European Union Reference Laboratory (EURL)
Proficiency Testing Scheme

Enumeration of *Escherichia coli* in bivalve molluscan shellfish

EURL PT reference number: PT 60

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Contents **Page number**

This scheme is intended to provide proficiency testing samples for laboratories undertaking examination of live bivalve molluscs from production areas in accordance with Regulation (EC) No. 854/2004 and from throughout the production chain in accordance with Regulation (EC) No. 2073/2005.

The scheme is organised by the European Union Reference laboratory (EURL) for monitoring bacteriological and viral contamination of bivalve molluscs. The EURL is designated by the European Union in accordance with Regulation (EC) No. 882/2004. The scheme is intended to compliment the EURL/PHE Shellfish Scheme (http://www.hpa.org.uk/ProductsServices/MicrobiologyPathology/ExternalQualityAssessmentProficiencyTesting/EQAPTForFoodWaterAndEnvironmentalMicrobiology/ShellfishScheme/ through examination of aspects of the methods not covered under the Shellfish Scheme (initial sample preparation and preparation of initial dilutions) and to provide additional data for laboratories for ISO 17025 accreditation purposes.

The EU stipulated reference method for enumeration of *E. coli* in live bivalve molluscs in ISO TS 16649-3, Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Anon 2005). The EU reference method for detection of *Salmonella* spp. in live bivalve molluscs is ISO 6579, Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. (Anon 2002).

A scoring system is used to help assess participants' performance. Details of this system are included as Appendix I of this report. The purpose of scoring is to help the EURL, NRLs and other participating laboratories identify incorrect or outlying results. Further information on the use of scoring in proficiency testing and on recommended procedures for following up poor performance can be accessed via the EURL website (www.eurlcefas.org) or obtained by contacting the EURL. The European Union has produced a protocol for management of underperformance in comparative testing and/or lack of collaboration of NRLs with EURLs activities.

If you are experiencing problems with any aspects of these distributions please contact the EURL (contact details below), or alternately refer to the troubleshooting guide included as Appendix II of this report.

Sample preparation

Sample 1

Approximately 1,500 common mussels (*Mytilus edulis*) comprising a single batch were collected from a UK commercial harvesting area. Sample 1 provided to participating laboratories comprised of 25 randomly selected Manila clams from this bulk material.

Sample 2

Approximately 1,000 Pacific oysters (*Crassostrea gigas*) comprising a single batch were collected from a UK commercial harvesting area. Sample 2 provided to participating laboratories comprised of 15 randomly selected common mussels from this bulk material.

Sample distribution and examination

Each batch of shellfish were mixed thoroughly in a large container before being subsampled and packed in accordance with Cefas protocol for packaging shellfish for transportation and distributed at 12:30 on the 30th November 2015 to 37 participating laboratories. Participants were requested to analyse the material in duplicate immediately on receipt using their routine laboratory procedures for the enumeration of *E. coli*.

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*

Ten randomly selected samples were analysed in duplicate on 2 consecutive days (01.12.15 and 02.12.15) for *E. coli* using EURL SOP No. 1175 (Table 1). Sample homogeneity was assessed following the procedure described in ISO 22117.

Table 1: *E. coli* MPN/100g reference results

Sample type	Analyses dates	Range	Median	GM	Median ±3*SD_T
Sample 1 - Mussels	01.12.15	$2.0 \times 10^1 – 7.8 \times 10^2$	2.3×10^2	2.3×10^2	$4.4 \times 10^1 – 1.2 \times 10^3$
	02.12.15	$7.8 \times 10^1 – 4.9 \times 10^2$	2.2×10^2	1.9×10^2	$4.2 \times 10^1 – 1.2 \times 10^3$
Sample 2 - Oysters	01.12.15	$<1.8 \times 10^1 – 3.3 \times 10^2$	4.5×10^1	4.6×10^1	$<1.8 \times 10^1$ - 2.4×10^2
	02.12.15	$<1.8 \times 10^1 – 7.8 \times 10^1$	2.0×10^1	2.3×10^1	$<1.8 \times 10^1$ - 1.1×10^2

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

Participants' results

Performance assessment was according to the procedures described in the EURL/PHE EQA shellfish scheme for a single distribution, with minor modifications (Appendix II). Participants' results and scores allocated for PT 60 are shown in Tables 3, 4, 5 and Figure 1.

