European Community Reference laboratory
for monitoring bacteriological and viral
contamination of bivalve molluscs

Report on the whole animal bivalve shellfish ring trial:

Enumeration of *Escherichia coli* and the detection of S*almonella* spp..

CRL ring trial reference: RT 28 (*E. coli/Salmonella* 2008)

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1.0 Introduction

In November 2008 the CRL organised a distribution of naturally contaminated common mussels (*Mytilus edulis*) for enumeration of *E. coli* and detection of *Salmonella* spp.

2.0 Proficiency testing sample

2.1 Sample preparation and distribution

One batch, consisting of approximately 1500 *M. edulis*, was collected from a UK commercial producer. On arrival at the Cefas laboratory sub-samples of approximately 35 mussels were selected at random and placed in clean plastic sample bags containing a numbered temperature logger (Thermotrack, Progress Plus). Individual samples were placed in 10 litre Biotherm thermal control units and packaged according to Cefas procedure for use of 10L Biotherm boxes and IATA regulations. Fifty-five samples were distributed under refrigeration conditions by CitySprint courier services on 24th November 2008. The remaining mussels were stored at 3±2˚C for reference testing. On receipt, participants were requested to analyse the material immediately and to return the temperature logger to the CRL.

2.2 Sample temperature

Participants were asked to manually record the internal air and sample temperature on arrival and return the temperature logger. The temperature range (as determined from downloaded loggers), and participants' temperature measurements are given in Appendix I.

3.0 Results

3.1 Confidentiality of results

Each laboratory was provided with a personal identification number to preserve anonymity.

3.2 Reference results

Twelve sub-samples were analysed using the CRL standard methods (ISO 16649-3 and ISO 6579). The reference results are summarised in Table 1 and included, for *E. coli*, in Figure 1.

Table 1: The *E.coli* range and *Salmonella* spp. presence / absence from the reference results

Sample description	*E. coli* MPN/100g	*Salmonella spp..* Present in 25g	Absent in 25g
M. edulis	1.7×10^3 – $>1.8 \times 10^4$	4 from 12	8 from 12

3.3 Analysis of results

Participants duplicate *E. coli* and *Salmonella* results are given in Table 2. The median and upper and lower limits (±3 standard deviations (SD) and ±5 SD) were calculated from participants' results. SD calculations were based on the expected inherent variability of the five tube MPN method which is 0.26 $\log_{10}$. The results chart was compiled using $\log_{10}$ transformed MPN values (Figure 1). Reference results were omitted from this calculation. Performance assessment was carried out according to the procedures described in the CRL/HPA EQA shellfish scheme, with minor modifications to reflect replicate analyses of a single sample (Appendix II). The participants' and reference results median and SD and the expected range for *E. coli* estimated from participants' results are given in Tables 3 and 4. Scoring for detection of *Salmonella* spp. was not undertaken.

Table 2: Results reported by participants for *E.coli* and *Salmonella* spp..

