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

Fourth proficiency testing distribution for the detection of *Escherichia coli* in shellfish and the detection of faecal coliforms in water (PT 103)

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Cefas Document Control

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Version	Author	Date	Comment
0.01	L. Stockley	13/10/25	Sent to JL for QA
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V1	L. Stockley	24.10.25	Amendments approved and sent to participants
V2	L. Stockley	02.12.25	Minor content changes and additional information added to sample analysis following comments from participants

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 (RC) for Bivalve Mollusc Sanitation. The aim of the FAO RC is to provide support to FAO member countries in the development of bivalve shellfish production.

Part of the FAO RC 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 Fourth PT distribution contained Lenticule™ discs originating from the UK Health Security Agency (UK HSA). Each Lenticule disc contains known levels of fully characterised *Escherichia coli* NCTC 9001 (*E. coli*) or faecal coliforms (FC) (supplied as certified reference material) in a solid water-soluble matrix. The discs are very stable and short periods at ambient temperature, such as during shipment, is not an issue for product stability. Participants were requested to store all samples at <-20°C on arrival until testing begun.

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 RC 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.

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 per 100 g 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 FC in water and/or the FAO RC generic protocol [Generic Protocols] based upon the approach set out in US FDA BAM Chapter 4.

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 Worldnet on the 24th June 2025 to 17 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 FC, 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 RC generic protocol based on ISO 16649-3 (Anon, 2015). Three randomly selected Lenticules were examined in

duplicate under repeatability conditions by the FAO RC. 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 RC generic protocol based on the approach set out in US FDA BAM Chapter 4 (Anon, 2020). Three randomly selected Lenticules were examined under repeatability conditions by the FAO RC. 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* value (*E. coli* / 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 UK HSA for PT performance assessments of MPN tests and Lenticule discs (FEPTU562.13.pdf (publishing.service.gov.uk). Reported *E. coli* 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 (±2.68 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 a 5 x 3 MPN method (0.24 log_{10}). Reference values were excluded from the calculation of the participants' median.

Note: To enable reported results to be statically analysed, those results reported containing a < or >, a value of 1 was added or subtracted to the final result. Results reported as >1600 *E. coli* / 100 g were assigned a value of 1601 for statistical analysis. Results reported as <3, <18 and <180, treated as 2, 17 and 179 respectively. Results reported as zero were not included in statistical analysis.

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

- **Sample contents**

E. coli (certified reference material) $5.0 \times 10^2 – 5.0 \times 10^4$ 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'	$<1.8 \times 10^1$ - 1.7×10^4	2.3×10^3	1.3×10^3	5.2×10^2 - 1.0×10^4	2.5×10^2 - 2.1×10^4
Reference	2.3×10^3 - 1.3×10^4	4.9×10^3	5.5×10^3	-	-

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

Table 2: Participants' results returned

Lab ID.	*E. coli* (per 100 g)	
	Replicate 1	Replicate 2
40	1700	17000
136	4900	3300
171	260	270
264	3300	4900
285	2300	2200
290	1300	4900
328	3300	3300
337	2300	4900
359	9200	9200
464 [a]	<18	<18
492 [a]	>1600	>1600
530	4000	4000
540	2300	1500
588	15	23

Key:

a; results reported as <18 and >1600, treated as 17 and 1601 respectively for statistical analysis.

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

- **Sample contents**

E. coli (certified reference material) $3.0 \times 10^1 – 1.2 \times 10^2$ 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'	0 – <1.8 x 10^2	2.0 x 10^1	2.5 x 10^1	5.0 x 10^0 – 8.8 x 10^1	2.0 x 10^0 – 1.8 x 10^2
Reference	<1.8 x 10^1 – 6.8 x 10^1	4.5 x 10^1	3.8 x 10^1	-	-

Note: Results are in *E. coli* per 100 g. 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]	40	<18
136	45	45
171 [a]	<18	<18
264	40	20
285	68	45
290	20	20
328 [a]	<180	<180
337 [a]	20	<18
359	20	45
464 [a]	<18	<18
492	49	33
530	0	0
540	9.2	23
588 [a]	<3	<3

Key:

a; results reported as <3, <18 and <180, treated as 2, 17 and 179 respectively for statistical analysis.