Note: The median and upper and lower limits (±3 SD and ±5 SD) were calculated from participants' 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 participants' median.

Table 3: Participants' results

Sample type	*E. coli* MPN/100g			
	Range	Median	GM	Median±3*SD_T
Sample 1 - Mussels	<1.8 x 10^1 – 2.5 x 10^3	2.3 x 10^2	2.5 x 10^2	4.4 x 10^1 – 1.2 x 10^3
Sample 2 - Oysters	<1.8 x 10^1 – 3.3 x 10^2	4.5 x 10^1	4.4 x 10^1	9.0 x 10^0 – 2.4 x 10^2

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

Table 4: Summary statistics of participants' results

E. coli	Sample 1 - Mussels	Sample 2 - Oysters
Participants reporting duplicate results for *E. coli* MPN	35	36
Participants reporting a single MPN result	1	0
Participants reporting MPN results within the expected range for both replicates[1]	32	31
Participants reporting MPN results outside the expected range for one replicate	1	3
Participants reporting MPN results outside the expected range for both replicates	2	2
Participants reporting MPN results as censored results for one replicate	0	0
Participants reporting MPN results as censored results for both replicates	1	8
Participants reporting MPN results inconsistent with ISO 7218 (Anon 2007)[2]	9	8
Participants not returning results	0	0
Participants not receiving material due to problems at customs	0	0

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

[2] points deducted from participants returning results inconsistent with ISO 7218.

Table 5: Participants' results and allocated scores

Lab ID	Sample 1			Sample 2		
	Rep 1	Rep 2	Score	Rep 1	Rep 2	Score
3 *	690	330	12	230	330	9
7 *	2300	1700	6	110	45	12
9 *	NE	NE	-	230	170	12
10 *	230	110	12	78	110	12
13 *	330	330	12	78	20	12
17 *	490	220	12	330	140	9
19 * [c]	780	330	12	0	0	12
21 *	230	490	12	20	45	12
22 *	780	920	12	40	78	12
23 * [a b c]	45	68	8	0	0	12
27 *	330	130	12	20	20	12
32 *	170	330	12	<18	45	12
33 * [a]	490	1100	8	45	78	8
35 *	170	170	12	130	78	12
39 *	330	450	12	68	40	12
41 *	330	220	12	130	330	9
42 * [a]	130	230	8	20	68	8
43 *	490	220	12	40	40	12
44 * [a]	45	68	8	20	20	8

Lab ID	Sample 1			Sample 2		
	Rep 1	Rep 2	Score	Rep 1	Rep 2	Score
47 *	330	230	12	78	78	12
50 [a]	230	230	8	<18	<18	8
68 * [a]	230	110	8	NE	NE	-
69	780	230	12	<18	170	12
72	260	230	12	45	20	12
83 *	330	Void	7	45	130	12
90 *	230	220	12	45	20	12
92	230	330	12	68	45	12
96 [a]	330	130	8	<18	<18	8
98 [a]	110	140	8	20	20	8
100	330	220	12	20	20	12
147 *	20	<18	4	130	78	12
170 *	<200	<200	12	<200	<200	12
189	130	230	12	<18	<18	12
203	68	230	12	20	20	12
212 *	760	2500	9	<67	<67	12
236	230	330	12	<18	20	12
245 [a]	460	490	8	50	50	8

* Designated NRL's,

[a] Scores deducted as tube combination inconsistent with rules specified in ISO 7218.

[b] Score deducted as incorrect MPN value given for recorded tube combination.

[c] Scores were not deducted on this occasion as further discussion is required regarding the value given by the MPN calculator reported for tube combinations of 0 0 0.

NE – Not examined.