Lab ID	*E.coli* MPN/100g			*Salmonella* spp. in 25g	
	Replicate 1	Replicate 2	Score	Replicate 1	Replicate 2
3	1700	2400	12	absent	absent
7	1300	790	12	absent	absent
9	1100	1400	12	absent	absent
10	3500	1300	12	absent	absent
11	1100	1100	12	absent	absent
12	750	2000	12	absent	absent
13	5400	2400	12	absent	absent
19	1100	3500	12	absent	absent
21	90	310	9	absent	absent
22	1100	1100	12	absent	absent
23	310	950	12	absent	absent
26	790	490	12	absent	absent
27	750	2400	12	absent	absent
30	750	1700	12	absent	absent
32	750	1300	12	absent	absent
33	1300	2400	12	absent	absent
35	1100	1700	12	absent	absent
36	2400	1300	12	absent	absent
39	1400	2100	12	absent	absent
41	1300	2400	12	absent	absent
42	5400	3500	12	absent	absent
43	1400	1300	12	absent	absent
44	330	490	12	absent	absent
47	2100	330	12	absent	absent
56	310	750	12	NE	NE
58	1300	700	12	absent	present
61	3500	1300	12	absent	absent
63	700	750	12	absent	absent
64	790	4600	12	NE	NE
68	490	330	12	absent	absent
70	790	790	12	absent	absent
77	2400	2200	12	absent	absent
79	1700	1700	12	absent	absent
85	5400	750	12	absent	absent
86	1300	2000	12	absent	absent
88	2400	2400	12	absent	absent
90	1300	1700	12	absent	absent
96	700	230	12	absent	absent
101	1700	2400	12	absent	absent
103	1300	490	12	absent	absent
108	1700	1300	12	absent	absent
109	310	500	12	absent	absent
112	NR	NR	2	NR	NR
117	3500	2400	12	absent	absent
120	3500	1100	12	absent	absent
125	3500	2200	12	absent	absent
126	3500	1100	12	absent	absent
131	500	500	12	NE	NE

Lab ID	*E.coli* MPN/100g			*Salmonella* spp. in 25g	
	Replicate 1	Replicate 2	Score	Replicate 1	Replicate 2
135	2200	2200	12	absent	absent
139	5400	3500	12	absent	absent
142	1300	1300	12	absent	absent
144	500	500	12	absent	absent
148	1100	1100	12	absent	absent
149	1300	1100	12	absent	absent
150	5400	5400	12	absent	absent

NE - Not examined
NR - Not reported

3.4 Summary participation statistics for RT 28

Total participants reporting duplicate results for *E. coli* MPN	54
Participants reporting single MPN	0
Participants reporting MPN results within the expected range[1]	53
Participants reporting MPN results outside the expected range for one replicate	1
Participants not returning results	1

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

Table 3. Participants and reference results for *E. coli* MPN – replicates combined

	Median MPN/100g	Standard deviation
Reference results	$2.6 \times 10^{4*}$	1.5×10^{4}
Participants results	1.3×10^{3}	1.3×10^{3}

* censored results were allocated a nominal score of 3.6×10^{4}

Table 4. Expected range for *E.coli* obtained from participants' results

	Expected range for *E. coli* MPN/100g (median ±3 SD)
Participants results	$2.2 \times 10^{2} – 7.8 \times 10^{3}$

4. Conclusion and recommendations

4.1 General comments

Fifty-four laboratories (20 NRLs and 34 in country Official Control laboratories) returned results for the distribution. Fifty-six percent of samples arrived within 24 hr of dispatch. Thirty-eight laboratories analysed the samples on the day of arrival. Of the remaining 16 laboratories, 15 analysed on the following day (i.e. within 48-72 hr of dispatch).

4.2 *E. coli* analysis and methodology

Fifty-three laboratories returned replicate results between ±3 SD of the participants' median. Laboratory 21 returned a single replicate between 3 and 5 SD of the participants' median. Laboratory 112 did not examine the sample within the specified timeframe. The laboratory indicated that the sample had arrived too late for analysis to be carried out on the day of arrival. On request of the CRL the courier (CitySprint) provided records indicating that the delivery time at the institute was 14:20. It is recommended that laboratory 112 examine their internal quality procedures for receipt of samples.

Laboratories 12, 47, 64, 86 and 144 returned MPN results that were not consistent with the CRL guidance on interpretation of 5x3 MPN tables. For CRL guidance on the interpretation of MPN tube combinations see Appendix III. Laboratory 39 gave a tube combination (5,3,4) which would, using CRL guidance on use of MPN tables, be considered void. The laboratory reported an MPN value of 2100 which was within the expected range.

Fifty-three laboratories from the fifty-four returning results reported the use of the *E. coli* reference method (ISO TS 16649-3) or Donovan *et al* 1998. Laboratory 64 reported the use of NMKL 96 for enumeration of *E. coli.* This method has not been formally validated according to ISO 16140 for bivalve shellfish.

