# European Union Reference Laboratory (EU-RL)
# Proficiency Testing scheme

## Enumeration of *Escherichia coli* and the detection of *Salmonella* spp. in Pacific oysters (*Crassostrea gigas*)

**EURL proficiency testing reference number: RT 41**

**Sample number: RT 41**

**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 NRL is designated by the European Union in accordance with Regulation (EC) No. 882/2004. The scheme is intended to compliment the EURL/HPA Shellfish Scheme (www.hpa.org.uk) 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 stipulated 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 II of this report. The purpose of scoring is to help the EURL, member state 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.co.uk) 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 III of this report.

Further advice on microbiological testing of bivalve mollluscan shellfish can be obtained via the EURL website (www.eurlcefas.co.uk)

Due to the nature of this scheme repeat samples are not available.

| | |
|---|---|
| **Distribution date:** | **28th November 2011** |
| **Report date:** | **10th January 2011** |
| **Report compiled by:** | **Louise Stockley**<br>**Rachel Rangdale** |
| **Authorisation by:** | **Rachel Rangdale** |

## Samples

### Sample preparation

One batch consisting of approximately 1200 (120kg) Pacific oysters (*Crassostrea gigas*) was collected from a UK commercial harvesting area. Approximately 100 oysters were evenly spread on individual trays and immersed in a small scale depuration unit that had been filled with 500 litres of filtered (50 micron filter) seawater and maintained at a temperature of 12°C (approximate temperature of seawater in harvesting area). Six trays were prepared for each depuration tank. Seawater was re-circulated at 28 litres per min (with UV) for 96 hours to allow the shellfish to acclimatize. *E. coli* previously isolated from shellfish matrix was exposed to stress conditions in seawater to mimic the natural marine environment and screened raw sewage collected from a local sewage treatment works were analysed to determine the *E. coli* levels using membrane filtration (Anon 2000). All oyster trays were removed from the tanks and 1 litre of screened raw sewage (≈1 x $10^8$ cfu/100ml) and 100ml of stressed *E. coli* cells (≈1.5 x $10^9$ cfu/100ml) was added to each tank and thoroughly mixed. The oysters were re-immersed in the tank and the temperature of the seawater was increased to 16°C with constant re-circulation (without UV). After 3 hours of exposure the oysters were removed. Each sample comprised of 35 randomly selected oysters.

**Note:** Repeat samples were not available for this scheme

### Sample distribution and examination

Samples were packed in accordance with Cefas protocol for packaging shellfish for transportation and distributed on 28th November 2011 to 29 participating laboratories. Participants were required to analyse the material in duplicate immediately on receipt using their routine laboratory procedures. Supplementary advice on sample acceptance, receipt and processing is available via the EURL website (www.eurlcefas.org).

### Sample temperature

Temperature recorders (Thermotrack, Progress Plus) were included in each consignment. Participants were required to record the internal air and sample temperature on arrival and to return the recorder. Temperatures recorded by participants are shown in Appendix I.

## Results

### Reference results - *E. coli*

Ten randomly selected sub-samples were analysed in duplicate on 2 consecutive days (29.11.11 and 30.11.11) for *E. coli* using EURL SOP No. 1175 http://www.eurlcefas.org/InformationCentre/docs/CRL_SOP_*E. coli*_04_04_08.pdf (Table 1).

**Table 1: Reference results**

| Sample analyses dates | *E. coli* MPN/100g | | | |
|---|---|---|---|---|
| | Range | Median | GM | Median ±3*$SD_T$ |
| **29.11.11** | 1.6 x $10^6$ – 1.6 x $10^6$ | 5.4 x $10^5$ | 6.1 x $10^5$ | 9.0 x $10^4$ - 3.3 x $10^6$ |
| **30.11.11** | 1.6 x $10^6$ – 1.6 x $10^6$ | 5.4 x $10^5$ | 6.1 x $10^5$ | 9.0 x $10^4$ - 3.3 x $10^6$ |

GM- geometric mean, $SD_T$ – theoretical standard deviation

### Reference results – *Salmonella* spp.

Ten randomly selected sub-samples were analysed on 2 consecutive days (29.11.11 and 30.11.11) for *Salmonella* spp. based on the mini-MSRV MPN technique (Fravalo *et al,* 2003) (Table 2).