General comments

Thirty-seven laboratories (26 NRL and 11 other laboratories) were sent material with all laboratories returning results. Information provided by laboratories on the arrival time of the material showed that 76% (28) of laboratories received the material the day after dispatch (01.12.15), with 41% (15) of these laboratories analysing the material on arrival. Sixteen percent (6) of laboratories received material within 48 hours of dispatch, with the remaining 8% (3) of laboratories receiving the material within 72 hours after dispatch. Forty-one percent (15) of laboratories analysed the material the day after receiving the material with exception of laboratory 23 who analysed their material 42 hours after receipting the material. Temperature record range of 0.5 – 7.0°C. All temperature data, arrival and analysis dates and times recorded by participants are shown in Appendix I.

Sample analyses

Thirty-seven laboratories returned the report form for this PT distribution. Laboratory 9 and 68 did not examine one of the samples due to laboratory error.

Sample 1 – Mussels

Thirty-two laboratories returned duplicate *E. coli* MPN/100g results falling between ±3 SD of the participants' median with 24 laboratories obtaining a score of 12. Laboratory 212 reported 1 replicate result between ±3 and ±5 SD of the participants' median. Laboratory 7 reported both replicate results between ±3 and ±5 SD of the participants' median. Laboratory 147 both replicate results outside ±3 SD of the participants' median with 1 replicate falling between ±3 and ±5 SD and the other falling outside ±5 SD of the participants' median.

Nine laboratories (laboratories 23, 33, 42, 44, 50, 68, 96, 98 and 245) were deducted points for reporting tube combinations inconsistent with the guidance given in ISO 7218 for interpretation of 5 x 3 MPN tables. Participants' are reminded that for official control testing of live bivalve molluscs in the EU, the 5 x 3 MPN tables or MPN calculator in ISO7218:2007/Amd1:2013 and the EURL generic protocol for enumeration of *E. coli* in bivalve molluscs (Issue 11) should be used.

Sample 2 - Oysters

Thirty-one laboratories returned duplicate *E. coli* MPN/100g results falling between ±3 SD of the participants' median with 26 laboratories obtaining a score of 12. Laboratories 3, 17 and 41 reported one replicate result within ±3 SD of the participants' median. Laboratories 19 and 23 reported both replicate results as 0. Scores were not deducted on this occasion as further discussion is required regarding the value given by the MPN calculator reported for tube combinations of 0 0 0 is 0 – previous discussions at an EURL workshop identified that this could be confusing (a zero is given whatever dilution set is used). The tables in the EURL generic protocol give the value for the standard dilution series as <18/ 100g, as agreed at that workshop.

Eight laboratories (laboratories 23, 33, 42, 44, 50, 96, 98 and 245) were deducted points for reporting tube combinations inconsistent with the guidance given in ISO 7218 for interpretation of 5 x 3 MPN tables. Laboratory 245 reported MPN values inconsistent with the tube combination provided. Participants are reminded that for official control testing of live bivalve molluscs in the EU, the 5 x 3 MPN tables or MPN calculator in ISO7218:2007/Amd1:2013 and the EURL generic protocol for enumeration of *E. coli* in bivalve molluscs (Issue 11) should be used.

Summary

Twenty-one laboratories (57%) achieved full marks for both samples tested for the enumeration of *E. coli*. For this distribution the EURL recommended participants to analyse the sample with 4 dilutions. Laboratories who incurred a deduction (7 laboratories) in scores were due to the advice given in ISO7218:2007/Amd1:2013 and/or the EURL generic protocol for calculating the MPN value for *E. coli* was not followed. Laboratories are requested to note in

ISO ISO7218:2007/Amd1:2013 it states '*In any circumstance when more than three dilutions are made, it is essential that all measured data values be used. It is not scientifically correct to "select" any combination of values on the premise that these values are more "correct" than other combinations. The results from all possible combinations of positive tubes should be recorded and the MPN calculator (http://standards.iso.org/iso/7218/) used to derive MPN values*'.

Those laboratories who achieved <40% of the maximum possible score in this distribution for *E. coli* enumeration (<5 out of the maximum 12 score) should review their laboratory procedures. In the first instance refer to the troubleshooting guide included as Appendix III. However, further guidance is available from the EURL.

References

Anon 2015. ISO TS 16649-3. Microbiology of food and animal feeding chain - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Geneva, Switzerland.

Anon 2002. ISO 6579. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva, Switzerland.

Anon 2013 ISO 7218:2007/FDAM 1:2013, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - Amendment 1. International Organization for Standardization, Geneva.