4.3 *E. coli* enumeration - reference results

Reference results generated by replicate sample testing by the NRL indicated a high level of between sample variability, with 6 sub-samples returning *E. coli* MPN/100g of >1.8 $x10^4$ (range 1.7 x 10^3 –>1.8 x 10^4). For estimation of the reference result median and graphical representation of data points censored values were allocated a nominal score of 3.6 x 10^4.This high level of variability was not observed in participant's results. The reasons for this sample to sample variation were not clear, however on investigation it was identified that all reference sub-samples were derived from one of three large boxes containing the total consignment, whereas participants' sub-samples were extracted from the two remaining boxes. The mussels were harvested by the producer and collected by a commercial courier from the site already boxed. It is speculated that mussels from the third box (reference material) were sampled from a different lateral or longitudinal position across the bed that had been exposed to different levels of faecal contamination. This degree of between sample variation is substantially outside the observed measurement uncertainty of a 5-tube, 3-dilution MPN assay for live bivalve shellfish (Anon 2003), thus it is suggested that unusual environmental factors influenced sample content on this occasion.

The CRL will produce written procedures for randomisation of ring trial sub-samples to reduce the potential for reoccurrence of this anomaly.

4.4 *Salmonella* spp. analysis and methodology

Only one laboratory detected *Salmonella* spp in a single replicate.

Seventy-six percent of laboratories used the EU specified reference method for *Salmonella* spp (ISO 6579). Three laboratories referenced NMKL 71: *Salmonella* detection in foods. Two laboratories referenced Vidas and BAX (PCR method) to detect for *Salmonella* analysis. These methods have not been formally validated according to ISO 16140 for bivalve shellfish.

4.5 *Salmonella* spp. analysis - reference results

Salmonella spp. was detected in a third of the reference samples. At low levels *Salmonella* may have been absent in a proportion of samples and potentially below or at the limit of detection of the assay in others. Previous studies at the CRL have estimated that the limit of detection of ISO 6579 in seeded bivalve shellfish matrix is 4CFU/25g. However, given the elevated *E. coli* levels in a sub-population of the mussel samples, it is considered likely that detection of *Salmonella* spp. in the reference results was associated with exposure to high levels of faecal contamination. All 4 *Salmonella* spp. positive reference samples returned *E. coli* MPN/100g >1.8 x 10^4.

4.6 Transit time and temperature

The maximum internal temperature of samples during transit as determined by the automatic temperature loggers did not exceed the maximum receipting of 10°C as recommended by the CRL (Anon 2007).

5. References

Anon 2004 ISO/TS 16649-3:2004. '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 2003 Report (WD SANCO/58/2003) upon Measurement uncertainties in microbiological analyses- with special reference to the uncertainties linked to the proposed microbiological criteria in the document SANCO/4198/2001, rev.5 .

