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## **FAO Reference Centre for Bivalve Mollusc Sanitation**

Pilot Proficiency Testing distribution for the detection of *Escherichia coli* in shellfish and the detection of Faecal Coliforms in water (PT 88)

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### **Cefas Document Control**

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# 1. Introduction

In 2019, Cefas (The Centre for Environment, Fisheries and Aquaculture) 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 in the development of bivalve shellfish production.

Part of the FAO Reference Centre work programme is to organise a pilot 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 Pilot PT exercise comprised of Lenticule™ discs (samples originated from the UK Health Security Agency (UK HSA) External Quality Assessment (EQA) containing 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 laboratories in house method for the enumeration of *Escherichia coli* (*E. coli*) and/or use the FAO Reference Centre generic protocol based upon ISO 16649-3, Microbiology of the food chain – Horizontal method for the enumeration of  $\beta$ -glucuronidase-positive *Escherichia coli* Part 3:

Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl- $\beta$ -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 [[Generic Protocols](#)]. 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 coliform in water

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

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

## 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 22<sup>nd</sup> March 2021 to 29 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 coliform, 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). Six randomly selected Lenticules were examined under repeatability conditions. The reference results are given in Figure 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. Six randomly selected Lenticules were examined under repeatability conditions. The reference results are given in Figure 1 and 2, and Tables 1 and 3.

## 3. Analysis of participants results

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

Each participants *E. coli* Most Probable Number (MPN) value (MPN/100g) reported was compared against the calculated median MPN from all participants' results, reference results were omitted from the calculation. The acceptable limits were calculated as the participants' median  $\pm 2.68$  standard deviation (SD) and  $\pm 4$  SD above and below the participants' median for the same sample. 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 ( $\pm 2.68$ SD and  $\pm 4$  SD) were calculated from participants' results. SD<sub>T</sub> calculations were 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:** When calculating the geomean all results reported as zero were removed from the calculations.

**Note:** Values reported as >16,000 MPN/100g or <18 MPN/100g was assigned a value of 32,000 or 9 respectively.

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

- **Sample contents**

*Escherichia coli*  $2.3 \times 10^3 - 1.7 \times 10^4$  (wild strain), *Lactobacillus paracasei*  $1.3 \times 10^4$  (wild strain), *Proteus mirabilis*  $2.8 \times 10^3$  (wild strain)

- **Sample results**

**Table 1: Participants' and reference results median, median  $\pm 2.68$  and  $\pm 4$  SD**

	Range	Median	GM	Median $\pm 3 \cdot SD_T$
<b>Participants Results</b>	0 – $3.2 \times 10^4$	$4.9 \times 10^3$	$1.9 \times 10^3$	$9.9 \times 10^2 - 2.4 \times 10^4$
<b>Reference Results</b>	$2.3 \times 10^3 - 1.7 \times 10^4$	$1.1 \times 10^4$	$9.0 \times 10^3$	$2.2 \times 10^3 - 5.5 \times 10^4$