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

Note: Median value calculated using all participants' results

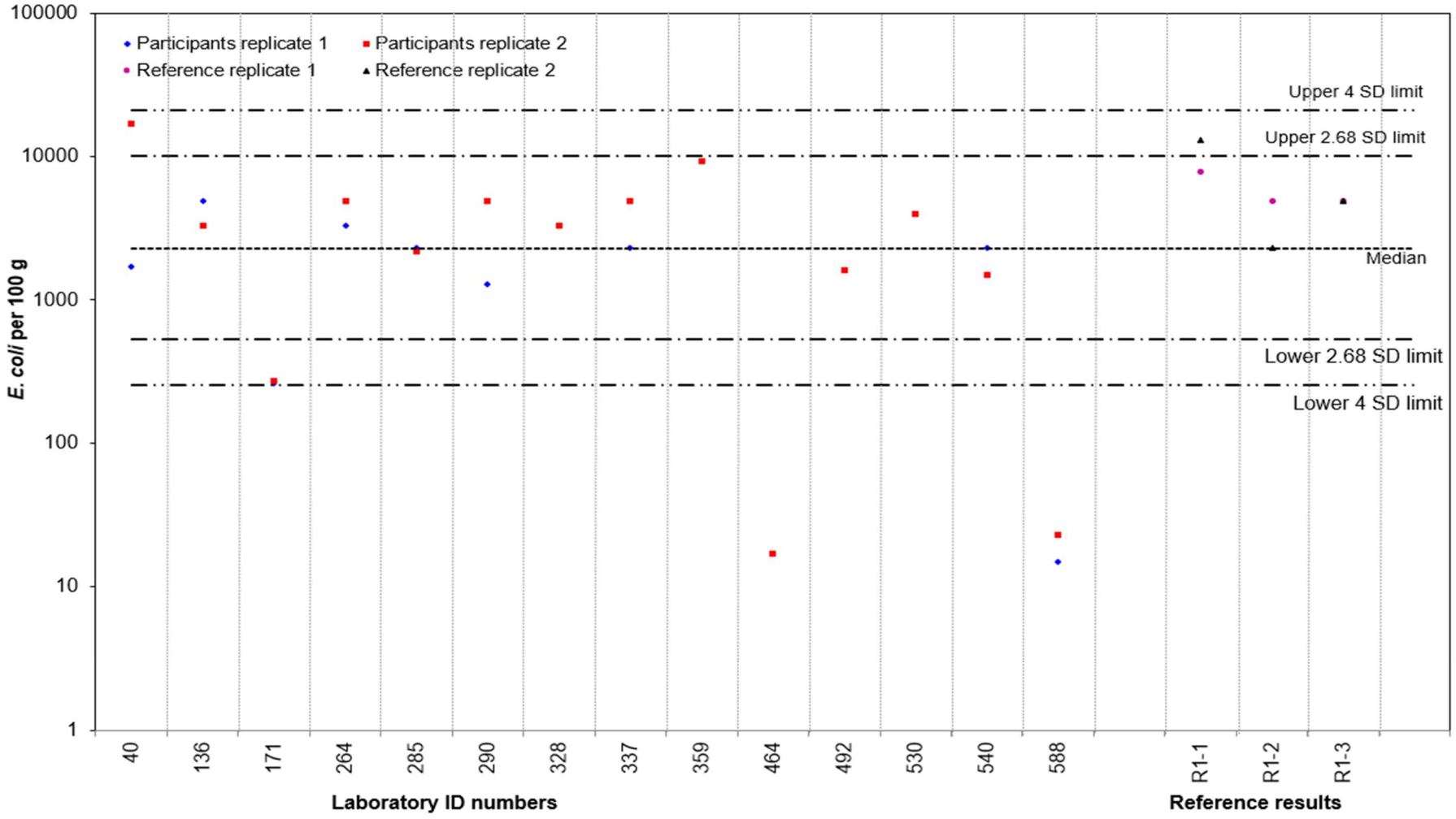
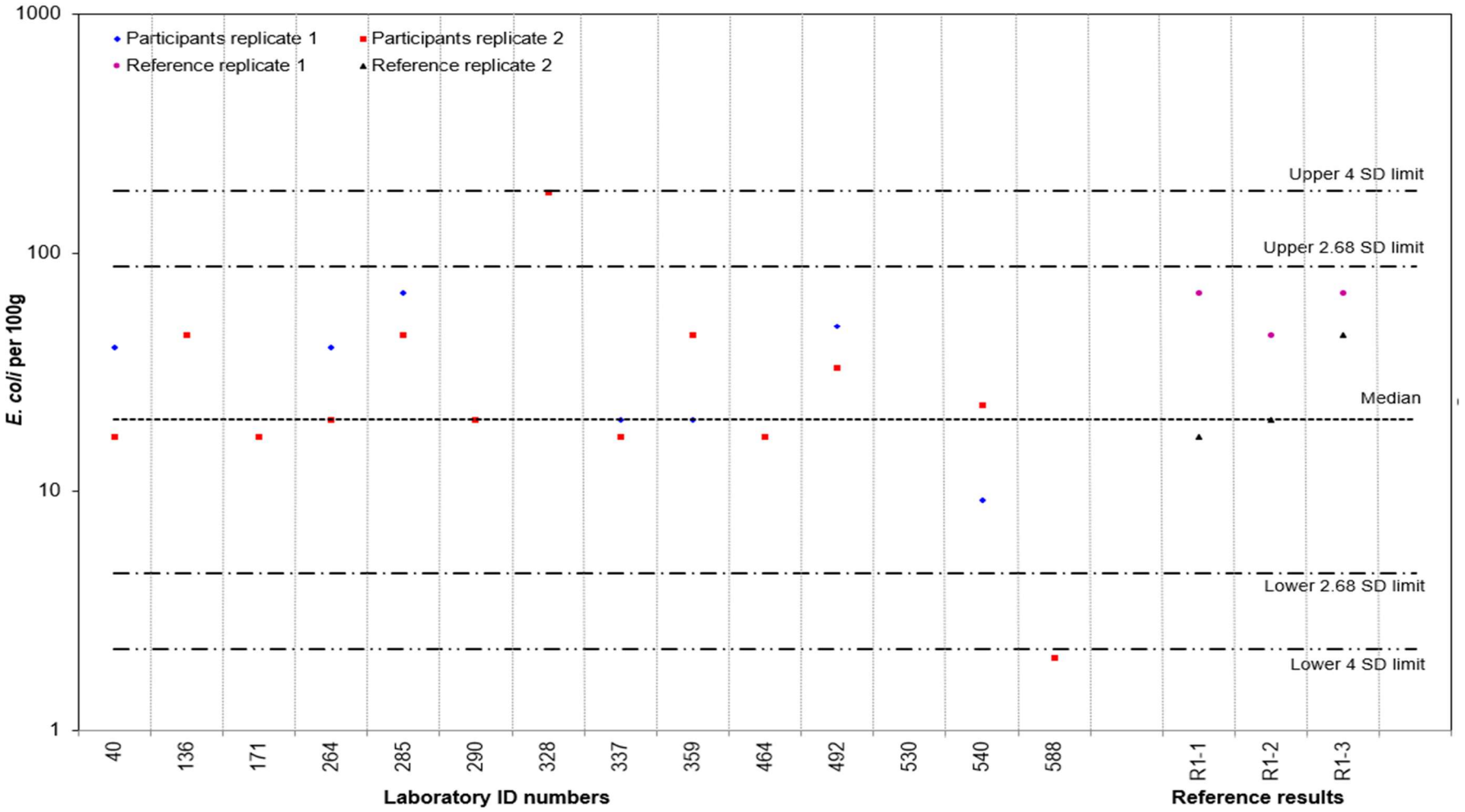


