CEFAS
The Centre for Environment, Fisheries & Aquaculture Science

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

# Report on *E.coli* shellfish homogenate ring trial, 2003

**Author : Louise Stockley, 17th March 2004**

**CRL ring trial reference : RT5 (*E.coli* in shellfish, 2003)**

CEFAS Weymouth Laboratory,
Barrack Road, The Nothe,
Weymouth, Dorset, DT4 8UB, UK
Telephone : +44 (0) 1305 206600
Fax : +44 (0) 1305 206601
E-mail : fsq@cefas.co.uk
Website : www.crlcefas.org

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## Background and purpose of ring trial

The Centre for Environment, Fisheries and Aquaculture (CEFAS) Weymouth was designated by the European Council on 29 April 1999 to be the co-ordinating European Community Reference Laboratory (CRL) for monitoring bacteriological and viral contamination of bivalve molluscs. Member states were also each required to designate a National Reference Laboratory (NRL) to liase with the CRL and to co-ordinate activities in their country. One of the responsibilities of the CRL is to organise comparative testing among NRLs. The 2002 workshop of NRLs supported the use of the CRL/HPA Collaborative Shellfish EQA Scheme as the primary means of comparative bacteriological testing between NRLs. However, at the 2003 workshop it was agreed that the CRL would organise a ring trial among NRLs of either whole shellfish, or shellfish homogenates, to challenge further aspects of the *E.coli* method. This ring trial was also intended to generate comparative data from analysis of naturally contaminated shellfish for comparison with the ongoing EQA scheme which uses fully characterised freeze dried bacterial cultures representative of shellfish flora. It is important that the performance of laboratories and faecal indicator methods in the EQA scheme can be regarded as representative for naturally contaminated shellfish.

The CRL distributed under controlled temperature conditions 3 individual samples of oyster homogenates, A, B and C, that required examination on 9th October 2003.

## Sample preparation

Batches of approximately 200 Pacific oysters (*Crassostrea gigas*) were collected from 2 UK commercial production areas with differing classifications under Directive 91/492 - a classification B area and a classification C area. For the classification B area a sample was also obtained following commercial depuration. Each batch was cleaned, shucked and homogenised into groups of approximately 25 shellfish. The homogenates from the groups of a single batch were then combined and mixed well. A sample of each batch was then tested in duplicate by the CRL to determine the concentration of *E.coli* present in

the sample. The method used for analysis of *E.coli* was the CRL reference method (Donovan *et al* 1998, see www.crlcefas.org for method details). Table 1 shows the concentration of *E.coli* present in each of the samples following initial analysis.

| Sample code | Description | Replicate 1 | Replicate 2 |
|---|---|---|---|
| A | Classification B site | 500 | 200 |
| B | Classification C site | 3500 | 2400 |
| C | Depurated sample | <20 | <20 |

Table 1: The concentration of *E.coli* present in each sample at initial analysis.

**Distribution of samples and reference results**

The samples from each batch were dispensed into labelled plastic centrifuge tubes in 50ml volumes. Each laboratory undertaking the homogenate ring trial was supplied with 2x50ml of homogenate of each sample in its neat form. These were placed in a sealed container with cool packs to the specification recommended by the courier. The containers were sealed with the relevant documentation and were transported by courier. All samples were dispatched on the 3rd October 2003.

To control sample transport conditions the CRL specified to the courier that the samples should be kept refrigerated throughout the duration of their transportation and arrive within the period specified by the courier. The latter depended on the country and ranged from 1 to 3 days.

**Analysis of samples by participants**

All NRLs were contacted to see whether they wished to take part in the *E.coli* homogenate ring trial. 13 NRLs indicated agreement to participate and were distributed samples. 11 NRLs returned results. Details of NRLs participating in the ring trial are given in table 2.

