levels of *E. coli* within each sample should carry out an investigation to identify the possible cause.

2.6.1. Faecal coliforms in water – Sample 1

- **Sample content**

Enterococcus gallinarum (59) (NCTC 11428), *Salmonella* Senftenberg 1,3,19:g,[s],t: ($1.76x10^2$) (wild strain), *Pseudomonas aeruginosa* (38) (NCTC 12951).

- **Sample results**

Reference results were obtained by testing six randomly selected Lenticules under repeatability conditions and are included in Table 5.

Table 5: Participants' and reference ranges

Results	Range (cfu/100ml)
Participants'	0 – 13
Reference	0

Table 6: Participants' results

Lab ID	CFU / 100ml	
	Replicate 1	Replicate 2 *
118	0	
136	0	
151	0	0
164-1	0	
164-2	0	
164-3	0	
292	<1.8	
295	13	
299	0	
307 **	1	0
325	0	
492	0	
503	2	<1.8
508	0	
530	0	

* Replicate results were not required.

** Laboratory 307 experienced a power outage during the PT exercise.

2.6.2. Faecal coliforms in water – Sample 2 results

- **Sample content**

Escherichia coli (17*)* (wild type), *Enterococcus faecalis* (98) (wild type), *Salmonella* Liverpool 1,3,19:d:e,n,z_{15} (65 per disc) (wild strain), *Leclercia adecarboxylata* (34) (NCTC 10599).

- **Sample results**

Reference results were obtained by testing six randomly selected Lenticules under repeatability conditions and are included in Table 7 and Figure 3.

Table 7: Participants' and reference ranges

Results	Range (cfu/100ml)
Participants'	0 – 37.6
Reference	2 - 17

Table 8: Participants' results

Lab ID	CFU / 100ml	
	Replicate 1	Replicate 2 *
118	0	
136	13	
151	0	0
164-1	0	
164-2	0	
164-3	0	
292	23	
295	0	
299	1	
307 **	2	1
325	1	
492	0	
503	6.8	2
508	20	
530	37.6	

* Replicate results were not required.

**Laboratory 307 noted that Sample 2 Lenticule was received broken and also experienced a power outage during the PT exercise.

3. Analysis of results

3.1. General comments

The methods used to test the samples are shown in Table 9 and 10 with the number of laboratories citing the method.

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

Method reference	No. of Labs.
ISO 16649 - 3 (MPN - 5 tubes, 3 dilutions) (FAO Reference Centre Generic protocol)	6
Bacteriological Analytical Manual, Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria	3
MPN – Media used MacConkey Broth Pour Plate Method-Media used Tryptone Bile X-Glucuronide (TBX) Medium	1
NCH 3056:2007	1
ISO 7218:2007	1
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)	4
Standard Methods for the examination of water and wastewater 23th Edition 2017.	3
ISO 9308-1: 2014	2
Membrane filtration APHA 9222B	1
Membrane filtration; SANS 5221, 4.5:2018	1
MPN-Media used MacConkey Broth.	1
COLILERT-18	1

3.2. Sample analyses

Fourteen laboratories returned results for the second PT distribution (13 labs each for *E. coli* and FC).

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

3.2.1. *E. coli* in shellfish

Laboratory 292 only returned a single replicate result for both samples. Laboratory 164 analysed three sets of samples, returning duplicate results for each set analysed.

3.2.1.1. Sample 1

Seven laboratories returned duplicate *E. coli* MPN/100g results falling within the expected range (between ±2.68 SD_T of the participants' median) including Laboratory 164 which returned three sets of results falling within the expected range (Figure 1). Laboratory 292 reported a single replicate result and Laboratory 492 reported both results that fell between ±2.68 SD_T and ±4 SD_T of the participants' median. Laboratory 503 reported one replicate result which fell within the expected range and one replicate result falling outside ±4 SD_T of the participants' median. Laboratories 299, 325 and 530 reported the absence of *E. coli* in the sample.

3.2.1.2. Sample 2

Six laboratories returned duplicate *E. coli* MPN/100g results falling within the expected range (between ±2.68 SD_T of the participants' median) including Laboratory 164 which returned three sets of results falling within the expected range (Figure 2). Laboratory 156 reported one replicate result within the expected range while the second replicate result fell between ±2.68 SD_T and ±4 SD_T of the participants' median. Laboratories 136 and 151 reported duplicate results between ±2.68 SD_T and ±4 SD_T of the participants' median, and laboratories 299, 325 and 492 reported both replicate results outside ±4 SD_T of the participants' median. Laboratory 292 only reported a single replicate result that fell outside ±4 SD_T of the participants' median.

3.2.2. Faecal coliforms in water

Due to the low numbers of zero results reported for this distribution, no statistical analysis was performed on these samples.

4. References

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 (2015)

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

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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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