Figure 2: *E. coli* in shellfish - Sample 2

Note: Median value calculated using all participants' results



3.2. Faecal coliforms in water – Samples 1 and 2

Each participant's reported results for FC 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 SD_T and ±4 SD_T following the recommended approach described by the UK HSA 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 (±2.68 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 a 5 x 3 MPN method (0.24 log_{10}). Reference values were excluded from the calculation of the participants' median.

Note: To enable reported results to be statically analysed, those results reported containing a < or >, a value of 1 was added or subtracted to the final result. Results reported as <3 and <18 were assigned a value of 2 and 17 respectively for statistical purposes. Results reported as >1600 and 1800+ were assigned a value of 1601 and 1801 respectively. Results reported as zero were not included in statistical analysis.

3.2.1. Faecal coliforms in water – Sample 1

- **Sample content**

E. 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 - 2.5 x 10^3	1.2 x 10^1	2.1 x 10^1	2.8 x 10^0 – 5.5 x 10^1	1.4 x 10^0 – 1.1 x 10^2
Reference	2.2 x 10^1 - 3.3 x 10^1	2.7 x 10^1	2.7 x 10^1	-	-

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

Table 6: Participants' results reported

Lab ID	Faecal coliforms per 100ml
40 [a]	<18
136	33
171	2500
264	4.5
285	17
290	4.5
328	0
464	0
492 [b]	1
492 [b]	7.8
540	1900
588 [a]	<3

Key

a; results reported as <3 and <18, treated as 2 and 17 respectively for statistical analysis.

b; laboratory 492 participated using 2 different methods.

3.2.2. Faecal coliforms in water – Sample 2

- **Sample content**

E. coli (certified reference material) $1.0 \times 10^3 – 1.0 \times 10^4$ 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'	$0 - 9.4 \times 10^3$	2.7×10^2	2.1×10^2	$6.0 \times 10^1 – 1.2 \times 10^3$	$2.9 \times 10^1 – 2.4 \times 10^3$
Reference	$1.3 \times 10^3 - 9.2 \times 10^3$	2.4×10^3	3.1×10^3	-	-

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

Table 8: Participants' results reported

Lab ID	Faecal coliforms per 100ml
40	310
136	9400
171	0
264	220
285 [a]	1800+
290	490
328	14
464	0
492 [b]	11
492 [a b]	>1600
540	122
588	9.2

Key

a; results reported as >1600 and 1800+, treated as 1601 and 1801 respectively for statistical analysis.

b; laboratory 492 participated using 2 different methods.