On receipt of samples laboratories were asked to refrigerate the samples until the target analysis date of 9th October, records show that this was done. Participants were asked to

analysis each sample in triplicate in accordance with the NRLs routine method for the enumeration of *E.coli* (or faecal coliforms). The enumeration methods used in the ring trial by the individual participants' are shown in table 2. It should be noted that all laboratories participating in the ring trial used an MPN method of analysis.

| Country | Participation in ring trial | Delegates sent samples | Results sent to CRL | Routine method | Comments |
|---|---|---|---|---|---|
| Austria | Yes | Yes | Yes | Donovan | |
| Belgium/ Luxembourg | Yes | Yes | Yes | NF-V-08-600 | |
| Denmark | Yes | Yes | Yes | N.M.K.L. | |
| Finland | Yes | Yes | No | | Samples delayed, out of temp spec. |
| France | Yes | Yes | Yes | NF-V-08-600 | |
| Germany | Yes | Yes | Yes | Donovan | |
| Greece | Yes | Yes | Yes | Donovan | |
| Ireland | Yes | Yes | Yes | Donovan | |
| Italy | Yes | Yes | Yes | A1 medium | |
| Netherlands | Yes | Yes | No | | Not tested due to 'capacity problems' |
| Norway | Yes | Yes | No | | |
| Portugal | Yes | Yes | Yes | Donovan | |
| Spain | Yes | Yes | Yes | Donovan | |
| Sweden | No | No | No | | |
| UK | Yes | Yes | Yes | Donovan | |

Table 2: Participation in ring trial by NRLs and analytical methods used.

It was noted that the temperature recorded by the individual participants on sample receipt was generally well above the specified temperature (refrigeration temperature) and thus the courier did not comply with the specified temperature transport criteria. The temperatures and arrival dates recorded by the participants are shown in table 3.

| Country | Date of dispatch | Date of arrival at lab * | Arrival temp (°C) | Date analysed |
|---|---|---|---|---|
| Austria | 03.10.03 | 07.10.03 | 15.1 | 09.10.03 |
| Belgium/ Luxembourg | 03.10.03 | 07.10.03 | room temperature | 09.10.03 |
| Denmark | 03.10.03 | 08.10.03 | 13 | 10.10.03 |
| Finland | 03.10.03 | | | not tested |
| France | 03.10.03 | 08.10.03 | 16 | 09.10.03 |
| Germany | 03.10.03 | 06.10.03 | 15.2 | 09.10.03 |
| Greece | 03.10.03 | 09.10.03 | 20 | 10.10.03 |
| Ireland | 03.10.03 | 08.10.03 | 17 | 09.10.03 |
| Italy | 03.10.03 | 10.10.03 | 20 | 13.10.03 |
| Netherlands | 03.10.03 | 07.10.03 | | not tested |
| Norway | 03.10.03 | 08.10.03 | | not tested |
| Portugal | 03.10.03 | 08.10.03 | 24 | 09.10.03 |
| Spain | 03.10.03 | 09.10.03 | 15 | 13.10.03 |
| UK | 03.10.03 | 03.10.03 | 4 | 09.10.03 |

*Arrival dates reported by courier

Table 3: The receipt and analysis dates with arrival temperatures for each participating NRL.

**Reference analysis performed by the CRL**

Following distribution to participants the CRL refrigerated the remaining sample homogenates and performed reference analysis for *E.coli* on the same day as the participants in the ring trial (9th October). The elapsed period between initial analysis and reference analysis varied between 6 to 9 days (Table 4). 12 reference analysis were performed for each sample using the CRL reference method. The range of reference results obtained are summarised in table 4 and shown in figures 1, 2 and 3.

| Sample No. | Description | Elapsed time | Range of E.coli results MPN/100g |
|---|---|---|---|
| A | Classification B site | 6 days | 220 - 2200 |
| B | Classification C site | 8 days | 3500 - 17000 |
| C | Depurated sample | 7 days | <20 |

Table 4: The range of *E.coli* reference results analysed by the CRL on the 9th October 2003 (see also figures 1, 2 and 3) and the elapsed time between initial analysis and reference analysis.

**Analysis of results from participants**

Presentation of the results and subsequent analyses, were undertaken on the MPN values reported by the participants. Where MPN values had not been reported, the CRL assigned values to the reported tube combination using the approach given in Roberts. D and Greenwood, M. 2003, Practical food microbiology, 3rd Ed, Blackwell Oxford.