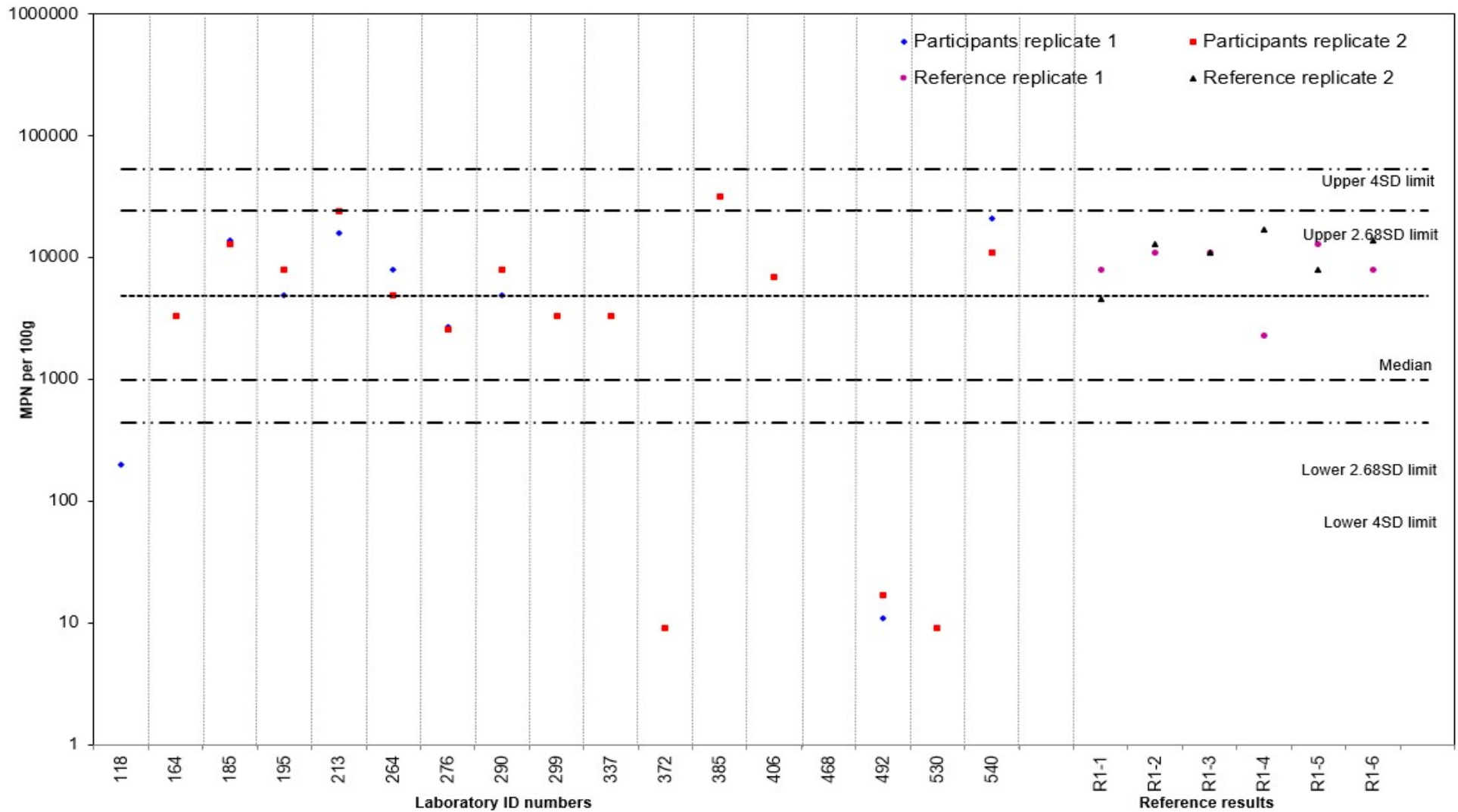
GM - geometric mean,  $SD_T$  - theoretical standard deviation ( $0.26 \log_{10}$ )

**Table 2: Participants results returned**

Lab ID.	<i>E. coli</i> (per 100g)	
	Replicate 1	Replicate 2
<b>118</b>	200	-
<b>164</b>	3300	3300
<b>185</b>	14000	13000
<b>195</b>	4900	7900
<b>213</b>	16000	24000
<b>264</b>	7900	4900
<b>276</b>	2700	2600
<b>290</b>	4900	7900
<b>299</b>	3300	3300
<b>337</b>	3300	3300
<b>372</b>	<18	<18
<b>385</b>	>16000	>16000
<b>406</b>	7000	7000
<b>468</b>	0	0
<b>492</b>	11	17
<b>530</b>	<18	<18
<b>540</b>	$2.1 \times 10^4$	$11.1 \times 10^3$



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



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

- **Sample contents**

*Escherichia coli*  $1.3 \times 10^2$  -  $7.8 \times 10^2$  (wild strain), *Salmonella* London 3, {10}{15}: I,v:1,6 -  $1.5 \times 10^2$  per disc (wild strain), *Aeromonas hydrophila*  $1.9 \times 10^2$  (wild strain), *Leuconostoc mesenteroides*  $4.5 \times 10^2$  (wild strain), *Serratia marcescens*  $1.1 \times 10^2$  (wild strain)