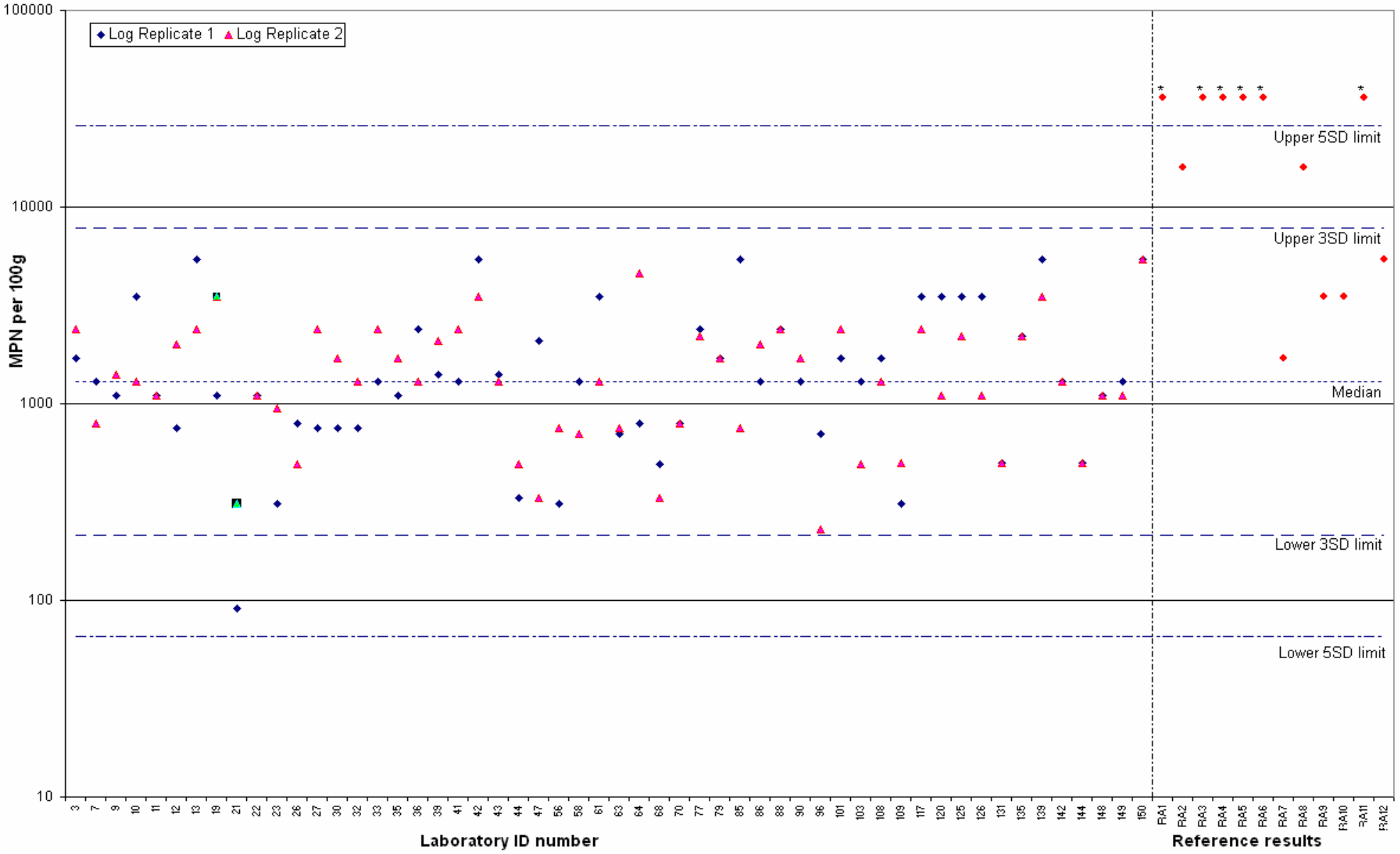
Anon. 2002. ISO TS 6579-2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella spp. ISO Copyright Office, Case Postale 56, CH-12111, Geneva 20, Switzerland.

Anon 2007. Microbiological monitoring of bivalve mollusc harvesting areas, a guide to good practice: technical application. CRL publication, issue 3, February 2007.

Donovan TJ, Gallacher S, Andrews NJ, Greenwood MH, Graham J, Russel JE, Roberts D, Lee R.1998. 'Modification of the standard method used in the united kingdom for counting *escherichia coli* in live bivalve molluscs'. Communicable disease and public health 1: 188-96.

Figure 1: Participants duplicate results for *E.coli* (MPN) on whole animal bivalve shellfish

Key - * *E.coli* reference results were given value of 36,000 as results reported were >18,000.



Appendix I: Sample temperatures

All samples were dispatched on the 24th November 2008.