The reported MPN values for each sample were compared with the median MPN from all reported results for that sample. The reference results were omitted from this calculation. The median was used instead of the mean as the latter may be more greatly affected by outlying results. For each distributed sample the log (base 10) of the median value was obtained and from this warning (± 3 x standard deviation) and action (± 5 x standard deviation) limits were calculated. Standard deviation (SD) calculations were based on the expected inherent variability of the five tube MPN method which is 0.26 $\log_{10}$. For sample C an arbitrary value of 10 was given to any results reported as being <18 or <20, for the purposes of calculating the median and plotting participants results. The results for participant 601, reported as <1.8 per gram, were plotted as 180 per 100 grams.

The triplicate results returned by the participants for each individual sample are shown in tables 5, 6 and 7 and plotted in figures 1, 2 and 3. The reference results are also shown.

**Results for Sample A**

| Lab ID | Replicate 1 | Replicate 2 | Replicate 3 |
|---|---|---|---|
| 593 | 2400 | 200 | 2400 |
| 594 | 490 | 1700 | 790 |
| 595 | 790 | 950 | 700 |
| 597 | 940 | 490 | 1100 |
| 598 | 2400 | 1100 | 600 |
| 599 | 310 | 320 | 380 |
| 600 | 70 | 70 | 320 |
| 601 | 1300 | 1100 | 450 |
| 603 | 1600 | 500 | 2000 |
| 604 | 310 | 750 | 700 |
| 608 | 750 | 500 | 310 |

Table 5: Results reported by the participants for sample A.