Anon 2001. ISO TS 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. Geneva, Switzerland.

Anon 2010 ISO 22117:2010 Microbiology of food and animal feeding stuffs – Specific requirements and guidance for proficiency testing by interlaboratory comparison. Geneva, Switzerland.

Figure 1: Results chart sample 1 – Mussels

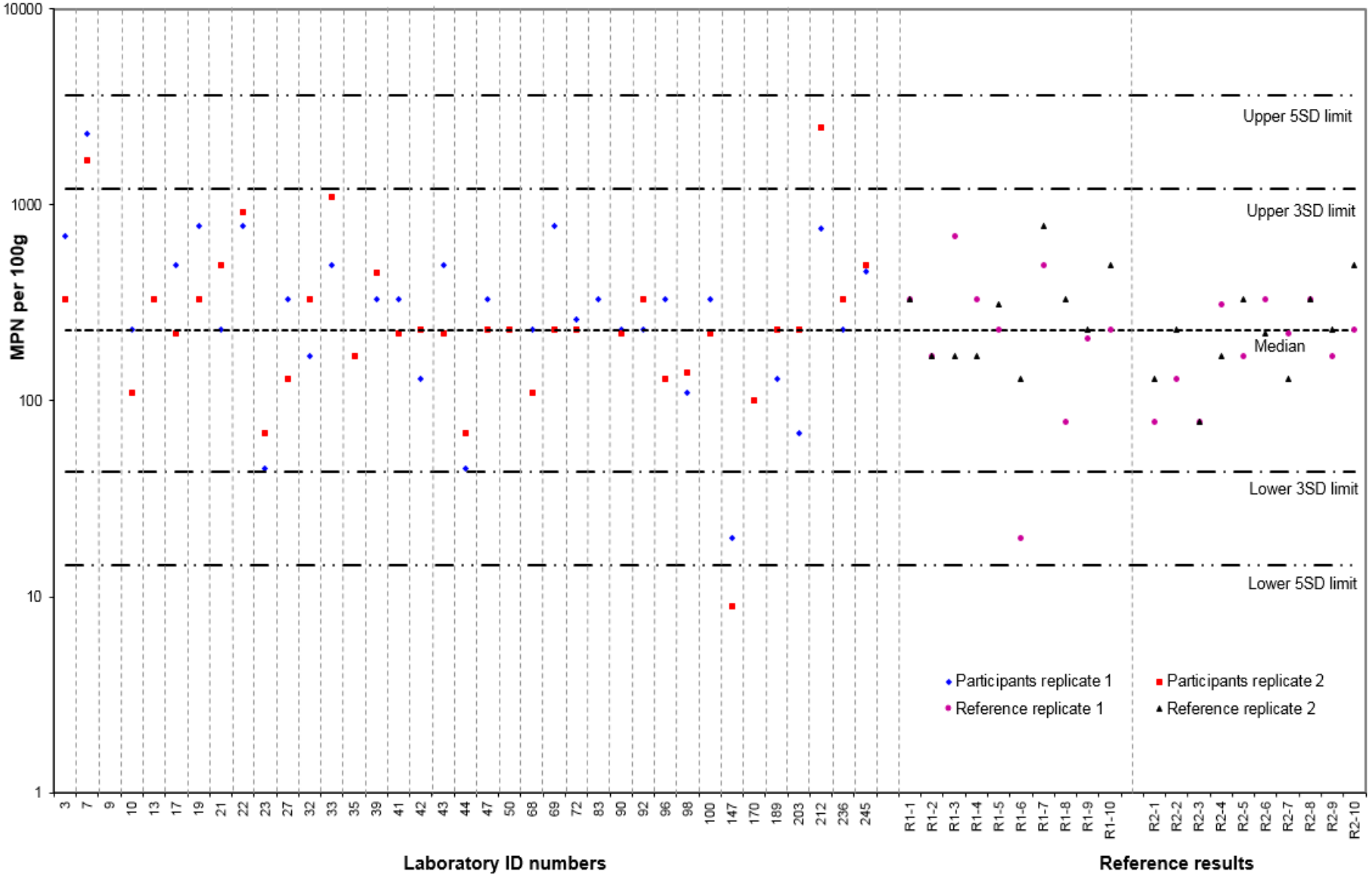
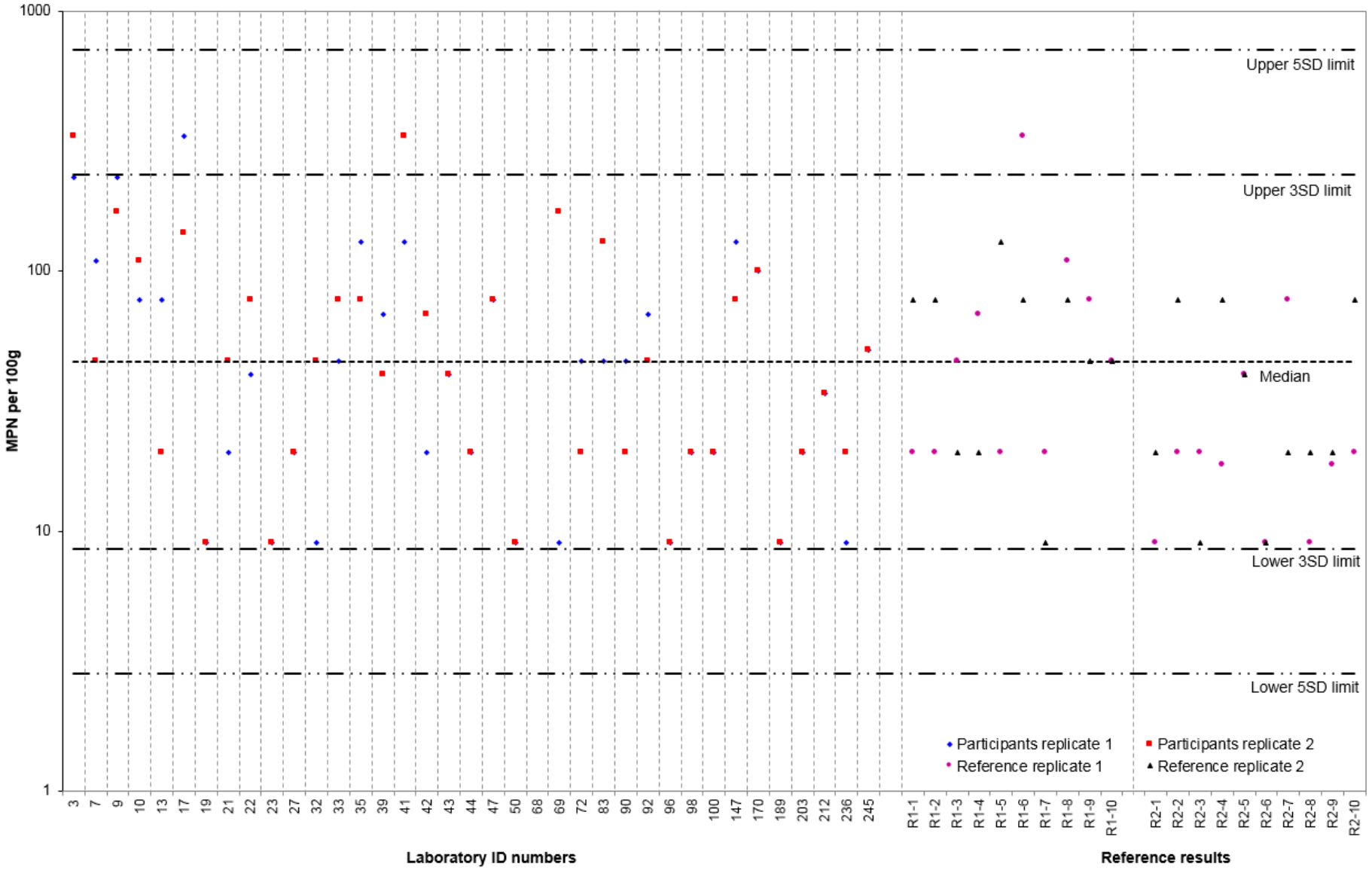