- **Sample results**

**Table 3: Participants' and reference results median, median  $\pm 2.68$  and  $\pm 4$  SD**

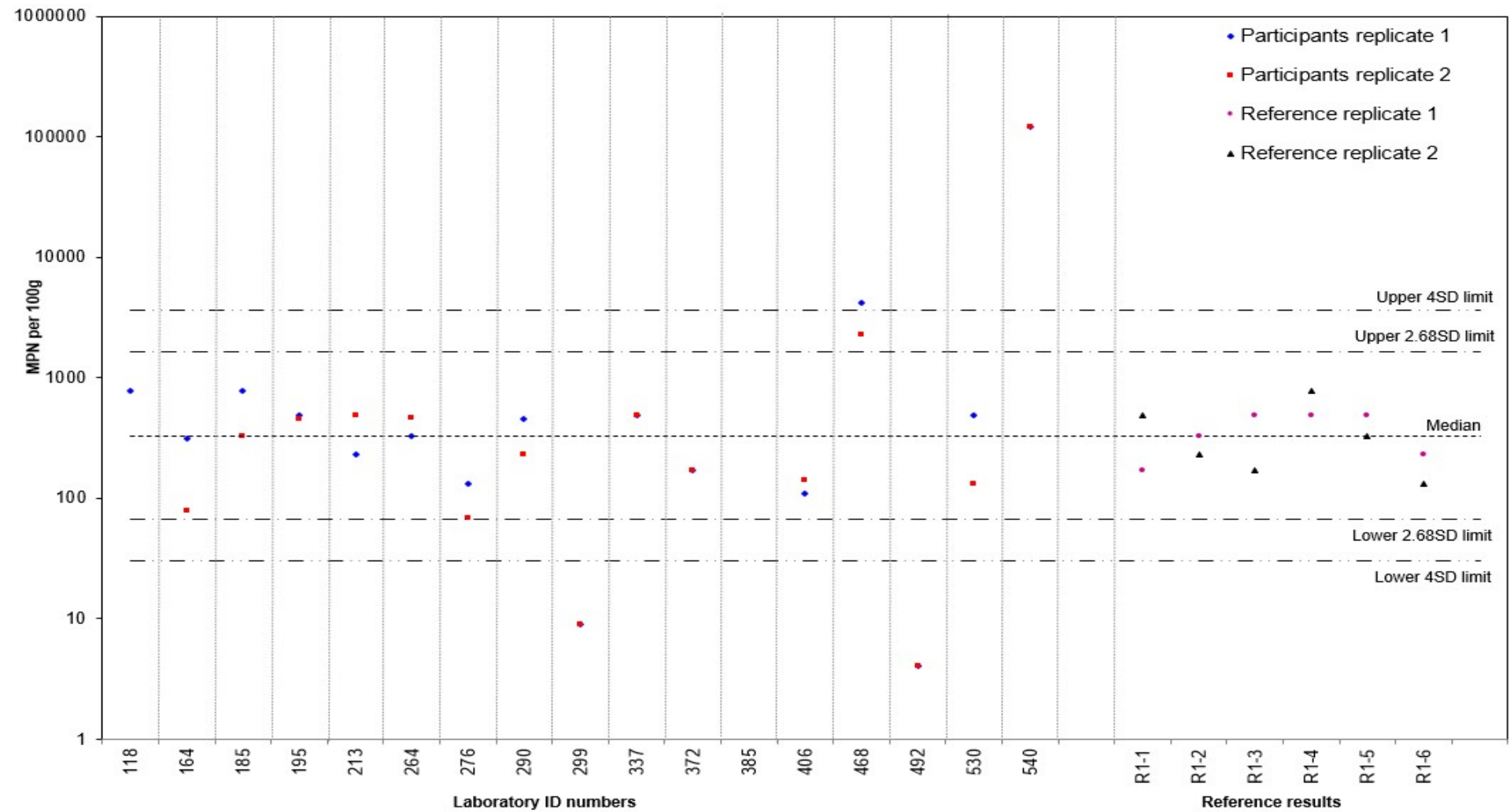
	Range	Median	GM	Median $\pm 3 \cdot SD_T$
<b>Participants Results</b>	4 – $1.2 \times 10^5$	$3.3 \times 10^2$	$3.0 \times 10^2$	$6.6 \times 10^1$ – $1.6 \times 10^3$
<b>Reference Results</b>	$1.3 \times 10^2$ – $7.8 \times 10^2$	$3.3 \times 10^2$	$3.2 \times 10^2$	$6.6 \times 10^1$ – $1.6 \times 10^3$

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

**Table 4: Participants results reported**

Lab ID.	<i>E. coli</i> (per 100g)	
	Replicate 1	Replicate 2
118	780	-
164	310	78
185	780	330
195	490	450
213	230	490
264	330	460
276	130	68
290	450	230
299	<18	<18
337	490	490
372	170	170
385	-	-
406	110	140
468	4200	2300
492	4	4
530	490	130
540	$12.1 \times 10^4$	$12.1 \times 10^4$

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



## 3.2. Faecal coliforms in water – Samples 1 and 2

Each participant's *E. coli* result reported was compared with the participants' median (calculated from all participants result's reported). Participants' results are shown in Table 5 and 6, and Figure 3, with acceptable limits being calculated as the participants' median  $\pm 2$   $\sigma$ -value and  $\pm 3$   $\sigma$ -value above and below the participants' median for the same sample.