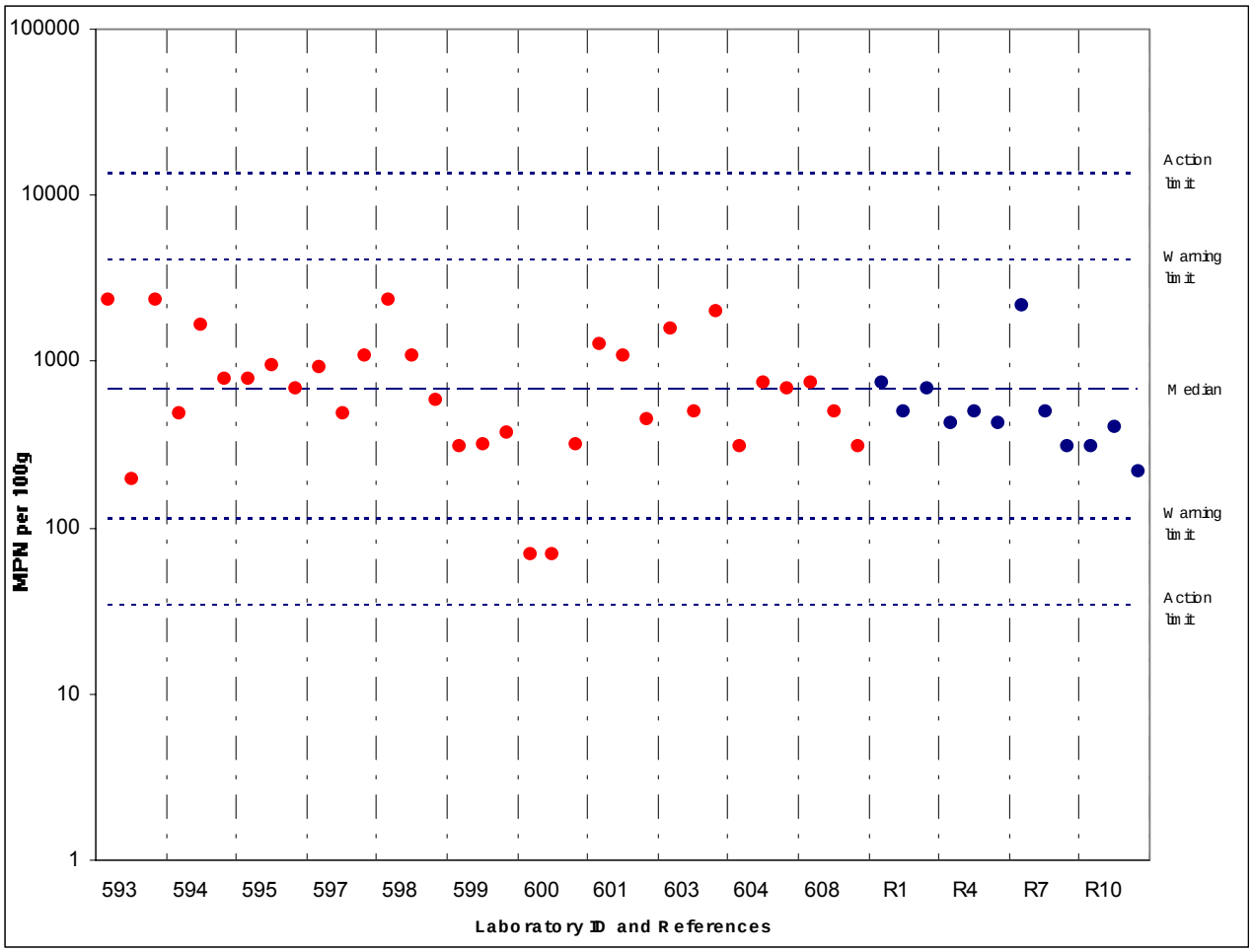


Figure 1: Sample A participants' and reference results compared to warning and action limits.

**Results for Sample B**

| Lab ID | Replicate 1 | Replicate 2 | Replicate 3 |
|---|---|---|---|
| 593 | 3500 | 16000 | 2200 |
| 594 | 4000 | 13000 | 4600 |
| 595 | 2400 | 16000 | 16000 |
| 597 | 7900 | 24000 | 17000 |
| 598 | 7000 | 2900 | 7500 |
| 599 | 2000 | 1400 | 1600 |
| 600 | 5000 | 13000 | 7500 |
| 601 | 13000 | 7900 | 2600 |
| 603 | 4300 | 17000 | 24000 |
| 604 | 13000 | 14000 | 7500 |
| 608 | 3500 | 5400 | 4300 |

Table 6: Results reported by the participants for sample B.