Figure 3: Faecal coliforms in water - Sample 1

Note: Median value calculated using all participants' results

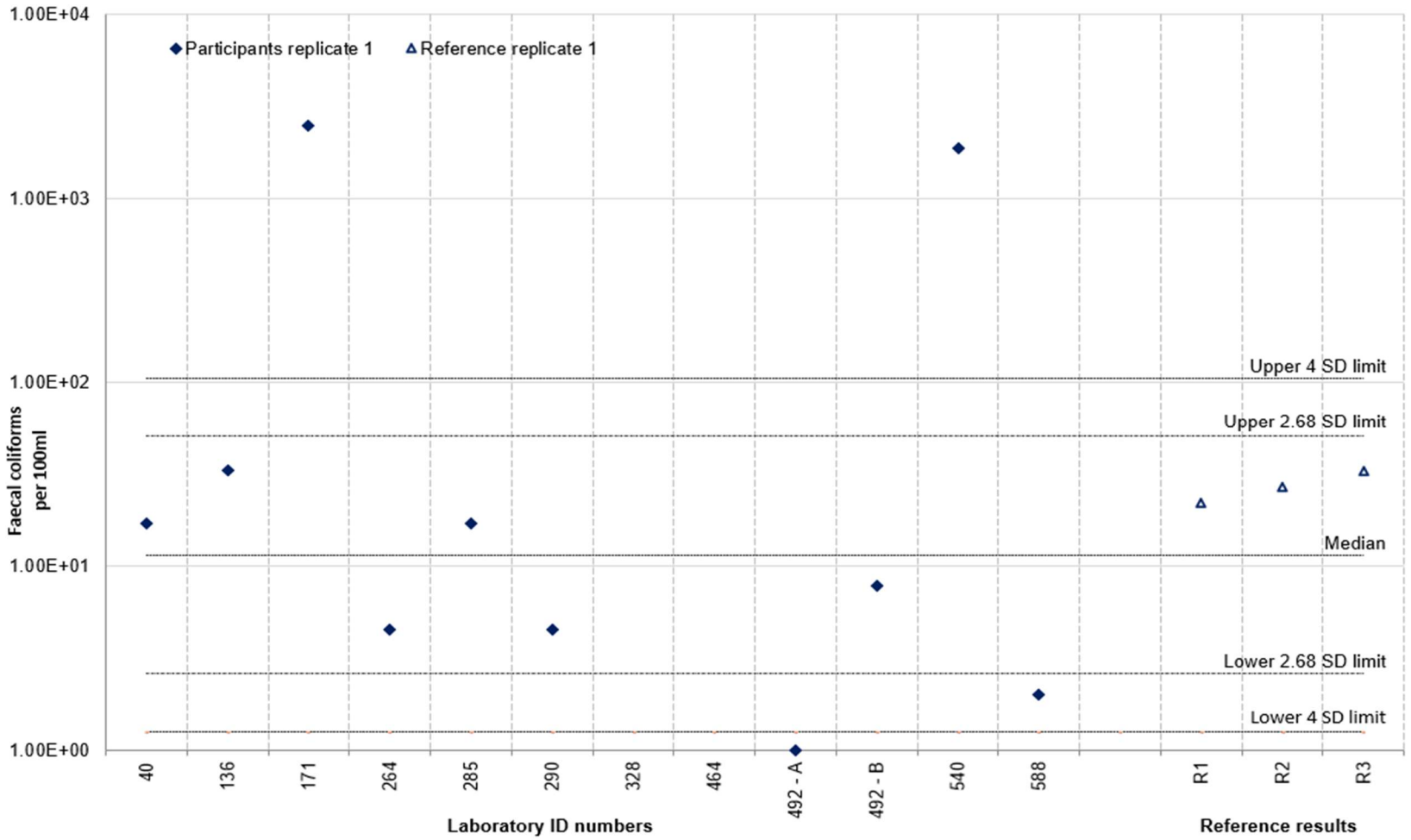
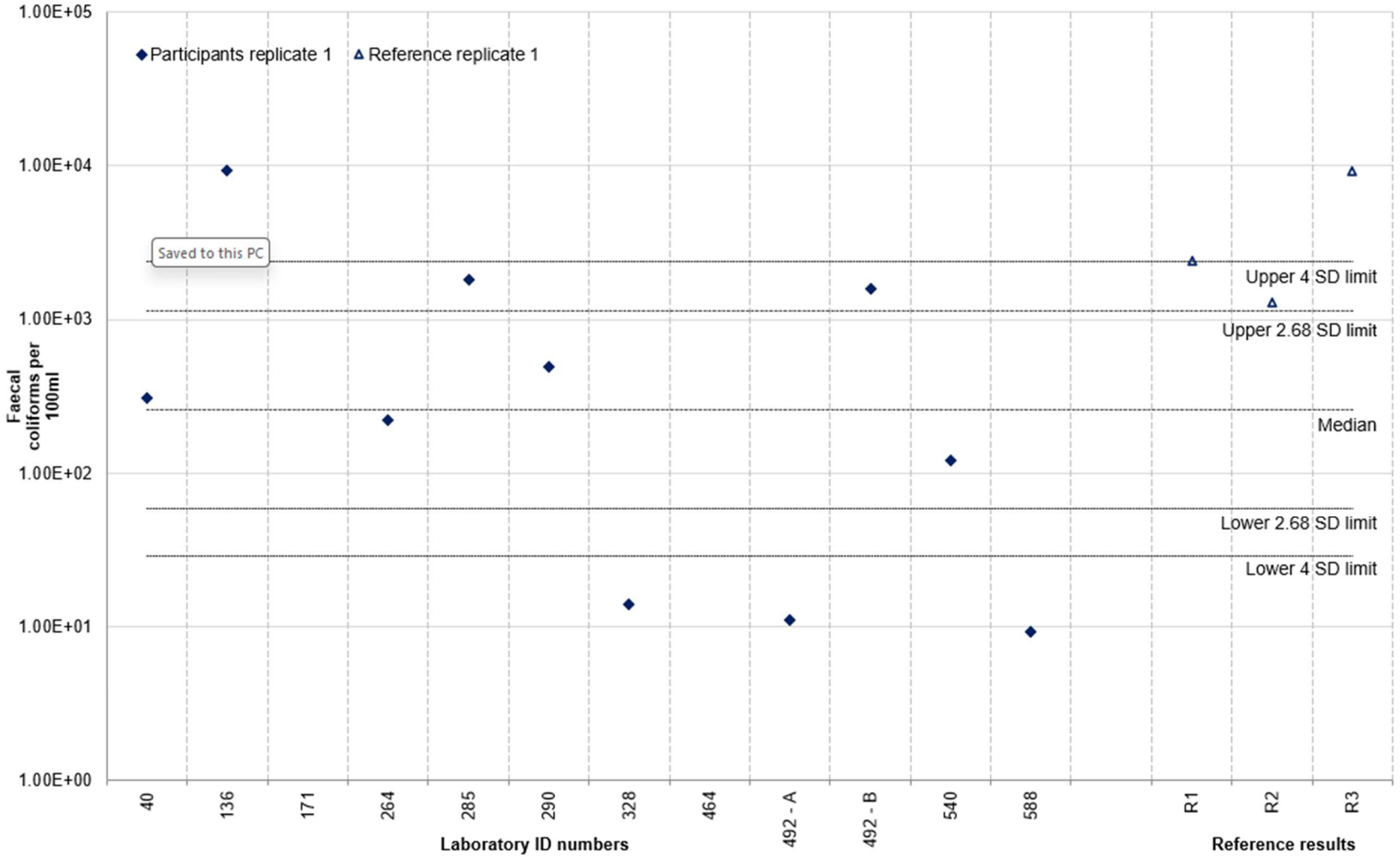