**Note:** Regulation (EC) No. 2073/2005 requires presence/absence testing for *Salmonella* spp. in live bivalve molluscs.

**Table 2: Reference results**

| Sample analyses dates | *Salmonella spp.* | No. of replicates giving positive results |
|---|---|---|
| **29.11.11** | Not detected in 25g | 12 |
| **30.11.11** | Not detected in 25g | 12 |

## Participants' results

Performance assessment was according to the procedures described in the EURL/HPA EQA shellfish scheme for a single distribution, with minor modifications (Appendix I). Participants' results and scores allocated for RT 41 are shown in Tables 3, 4 and Figure 1.

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

### *E. coli* summary statistics

| | |
|---|---|
| Participants reporting MPN results within the expected range[1] | 25 |
| Participants reporting MPN results outside the expected range for one replicate | 0 |
| Participants reporting MPN results outside the expected range for both replicates | 3 |
| Participants reporting MPN results inconsistent with ISO 7218 (Anon 2007)[2] | 4 |

[1]expected range = participants' median ± theoretical 3SD
[2] points deducted from participants returning results inconsistent with ISO 7218.

### *Salmonella* spp. summary statistics

| | |
|---|---|
| Participants reporting expected result | 28 |

**Table 3: Participants results and allocated scores**

| Lab ID | *E. coli* MPN/100g | | | *Salmonella* spp. in 25g | |
|---|---|---|---|---|---|
| | Replicate 1 | Replicate 2 | Score | Result | Score |
| **3 *** | 350000 | 540000 | 12 | Not detected | 2 |
| **7 *** | 540000 | 920000 | 12 | Not detected | 2 |
| **9 *** | 920000 | 540000 | 12 | Not detected | 2 |
| **10 *** | 1600000 | 540000 | 12 | Not detected | 2 |
| **13 *** | 350000 | 350000 | 12 | Not detected | 2 |
| **19 *** | 1600000 | 540000 | 12 | Not detected | 2 |
| **20** | 920000 | 350000 | 12 | Not detected | 2 |
| **21** | 540000 | 350000 | 12 | Not detected | 2 |
| **22** | 540000 | 350000 | 12 | Not detected | 2 |
| **27 *** | 540000 | 1600000 | 12 | Not detected | 2 |
| **30** | 170000 | 170000 | 10 | Not detected | 2 |
| **32 *** | 110000 | 160000 | 10 | Not detected | 2 |
| **33 *** | 170000 | 240000 | 12 | Not detected | 2 |
| **35 *** | 240000 | 240000 | 12 | Not detected | 2 |
| **41 *** | 920000 | 350000 | 12 | Not detected | 2 |
| **44 *** | 930000 | 430000 | 12 | Not detected | 2 |
| **47 *** | 240000 | 130000 | 10 | Not detected | 2 |
| **54** | 160000 | 140000 | 12 | Not detected | 2 |
| **68 *** | 1600000 | 1600000 | 12 | Not detected | 2 |
| **69** | 350000 | 170000 | 10 | Not detected | 2 |
| **76** [a] | >48000 | >48000 | 2 | Not detected | 2 |
| **86 *** | 920000 | 1600000 | 12 | Not detected | 2 |
| **90** | 920000 | 540000 | 12 | Not detected | 2 |
| **92** | 350000 | 540000 | 12 | Not detected | 2 |
| **106** [a] | >18000 | >18000 | 2 | Not detected | 2 |
| **111** | NR | NR | 0 | NR | 0 |
| **147 *** | 1600000 | 540000 | 12 | Not detected | 2 |

| Lab ID | *E. coli* MPN/100g | | | *Salmonella* spp. in 25g | |
|---|---|---|---|---|---|
| | Replicate 1 | Replicate 2 | Score | Result | Score |
| **170 *** | 15300 | 15960 | 2 | Not detected | 2 |
| **189** [a] | >18000 | >18000 | 2 | Not detected | 2 |