Country	Time of arrival	Date of arrival	Temp. logger (°C)[1]	Internal air (°C)[2]	Sample (°C)[2]	Storage (°C)[2]	Date analysed
Austria	09:30	26.11.08	<4.0	5.3	2.3	2 – 6	26.11.08
Bulgaria	14:40	25.11.08	<9.0	6.1	3.2	4	26.11.08
Croatia	12:15	27.11.08	<4.0	3.2	3.3	4	27.11.08
	11:24	27.11.08	<6.0	7.2	2.9	-	27.11.08
Denmark	13:30	26.11.08	<8.0	6.6	4.6	5	27.11.08
	11:15	26.11.08	<8.5	-	3	3	26.11.08
	11:20	26.11.08	<9.5	2.6	2.5	-	26.11.08
Finland	15:00	25.11.08	<5.0	-	-	5	26.11.08
France	09:30	26.11.08	<4.0	8.2	2.2	4.2	26.11.08
Germany	09:35	26.11.08	-	4	3	-	26.11.08
Greece	12:35	27.11.08	<8.0	3.4	3.2	5	27.11.08
Hungary	11:30	25.11.08	<3.5	5.5	3.2	3.5	26.11.08
Ireland	09:30	25.11.08	<5.0	6.3	3.1	-	25.11.08
	12:05	26.11.08	-	5.2	4.3	3.1	26.11.08
	11:30	26.11.08	-	4.5	4.2	3.6	26.11.08
	14:30	26.11.08	-	-	4	-	26.11.08
	15:00	25.11.08	<9.0	5	5	3	26.11.08
	10:00	26.11.08	<8.5	9.7	3.4	4	26.11.08
Italy	-	27.11.08	<7.5	3	1.5	4	27.11.08
Korea	18:00	27.11.08	<8.0	4	3.9	-	27.11.08
	15:40	28.11.08	<8.5	7.3	6.8	-	28.11.08
	12:57	27.11.08	<6.5	6	3.7	2.7	27.11.08
Latvia	15:30	25.11.08	<6.0	5.4	5	5	26.11.08
Lithuania	15:50	25.11.08	<5.0	0.4	4	4	26.11.08
Netherlands	11:00	27.11.08	<5.0	-	-	4	28.11.08
	10:30	26.11.08	<3.5	-	3.9	4	27.11.08
Norway	15:00	25.11.08	<2.5	-	-	-17	26.11.08
Poland	14:35	26.11.08	<6.5	7.5	6.2	2 – 8	27.11.08
Portugal	10:50	25.11.08	-	8	4	2 – 7	25.11.08
Romania	17:20	26.11.08	<6.0	3.2	2	4	27.11.08
Slovakia	11:00	26.11.08	-	-	-	3	27.11.08
Slovenia	12:00	25.11.08	<6.0	5	5	-	25.11.08
Spain	13:35	26.11.08	<9.5	5	4	1 – 3	27.11.08
Sweden	13:30	25.11.08	<8.0	-	3	5	25.11.08
	11:30	25.11.08	<6.5	3.8	1.6	4.2	25.11.08
UK		25.11.08	<6.0	-	-	2 - 5	25.11.08
	10:15	25.11.08	<3.0	-	3.7	4.1	25.11.08
	12:05	25.11.08	<4.5	2.9	0.8	4	25.11.08
	09:15	25.11.08	<7.0	-	3.6	0 - 5	25.11.08
	09:10	25.11.05	<9.5	6.5	3.5	-	25.11.08
	11:40	25.11.08	<4.0	1.8	3.4	4.5	25.11.08
	09:30	25.11.08	<5.0	10.8	3.8	-	25.11.08
	11:05	25.11.08	<6.5	2.2	2.5	5.6	25.11.08
	09:15	25.11.08	<6.0	5.8	4.4	4.2	25.11.08
	08:24	25.11.08	<2.0	6.3	3.8	4.9	25.11.08
	10:45	25.11.08	<7.5	3.7	-	4.6	25.11.08