Figure 4: Faecal coliforms in water - Sample 2

Note: Median value calculated using all participants' results



4. Analysis of results

4.1. General comments

Seventeen laboratories were sent material, with 14 laboratories returning results (14 returned results for *E. coli* in shellfish, 11 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 RC generic protocols.

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

Method reference	No. of Labs
ISO 16649 - 3 (MPN) (FAO RC Generic protocol)	7
SNI : 2332 : 1 : 2015	3
NF EN ISO 7251	1
NCh 3056 : 2007	1
NMKL 96 ed 4 2009	1
Simplate Method (Coliforms/*E. coli*)	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 RC Generic Protocol)	5
ISO 9308-1	4
NF T 90-413	1
SNI : 2332 : 1 : 2015	1
Standard Methods for the examination of water and wastewater 24 th Edition 2023.	1

4.2. Sample analyses against the participants' median

Fourteen laboratories returned results for the fourth FAO RC Lenticule™ PT distribution. The analyses given in section 4.2.1 and 4.2.2 shows participants' performance against the participants' median.

4.2.1. *E. coli* in shellfish

Fourteen laboratories reported duplicate results for both samples.

4.2.1.1. Sample 1

Ten laboratories returned duplicate *E. coli* / 100 g results falling within ±2.68 SD_T of the participants' median (Figure 1). Laboratory 40 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, Laboratory 171 reported both replicates >2.68 SD_T but <4 SD_T different from the participants' median, and laboratories 464 and 588 reported all replicates outside -4 SD_T of the participants' median.

4.2.1.2. Sample 2

Eleven laboratories returned duplicate *E. coli* / 100 g results falling within ±2.68 SD_T of the participants' median (Figure 2). Laboratory 328 reported both replicates >2.68 SD_T but <4 SD_T different from the participants' median, and laboratory 588 reported both results outside ±4 SD_T of the participants' median. Laboratory 530 reported a result of zero *E. coli* for this sample.