* Designated NRL's, [a] – high censored values doubled for data assessments, NR – Not returned

**Table 4: Participants results**

| | *E. coli* MPN/100g | | | |
|---|---|---|---|---|
| | Range | Median | GM | Median±3*$SD_T$ |
| **Participants results** | >1.8 x $10^4$ – 1.6 x $10^6$ | 3.5 x $10^5$ | 3.2 x $10^5$ | 5.8 x $10^4$ - 2.1 x $10^6$ |

GM- geometric mean, $SD_T$ – theoretical standard deviation

**General comments**

Twenty-nine laboratories (17 NRL and 12 other laboratories) received material for this distribution. Sixty-nine percent of samples arrived within 24 hr of dispatch. One sample arrived more than 48 hours after dispatch. Sixteen laboratories analysed the samples on the day of arrival. The remaining laboratories analysed the samples the following day. It is recommended in ISO 7218 that shellfish material should be analysed within 24 hr after sampling. Temperature loggers stored in each consignment showed an in transit temperature range of 0.5 - 8°C. All temperature data, arrival and analysis dates and times recorded by participants are given in Appendix II.

***E. coli* comments**

Twenty-eight laboratories returned results with 25 participants duplicate *E. coli* MPN/100g results falling between ±3 SD of the participants' median. Laboratories 76, 106 and 189 reported both replicate results as high censored values due to the analysis of insufficient dilutions. All high censored results were doubled to enable MPN values to be plotted. Laboratories 106 and 189 reported both replicate results between -3 SD and -5 SD of the participants' median and laboratory 170 reported both replicate results outside -5 SD of the participants' median. It is recommended that this laboratory critically examine its procedures for analysis of bivalve shellfish for *E. coli* and if necessary consult the EURL for assistance. Laboratory 111 did not return their results.

Laboratories 30, 32, 47 and 69 reported one or both MPN value(s) that were not consistent with the guidance given in ISO 7218 for interpretation of 5 x 3 MPN tables or those previously supplied to NRLs by the EURL. Laboratory 44 reported results as a 3 x 3 MPN. This form of MPN is not consistent with the guidelines given in ISO 16649-3 point 9.2.1 which states for live shellfish 5 tubes per dilution should be prepared.

Twenty-six laboratories cited ISO TS 16649-3 (Anon 2005), 1 laboratory cited Donovan *et al* (1998) and 1 laboratory cited ISO 16649-2 (Anon 2001) as their laboratory method for enumeration of *E. coli*. Laboratories are reminded that 5 x 3 MPN tables in Donovan *et al* (1998) and those contained in ISO 7251 (2005) Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique, differ slightly from those contained in ISO 7218. **Laboratories are reminded that for enumeration of *E. coli* in live bivalve molluscs for official control testing using ISO 16649-3 should use 5 x 3 MPN tables in ISO 7218 or those provided by the EURL should be used.**

***Salmonella* spp. comments**

Twenty-eight laboratories returned expected results for RT 41 with the exception of laboratory 111 who did not return their results.

Twenty-one laboratories used the EU specified reference method for detection of *Salmonella* spp (ISO 6579). Five laboratories referenced NMKL 71: *Salmonella* detection in foods and 1 laboratory reported the use of PCR for the *Salmonella* spp. detection. **Laboratories are reminded that for official control testing of live bivalve molluscs for *Salmonella* spp the EU reference method is ISO 6579, Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. (Anon 2002).**

**References**

Anon 2000. Microbiology of recreational and environmental waters 2000. Detection and enumeration of coliform organisms and faecal coliform organisms by membrane filtration.

Fravalo P, Hascoet Y, Le Fellic M, Queguiner S, Petton J and Salvat G. (2003). 'Convenient method for rapid and quantitative assessment of *Salmonella enterica* contamination: The mini-MSRV MPN technique.

Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs on the microbiological criteria relating to bivalve molluscs.