Figure 2: Results chart sample 2 – Pacific oysters



Appendix I

Sample arrival and temperature

Lab ID	Date arrived	Time of arrival	Sample (°C)	Storage (°C)	Date analysed	Time of analysis
3 *	01/12/2015	12:15:00	3	5	01/12/2015	16:00:00
7 *	01/12/2015	12:50:00	6		01/12/2015	13:30:00
9 *	01/12/2015	11:30:00	6	4	02/12/2015	10:30:00
10 *	01/12/2015	12:30:00	3		01/12/2015	14:00:00
13 *	01/12/2015	11:15:00	5	3	02/12/2015	13:00:00
17 *	01/12/2015	14:00:00	5		01/12/2015	15:00:00
19 *	02/12/2015	15:30:00	2.5	4	03/12/2015	09:00:00
21 *	01/12/2015	12:55:00	4.1		01/12/2015	13:30:00
22 *	01/12/2015	14:00:00	6.5		01/12/2015	14:10:00
23 *	01/12/2015	15:35:00	2.2	5	03/12/2015	09:30:00
27 *	01/12/2015	12:20:00	4.1		01/12/2015	13:00:00
32 *	01/12/2015	09:00:00	6.6		01/12/2015	
33 *	01/12/2015	13:15:00	3		01/12/2015	16:00:00
35 *	01/12/2015	13:15:00	2.8		01/12/2015	13:45:00
39 *	01/12/2015	12:00:00	2.8	3	02/12/2015	09:00:00
41 *	01/12/2015	11:00:00	3.9	4±2	01/12/2015	14:00:00
42 *	01/12/2015	16:45:00	7	5	02/12/2015	10:00:00
43 *	03/12/2015	08:50:00	1	4 - 8	03/12/2015	13:00:00
44 *	01/12/2015	16:30:00	2	3±2	02/12/2015	01:00:00
47 *	01/12/2015	11:15:00	5	4	02/12/2015	12:00:00
50	02/12/2015	11:20:00	3.2	3.1	02/12/2015	11:30:00
68 *	02/12/2015	11:30:00	3.0 - 3.1	3±2	03/12/2015	09:30:00
69	01/12/2015	13:30:00	2.06	4	02/12/2015	13:00:00
72	01/12/2015	11:10:00	5	2.8	01/12/2015	13:30:00
83 *	03/12/2015	13:20:00	0.5	3.5	03/12/2015	15:40:00
90 *	03/12/2015	09:36:00	3.9		03/12/2015	
92	02/12/2015	13:00:00	4.4	4	02/12/2015	13:40:00
96	02/12/2015	16:00:00	5.8	4	03/12/2015	13:30:00
98	01/12/2015	11:45:00	1.5	3	01/12/2015	14:00:00
100	01/12/2015	13:45:00	6	3±2	02/12/2015	12:00:00
147 *	01/12/2015	15:30:00	4	3±2	02/12/2015	
170 *	02/12/2015	15:30:00	2.8	4	03/12/2015	00:00:00
189	01/12/2015	16:15:00	0.5	4	02/12/2015	13:00:00
203	01/12/2015	12:50:00	2.1 - 2.3		01/12/2015	13:00:00
212 *	01/12/2015	11:00:00				
236	01/12/2015	11:45:00		3±2	01/12/2015	
245	01/12/2015	13:00:00	1.1	2	02/12/2015	09:45:00

* Designated NRL's

Appendix II:

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

Reported results	Returning of results	Replicate 1	Replicate 2	Total score
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 replicates 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 replicates MPN results are outside the expected range. The first falls between the median ±3SD and median ±5SD values and the second falls outside the median ±5SD values	2	2	0	4
Both replicates MPN results is outside the median ±5SD value	2	0	0	2

***E. coli* MPN scores allocated to participants returning 1 single replicate results**

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 is outside the median ±5SD value	2	0	2

***E. coli* score deductions**

Result	Score deducted	
	Replicate 1	Replicate 2
Tube combination inconsistent with MPN reported and / or tube combination selected not consistent with rules given in ISO 7218:2007/FDAM1:2013 or MPN tables provided by the EURL.	2	2
High censored result (e.g. MPN = >18000 per 100g)	2	2
Sample not examined or results returned late - no explanation received	12	

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

    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 have you 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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