4.2.2. Faecal coliforms in water

Eleven laboratories reported results for both samples. Laboratory 492 reported results for both samples using 2 separate methods.

4.2.2.1. Sample 1

Six laboratories returned results (FC per 100 ml) that fell between ±2.68 SD_T of the participants' median (Figure 3). Laboratory 588 reported a result >2.68 SD_T but <4 SD_T different from the participants' median and laboratories 171, 492-A and 540 reported results that fell outside ±4 SD_T of the participants' median. Laboratories 328 and 464 reported results of zero faecal coliforms for this sample.

4.2.2.2. Sample 2

Four laboratories returned results (FC per 100 ml) that fell between ±2.68 SD_T of the participants' median (Figure 4). Laboratories 285 and 492-B reported a result >2.68 SD_T but <4 SD_T different from the participants' median and laboratories 136, 328, 492-A and 588 reported results that fell outside ±4 SD_T of the participants' median. Laboratories 171 and 464 reported a result of zero faecal coliforms in this sample.

Note: If laboratories experience problems please contact us (FAO RC) for assistance.

4.2.3. Sample assessment against the UKHSA certified reference data

To assess the homogeneity and stability of each sample batch, the UKHSA analysed 30 individual samples following ISO 17034 guidelines to provide reference data for each batch. Table 11 and figures 5, 6, 7 and 8 show the calculated geometric mean ±3 SD above and below the geometric mean from the UKHSA reference results. This analysis is provided for information only.

Table 11: UKHSA certified reference material analysis

Sample info	Geometric mean	Expected Range	Mean * ±3SD
***E. coli* – Sample 1**	4.3×10^3	$2.1 \times 10^3 – 8.9 \times 10^3$	$1.8 \times 10^3 – 1.0 \times 10^4$
***E. coli* – Sample 2**	5.3×10^1	$2.5 \times 10^1 – 1.1 \times 10^2$	$2.2 \times 10^1 – 1.3 \times 10^2$
Faecal coliform – Sample 1	5.3×10^1	$2.5 \times 10^1 – 1.1 \times 10^2$	$2.2 \times 10^1 – 1.3 \times 10^2$
Faecal coliform – Sample 2	5.0×10^3	$2.6 \times 10^3 – 9.8 \times 10^3$	$2.3 \times 10^3 – 1.1 \times 10^4$