Anon 2007. ISO 7218. Microbiology of food and animal feeding stuffs - General recommendations and guidance for microbiological examinations. Geneva, Switzerland.

Anon 2005. 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. Geneva, Switzerland.

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.

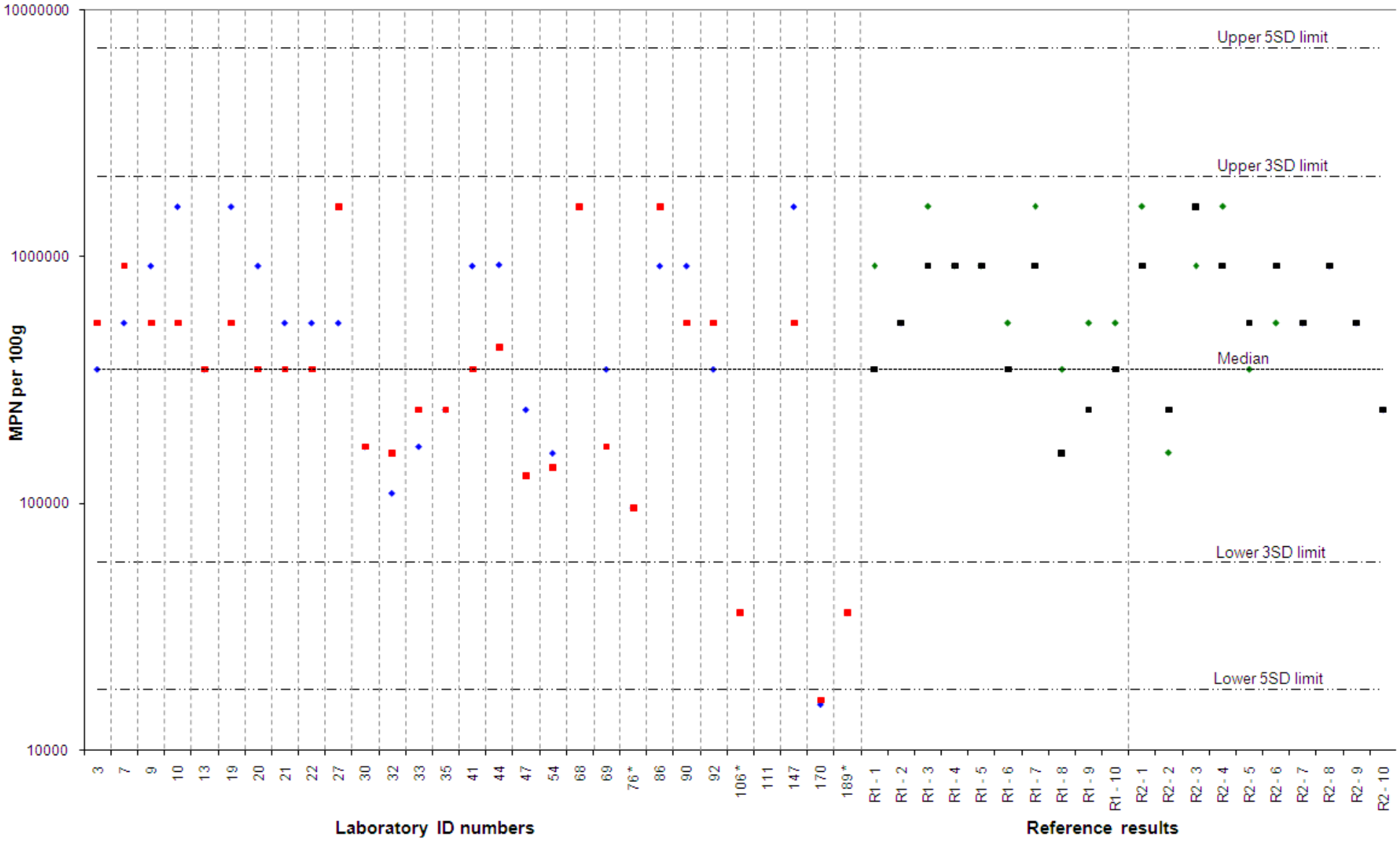
Anon 2001. ISO TS 16649-2. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 2: Colony-count technique at 44℃ using 5 -bromo-4-chloro-3-indolyl-β-D-glucuronide. Geneva, Switzerland.