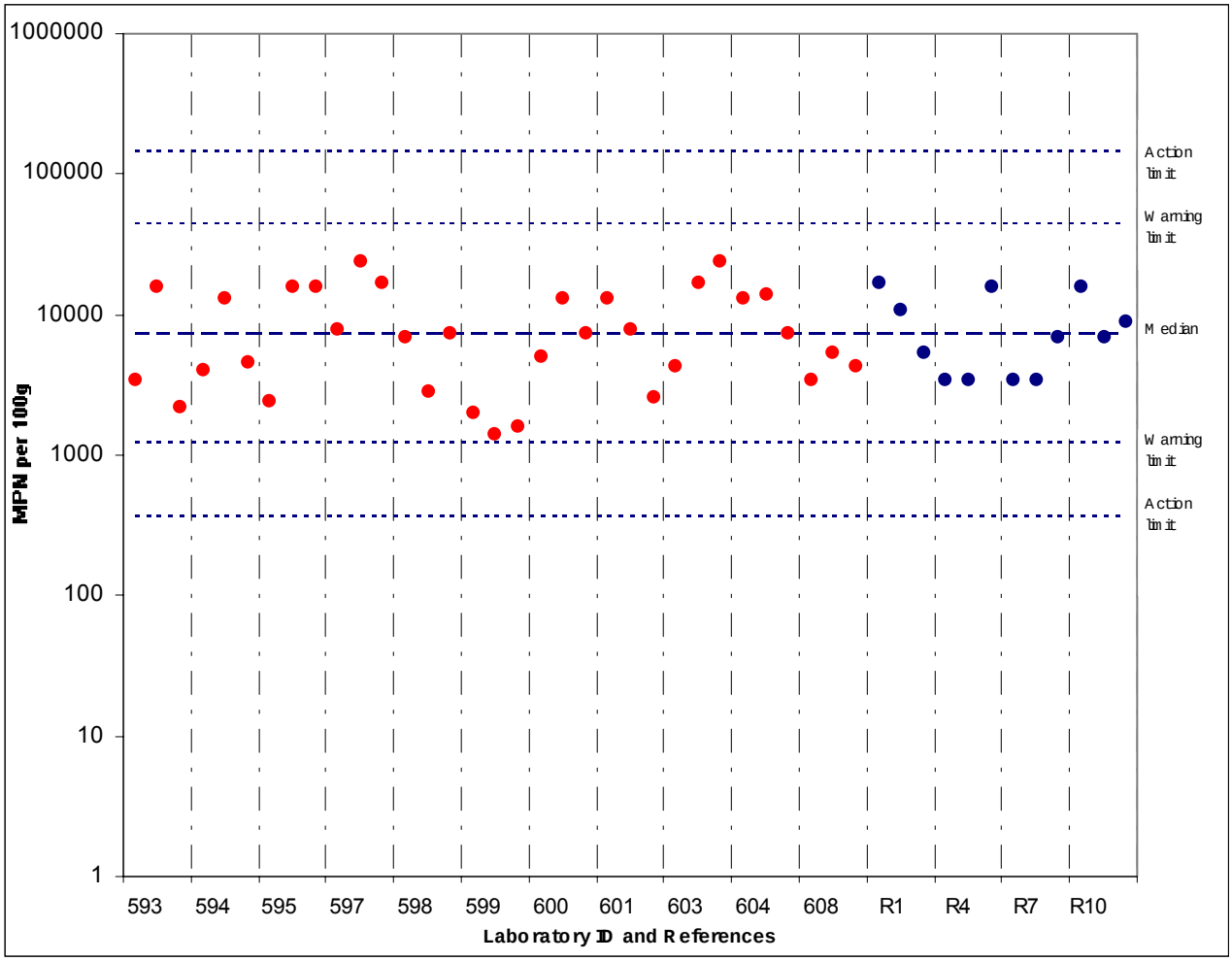


Figure 2: Sample B participants' and reference results compared to warning and action limits.

**Results for Sample C**

| Lab ID | Replicate 1 | Replicate 2 | Replicate 3 |
|---|---|---|---|
| 593 | <20 | <20 | <20 |
| 594 | <18 | <18 | <18 |
| 595 | <20 | <20 | <20 |
| 597 | <18 | <18 | <18 |
| 598 | 20 | 20 | <20 |
| 599 | <20 | <20 | <20 |
| 600 | 20 | <20 | <20 |
| 601 | <180 | <180 | <180 |
| 603 | <20 | <20 | <20 |
| 604 | <20 | <20 | <20 |
| 608 | <20 | <20 | <20 |

Table 7: Results reported by the participants for sample C.