Figure 5: *E. coli* in shellfish Sample 1

Note: UKHSA geometric mean ±3 SD

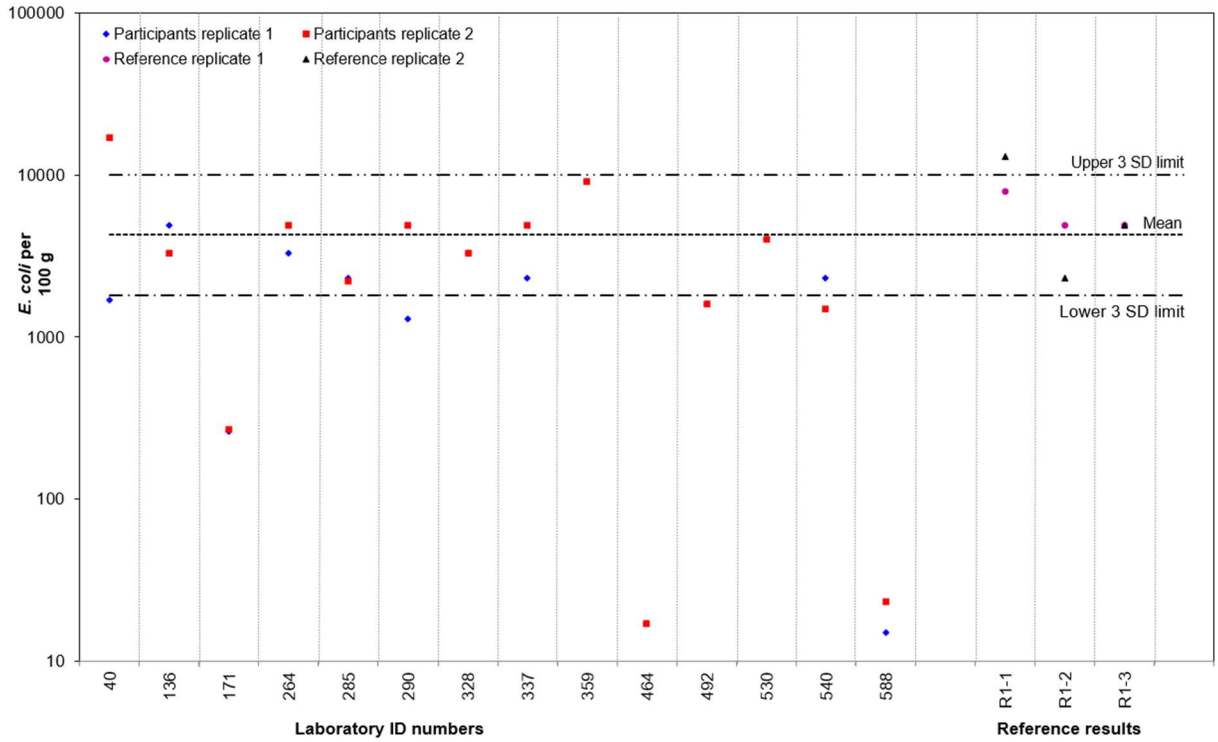


Figure 6: *E. coli* in shellfish Sample 2

Note: UKHSA geometric mean ±3 SD

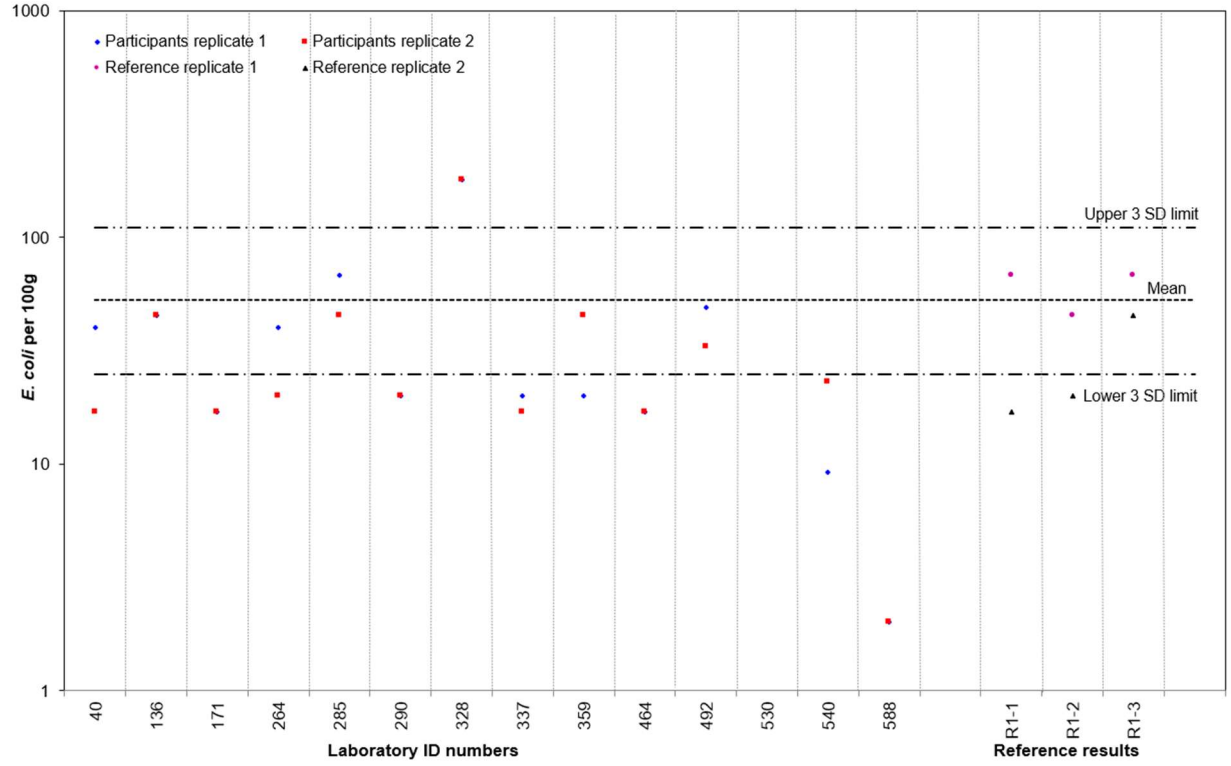


Figure 7: Faecal coliforms in water Sample 1