Anon 2005. ISO 7251. Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique.

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

## Results chart – RT 41

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



## 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 | |
| One replicate MPN result reported is outside the expected range and falls between the median ±3SD and median ±5SD value | 2 | 5 | 2 | 9 |
| Both replicates MPN results are outside the expected range and fall between the median ±3SD and median ±5SD value | 2 | 2 | 2 | 6 |
| One replicate MPN result reported 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 value and the second falls outside the median ±5SD value. | 2 | 2 | 0 | 4 |
| Both replicates MPN results reported 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 reported is within the expected range | 2 | 5 | 7 |
| Single replicate MPN result reported only and falls between the median ±3SD and median ±5SD value | 2 | 2 | 4 |
| Single replicate MPN result reported is outside the median ±5SD value | 2 | 0 | 2 |

***E. coli* score deductions**

| Result | Score deducted |
|---|---|
| Tube combination inconsistent with MPN reported, ISO 7218 or 5 x 3 MPN tables provided by the EURL. | 2 |
| Sample not examined or results returned late - no explanation received | 2 |
| High censored result (e.g. MPN = >18000 per 100g) | No score allocated |

***Salmonella* spp scoring**

| Result | Score allocated |
|---|---|
| Fully correct results | 2 |
| Misleading result, e.g. failure to isolate *Salmonella* | 0 |

## Appendix II

### Sample arrival and temperature

| Lab ID | Date arrived | Time of arrival | Temp. logger (°C) | Sample (°C) | Storage (°C) | Date analysed | Time of analysis |
|---|---|---|---|---|---|---|---|
| **3 *** | 29.11.11 | 09:45 | 3 - 7.5 | 4.1 | 4 | 29.11.11 | 12:30 |
| **7 *** | 29.11.11 | 13:20 | 2.5 - 7.5 | 6 | | 29.11.11 | 15:00 |
| **9 *** | 29.11.11 | 16:30 | 2 - 7.5 | 6 | 5.3 | 30.11.11 | 09:00 |
| **10 *** | 30.11.11 | | | 7.2 | | 30.11.11 | 14:00 |
| **13 *** | 29.11.11 | 12:25 | 5 - 8 | 4.8 | 2.9 | 30.11.11 | 13:15 |
| **19 *** | 29.11.11 | 13:30 | 2.5 - 7.5 | 9 | 4 | 30.11.11 | 10:30 |
| **20** | 29.11.11 | 10:00 | 3.5 - 7 | 2 | 2 | 29.11.11 | 11:00 |
| **21** | 30.11.11 | 11:30 | | 4.6 | 5 | 30.11.11 | 13:30 |
| **22** | 29.11.11 | 11:15 | 4.5 - 8 | 4.8 | 4.5 | 29.11.11 | 12:15 |
| **27 *** | 29.11.11 | 12:45 | 1 - 6 | 1.8 | 3 | 30.11.11 | 10:00 |
| **30** | 29.11.11 | 13:45 | 2.5 - 8 | 4.3 | 2 | 30.11.11 | 10:30 |
| **32 *** | 29.11.11 | 11:50 | 2.5 - 7.5 | 2.2 | 4 | 29.11.11 | 12:15 |
| **33 *** | 29.11.11 | 13:00 | 3 - 7.5 | 4.2 | 2 | 30.11.11 | 10:00 |
| **35 *** | 29.11.11 | 12:50 | | 2.8 | | 29.11.11 | 13:30 |
| **41 *** | 29.11.11 | 11:10 | 3 - 6.5 | 4.5 | 4 | 29.11.11 | 13:00 |
| **44 *** | 29.11.11 | 12:25 | 3 - 7 | 5 | 3 | 29.11.11 | 14:30 |
| **47 *** | 29.11.11 | 15:00 | 2 - 6.5 | 4.2 | 4 | 29.11.11 | 16:15 |
| **54** | 30.11.11 | 12:50 | 2 - 6.5 | 6 | 4 | 30.11.11 | 12:50 |
| **68 *** | 30.11.11 | | 0.5 - 6.5 | 4 | 2 | 1.12.11 | |
| **69** | 30.11.11 | 14:30 | 2.5 - 5 | 4.2 | | 30.11.11 | 14:45 |
| **76** | 30.11.11 | 10:15 | 3 - 6 | 5.1 | 3 | 30.11.11 | 13:00 |
| **86 *** | 29.11.11 | 11:10 | 2.5 - 8 | 3 | 4.8 | 30.11.11 | 09:00 |
| **90** | 02.12.11 | 12:10 | 1.5 - 7.5 | 3.2 | | 2.12.11 | 13:30 |
| **92** | 30.11.11 | 15:30 | 3.5 - 7 | 5.5 | 3.5 | 30.11.11 | 17:00 |
| **106** | 29.11.11 | 10:30 | 1.5 - 7 | 4 | 2 | 30.11.11 | 10:00 |
| **111** | 29.11.11 | | | | | | |
| **147 *** | 29.11.11 | 11:00 | 3.5 - 7 | 4 | 4 | 30.11.11 | 13:00 |
| **170 *** | 29.11.11 | 13:30 | 2.5 - 7.5 | 9 | 4 | 30.11.11 | 10:30 |
| **189** | 30.11.11 | 16:00 | | 3 | 3 | 01.12.11 | 08:00 |