Country	Time of arrival	Date of arrival	Temp. logger (°C)[1]	Internal air (°C)[2]	Sample (°C)[2]	Storage (°C)[2]	Date analysed
UK	14:20	25.11.08	-	-	-	-	-
	09:00	25.11.08	<4.5	1.5	0.9	1.5	25.11.08
	09:09	25.11.08	<6.5	3.62	3.62	3	25.11.08
	10:28	26.11.08	<5.0	7.9	2.9	4.6	26.11.08
	09:30	25.11.08	-	7.7	7.5	4	8.12.08
	16:00	25.11.08	<9.5	9.1	4	-	25.11.08
	12:45	25.11.08	-	3.2	4.5	2.6	26.11.08
	11:50	25.11.08	<2.0	3.3	2.6	3.7	25.11.08
	16:15	25.11.08	<3.0	4.7	2.9	3.5	25.11.08

[1] temperature indicated by sample temperature recorder
[2] temperature recordings by participants.

Appendix II: *E.coli* MPN scoring

Result	Points allocated
Return of results	2
All replicate MPN results within the expected range	10
Or	
One replicate MPN result reported is outside the expected range and falls between the median ±3SD and median ±5SD value	7
Or	
Both replicate MPN results are outside the expected range and fall between the median ±3SD and median ±5SD value	4
Or	
One replicate MPN result reported is outside the median ±5SD value	5
Or	
Both replicate MPN results reported is outside the median ±5SD value	0
Or	
Single MPN result reported only	5
Or	
Tube combination inconsistent with MPN reported (one replicate)	7
Or	
Tube combination inconsistent with MPN reported (both replicates)	5
Or	
Sample not examined or results returned late - no explanation received	0
Or	
High censored result (e.g. MPN = >18000 per 100g)	Score not allocated

Appendix III: Calculation of *E.coli* Most Probable Number (MPN) and reporting

Cefas Standard Operating Procedure – Enumeration of *Escherichia coli* in molluscan shellfish 6. www.crlcefas.org. Adapted from: ISO 7218:2007.

To calculate the most probable number (MPN), record the number of TBGA plate positives for each dilution. This gives a three figure tube combination number, which is used to calculate the MPN. MPN tube combinations fall into one of four categories. 95% of observed tube combinations fall in to category 1 with 4%, 0.9% and 0.1% in categories 2, 3 and 0 respectively. Both the category and MPN result can be determined from the MPN table (see Appendix 2) as follows:

- For dilutions of neat, 10–1 and 10–2 use MPN Table 1.
- For dilutions of 10–1, 10–2 and 10–3 use MPN Table 2.
- For dilutions of 10–2, 10–3 and 10–4 use MPN Table 3.
- For greater dilutions use MPN Table 3 and multiply the result by the extra number of dilution factors.

Where more than three dilutions have been tested for a sample, select the tube combination as stated in the following rules:

1.Select the combination of three consecutive dilutions having a category 1 profile to obtain the MPN index. If more than one combination having a category 1 profile is obtained, use the one with the highest number of positive tubes.

2. If no combination having a category 1 profile is available, use the one having a category 2 profile. If more than one combination having a category 2 profile is obtained, use the one with the highest number of positive tubes.

Results should be reported as the most probable number per 100g of shellfish. Negative samples should be reported as MPN <20/100g. Where the MPN tube combination is not given in the relevant table, the result should be reported as ‘Void’.

Note: The 5-tube 3-dilution MPN table given in ISO 7218:2007 includes all category 1 and category 2 combinations, and some (but not all) category 3 combinations. A note is included in the standard that: “Before starting testing, it should be decided which category will be acceptable, that is, only 1, 1 and 2 or even 1, 2 and 3. When the decision to be taken on the basis of the result is of great importance, only category 1, or at most 1 and 2, results should be accepted. Category 0 results should be considered with great suspicion”. Given that the NRL generic SOP will be referred to by official control laboratories, all of the category 3 combinations have been omitted from the version of the tables presented here.