Note: UKHSA geometric mean ±3 SD

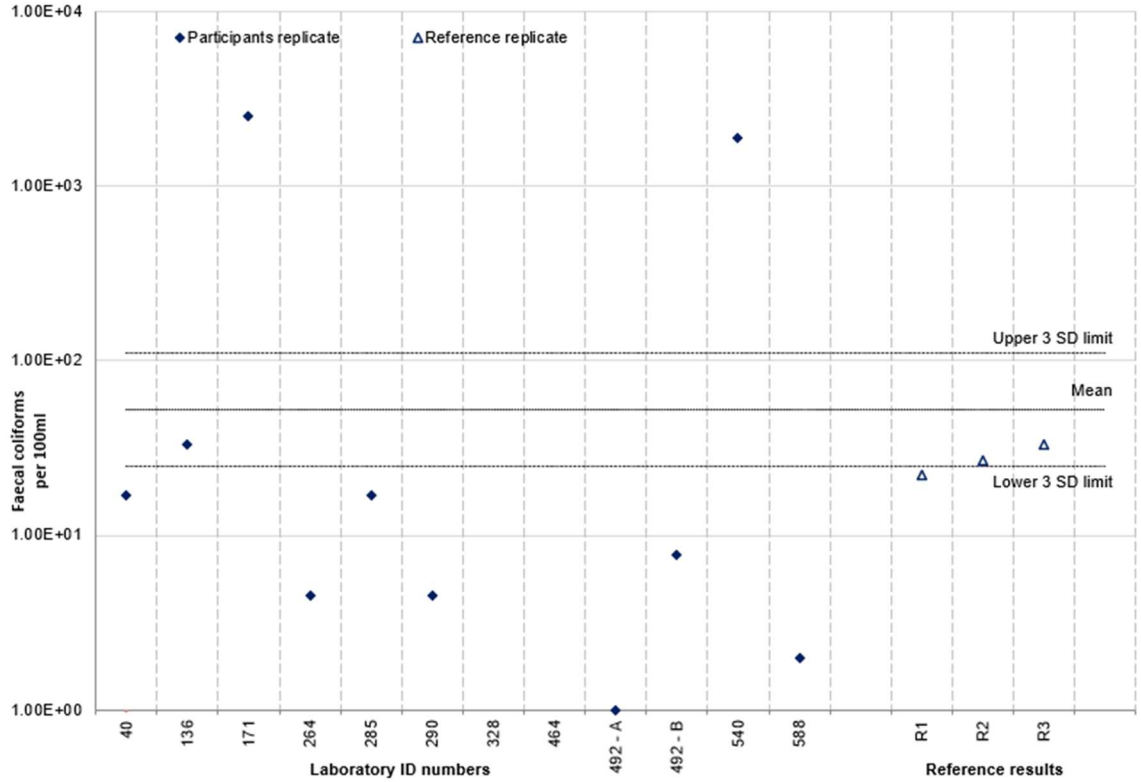
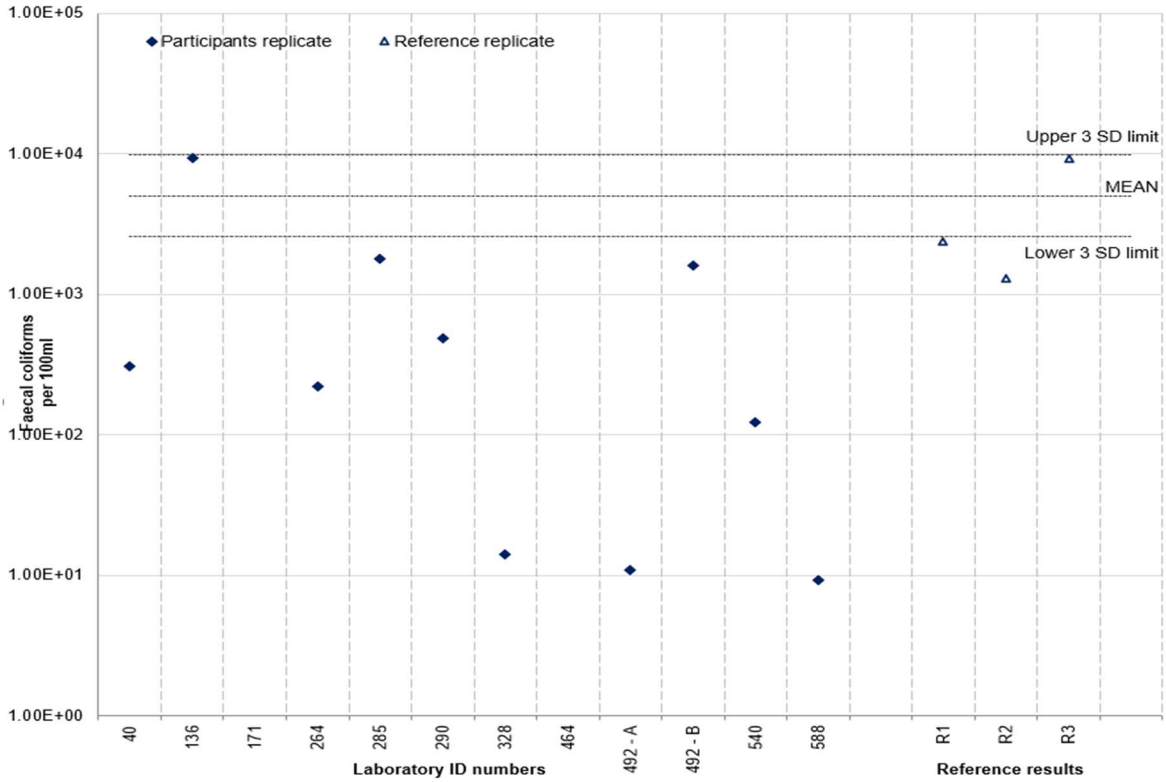


Figure 8: Faecal coliforms in water Sample 2

Note: UKHSA geometric mean ±3 SD



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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