* Designated NRL's

**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 MPN tables (if used) are interpreted correctly.

2. Culture Medium - 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 that adequate controls are in place and that and documentation for dealing with IQC failures is appropriate.

Further advice can be obtained from the EURL on request.

## About us

Cefas is a multi-disciplinary scientific research and consultancy centre providing a comprehensive range of services in fisheries management, environmental monitoring and assessment, and aquaculture to a large number of clients worldwide.

We have more than 500 staff based in 2 laboratories, our own ocean-going research vessel, and over 100 years of fisheries experience.

We have a long and successful track record in delivering high-quality services to clients in a confidential and impartial manner.
(www.cefas.co.uk)

Cefas Technology Limited (CTL) is a wholly owned subsidiary of Cefas specialising in the application of Cefas technology to specific customer needs in a cost-effective and focussed manner.

CTL systems and services are developed by teams that are experienced in fisheries, environmental management and aquaculture, and in working closely with clients to ensure that their needs are fully met.
(www.cefastechnology.co.uk)

.

## Customer focus

With our unique facilities and our breadth of expertise in environmental and fisheries management, we can rapidly put together a multi-disciplinary team of experienced specialists, fully supported by our comprehensive in-house resources.

Our existing customers are drawn from a broad spectrum with wide ranging interests. Clients include:

- international and UK government departments
- the European Commission
- the World Bank
- Food and Agriculture Organisation of the United Nations (FAO)
- oil, water, chemical, pharmaceutical, agro-chemical, aggregate and marine industries
- non-governmental and environmental organisations
- regulators and enforcement agencies
- local authorities and other public bodies

We also work successfully in partnership with other organisations, operate in international consortia and have several joint ventures commercialising our intellectual property

**Head office**
**Centre for Environment,**
**Fisheries & Aquaculture Science**
**Pakefield Road, Lowestoft,**
**Suffolk NR33 0HT UK**

**Tel +44 (0) 1502 56 2244**
**Fax +44 (0) 1502 51 3865**
**Web www.cefas.co.uk**

**Centre for Environment,**
**Fisheries & Aquaculture Science**
**Weymouth Laboratory,**
**Barrack Road, The Nothe, Weymouth,**
**Dorset DT4 8UB**

**Tel +44 (0) 1305 206600**
**Fax +44 (0) 1305 206601**

printed on paper made from a minimum 75% de-inked post-consumer waste