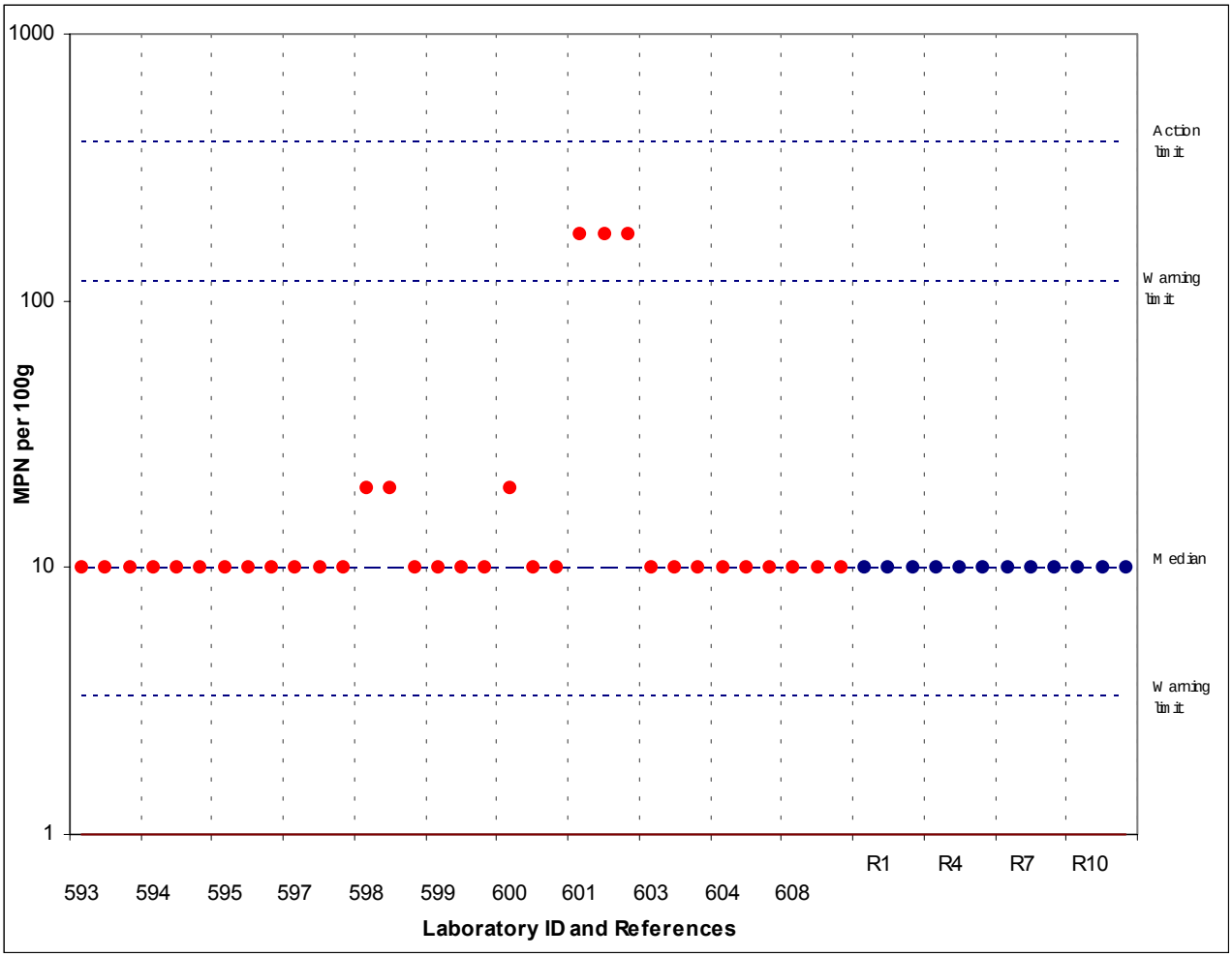


Figure 3: Sample C participants' and reference results compared to warning and action limits.

## Discussion

Transport criteria

Several participants noted that problems occurred during the transport of the samples. The elapsed time during transport often exceeded that agreed with the courier. Spoilage had occurred in several of the homogenates and the temperature of the containers were outside the limits specified to the courier. As naturally contaminated homogenates were used in the ring trial, such increases in temperature could have had a significant effect on the concentrations and relative amounts of the bacteria present in the samples.

Comparison with EQA

Standard deviations were determined for the log-transformed participant results for samples A and B. That for sample C could not be determined due to the large number of censored results (<20). For both samples, the values were approximately 0.37. The weighted mean standard deviation for the log-transformed results of all participants for 4 HPA Shellfish Scheme EQA samples was determined to be 0.42. Therefore, despite the distribution problems (elapsed time and maintenance of refrigeration temperatures) which occurred with the ring trial samples, the variability of the results was no greater than would have been expected from the EQA data. Other comparisons with the EQA, for instance whether specific laboratory performance was similar for both the EQA scheme and for analysis of naturally contaminated shellfish, were difficult to make because of the problems with temperature abuse of samples during this ring trial and the limited data set.

Laboratory performance

Sample A : All but one participant reported all results between the upper and lower warning limits. One participant reported 2 of 3 results below the lower warning limit. Despite the limited data available from the ring trial, it would be advisable for this laboratory to review its procedure.

Sample B : All participants reported results between the lower and upper warning limits.

Sample C : All participants reported results that conformed to the intended values. 1 laboratory reported results at a limit of detection that was higher than the calculated warning limit. Although this limit of detection was below the regulatory threshold for *E.coli* (230 *E.coli* per 100g) it did not permit comparison with the low values obtained by other participants for this depurated sample.

Tube combination

A number of the tube combinations reported by the participants did not coincide with the tube combinations specified on the MPN tables. Where MPN values had not been reported, the CRL assigned values to the reported tube combination. There are three ways that the tube combination can be interpreted. These are stated below using the 5,5,3,0 tube combination as an example.