**Note:** The standard deviation value ( $\sigma$ -value) used for all parameters is 0.35. Reference results were omitted from the calculation.

### 3.2.1. Faecal in water – Sample 1

- **Sample content**

*Escherichia coli* (19) (wild strain), *Enterococcus faecalis* (73) (wild strain), *Salmonella Heidelberg* 1,4,[5],12:r:1,2 (8) (wild strain).

- **Sample results**

**Table 5: Participants' results Participants' results and z-scores**

Lab ID	CFU / 100ml
118	4.5
164	0
195	49
213	8
269	Detected
295	20
406	150
468	0
530	6.9
540	123

**Note:** Lab 164 experience power issues in the laboratory.

### 3.2.2. Faecal in water – Sample 2 results

- **Sample content**

*Enterococcus faecalis* (85) (NCTC 5957), *Salmonella* *Wentworth* 11:z<sub>10</sub>:1,2 (8) (wild strain), *Klebsiella oxytoca* (41) (wild strain).

- **Sample results**

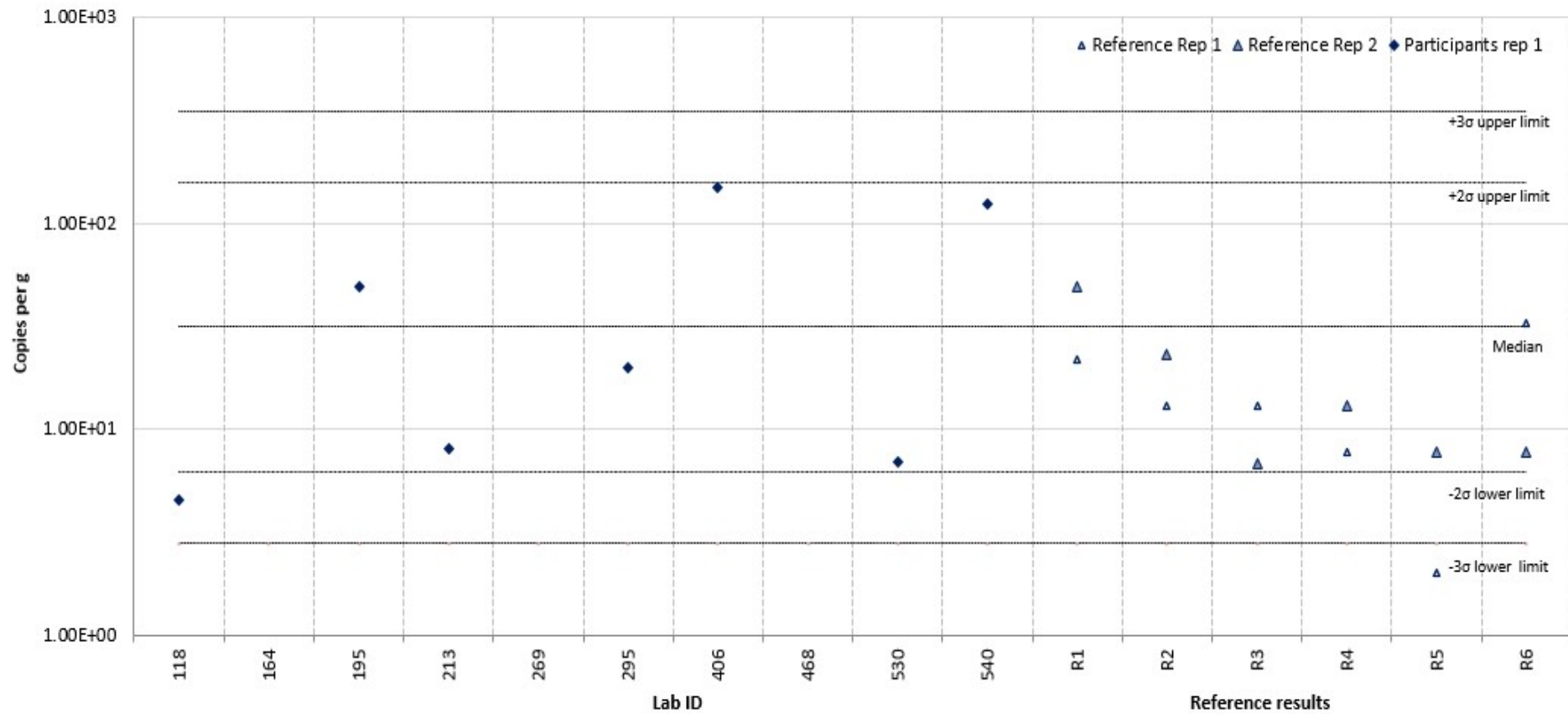
**Table 6: Participants' results**

Lab ID	CFU / 100ml
118	0
164	0
195	0
213	0
269	0
295	0
406	0
468	0
530 *	<1
540	52