1. Using the 5,5,3 combination = 9100 MPN/100g
2. Using 5,3,0 combination = 7500 MPN/100g
3. Finding the average from the two tube combinations = 8300 MPN/100g

For the purposes of this trial the CRL adopted the first process which uses only positive tubes to determine the MPN value when more than 3 dilutions are used. This requires clarification and will be raised during development of the ISO standard.

Method comparison

On the basis of the limited data obtained in these ring trials, the results reported by the participants using their routine methods for the enumeration of *E.coli* have reasonable consistency between the different methods. This is best demonstrated with the more contaminated samples with all the results reported being within the warning limits. Only 2 results for sample A fell below the warning limits and all results for sample B were within the action limits.

**Summary**

The ring trial was a qualified success. Although problems were experienced in the distribution of samples within specified temperature/time criteria this did not appear to grossly distort reported results. The ring trial has permitted comparison of results produced by different methods in use at NRLs on naturally contaminated samples. However, over analysis should be avoided because of the small number of data and the unsatisfactory conditions of sample transport.

The following conclusions can be drawn :

- Within the limits of the data generated by the ring trial all NRL participants appeared to have a similarly good level of performance and reported results were consistent with the reference values generated by the CRL.

- The method of analysis used by NRLs did not appear to grossly effect the reported result. It should however be noted that methods used by all participants in this ring trial were MPN procedures. This ring trial cannot therefore comment on the comparative performance of MPN and non-MPN methods.

- Before further ring trials of this nature are considered the sample distribution problems need to be addressed.

- The standard deviation of results reported in this ring trail was very similar to that reported in the CRL/HPA EQA scheme. Although this data set is limited it suggests that the EQA scheme may be considered as representative of naturally contaminated samples.

- Several of the results reported by the participants had unusual tube combinations. Not all tube combinations are presented on the MPN tables. There are also a variety of approaches that can be used to determine the MPN number. A standard method for determining the MPN number should be put in place.

- One participant reported results at a significantly higher limit of detection than the other participants. Harmonisation of assay detection limits could be desirable.

## Appendix

**Community Reference Laboratory shellfish homogenate ring trial- Autumn 2003**
Instruction sheet for shellfish homogenate samples A, B and C.

**General**

- The cardboard package contains a plastic container within which are six clearly labelled centrifuge tubes.
- The tubes are labelled either sample A, B or C, there are two tubes of each sample.
- On arrival the temperature inside the plastic container should be recorded but the tubes should **not** be opened until analysis starts.
- The tubes of homogenised shellfish (A, B and C) should be stored in the dark between 2 – 8$^{O}$C until there are analysed. Please record the storage temperature on the appropriate form.
- The homogenised samples should be tested on the 9$^{th}$ October (if this is not possible because the samples have not arrived in time on the 9$^{th}$ they should be tested as soon as possible but in all cases within 24 hours of arrival). If you are unable to complete the analysis on dates stated please inform the organisers immediately. Please record the start date and time of the analysis on the form.

**Sample Preparation and Examination**

- **The shellfish homogenates provided is not diluted.**
- The contents of the tubes labelled sample A should be pooled in a sterile container, this procedure should be repeated for samples B and C.
- Each sample (A, B and C) should contain approximately 100ml of homogenised shellfish flesh.
- The samples should be analysed in triplicate using your standard method.
- If you are undertaking the method comparison study please analyse using both your standard method and the CRL reference method (Donovan *et al* 1998) in triplicate. A copy of the CRL Standard Operating Procedure (SOP02) is included with these documents.
- The forms included with these documents are based upon a three by five tube/plate segment combination. Please indicate the combination used to determine the MPN from a MPN table. Please report the MPN per 100g of shellfish flesh for *E. coli*. [a]

  [a] If these forms are not appropriate to your method please attach your standard worksheet labelled with your laboratory identification and the sample.

**Reporting Results**
Please return your result sheets to Louise Stockley, CEFAS, Weymouth Laboratory, The Nothe, Barrack Road, Weymouth, DT4 8UB or electronically to l.stockley@cefas.co.uk by 14 November 2003.