\* Result is <1MPN /100 ml

**Note:** Lab 164 experienced power issues in the laboratory.

**Figure 3: Faecal coliform in water - Sample 1**



## 4. Analysis of results

### 4.1. General comments

Twenty-nine laboratories were sent material, with 19 laboratories (17 Shellfish laboratories and 10 Water laboratories) returning results. The methods used to test the samples are shown in Table 7 and 8 with the number of laboratories citing the method.

**Table 7: 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)	13
NMKL 96 (RAPID <i>E. coli</i> agar based chromogenic media)	1
APHA (1992). Compendium of Methods for the Microbiological Foods. 2nd Edition, American Public Health Association, Washington, DC. (MPN - 4 dilutions, 3 tubes)	1
Indonesian National Standard, SNI 2332.1: 2015	1
MPN and Simplate	1

**Table 8: 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
APHA (2001). Standard Methods for the Examination of Water and Wastewater. 20th Edition, American Public Health Association, Washington, DC. (MPN Method - 4 dilutions, 5 tubes)	1
COLILERT-18 Test Kit (MPN Method)	1
Membrane Filtration APHA 9222B	1
ISO 9308-1	1
SANS 5221	1
Membrane filtration (No reference given)	1
IDEXX (Q 2000)	1

## 4.2. Sample analyses

Nineteen laboratories returned results for the pilot PT distribution.

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

### 4.2.1. *E. coli* in shellfish

Laboratory 118 only return a single replicate result for both samples and Laboratory 385 was unable to analyse sample 2.

#### 4.2.1.1. Sample 1

Eleven laboratories returned duplicate *E. coli* MPN/100g results falling between  $\pm 2.68$  SD of the participants' median (Figure 1). Laboratory 385 reported both replicates between  $\pm 2.68$  SD and  $\pm 4$  SD of the participants' median, Laboratory 118 reported 1 replicate result outside  $\pm 4$  SD of the participants' median and laboratories 372, 492 and 530 reported both replicates outside  $\pm 4$  SD of the participants' median.

#### 4.2.1.2. Sample 2

Twelve laboratories returned (11 reported duplicate results and 1 reported a single replicate result) *E. coli* MPN/100g results falling between  $\pm 2.68$  SD of the participants' median (Figure 2). Laboratory 468 reported 1 replicate result between  $\pm 2.68$  SD and  $\pm 4$  SD of the participants' median and the second replicate result outside  $\pm 4$  SD of the participants' median. Laboratories 299, 492 and 540 reported both replicate results outside  $\pm 5$  SD of the participants' median.

### 4.2.2. Faecal coliforms in water

#### 4.2.2.1. Sample 1

Six laboratories returned FC results (cfu per 100ml) and fell between  $\pm 2$  opt-value of the participants' median (Figure 3). Laboratory 118 result reported fell between  $\pm 2$  opt-value and  $\pm 3$  opt-value of the participants' median and laboratories 164 and 468 did not detect *E. coli* in this sample.



#### 4.2.2.2. Sample 2

Eight laboratories correctly reported the absence of FC in sample 2. Laboratory 530 and 540 incorrectly reported the presence of FC in the sample.

## 5. References

ISO 16649-3, Microbiology of the food chain – Horizontal method for the enumeration of  $\beta$ -glucuronidase-positive *Escherichia coli* Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (2015)

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

## Appendix I:

### *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 $\pm 2.68SD$ and median $\pm 4SD$ values.	2	5	2	9
Both replicates MPN results are outside the expected range and fall between the median $\pm 2.68SD$ and median $\pm 4SD$ values.	2	2	2	6
One replicate MPN result is outside the median $\pm 4SD$ value.	2	5	0	7
Both replicates MPN results are outside the expected range. The first falls between the median $\pm 2.68SD$ and median $\pm 4SD$ value and the second falls outside the median $\pm 4SD$ values.	2	2	0	4
Both replicates MPN results reported is outside the median $\pm 4SD$ 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 $\pm 3SD$ and median $\pm 5SD$ values.	2	2	4
Single replicate MPN result reported is outside the median $\pm 5SD$ value.	2	0	2



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