# Community Reference Laboratory (CRL) Proficiency Testing Schemes

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

**NRL ring trial reference number: RT 31**

**Sample numbers: RT31A and RT31B**

**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 Community Reference laboratory (CRL) for monitoring bacteriological and viral contamination of bivalve molluscs. The NRL is designated by the European Commission in accordance with Regulation (EC) No. 882/2004. The scheme is intended to compliment the CRL/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).

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 I of this report. The purpose of scoring is to help the CRL, 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 is included as Appendix II, can be accessed via the CRL website (www.crlcefas.co.uk) or obtained by contacting the CRL. The European Commission has produced a protocol for management of underperformance in comparative testing and/or lack of collaboration of NRLs with CRLs activities. This is reproduced as Appendix II of this report or can be obtained by contacting the CRL.

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

Further advice on microbiological testing of bivalve mollluscan shellfish can be obtained via the NRL website (www.nrlcefas.org)

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

| | |
|---|---|
| **Distribution date:** | **12th October 2009** |
| **Report date:** | **22nd December 2009** |
| **Report compiled by:** | **Louise Stockley**<br>**Rachel Rangdale** |
| **Authorisation by:** | **Rachel Rangdale** |

## Samples

### Sample preparation

Two batches each consisting of 800 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area classified according to Regulation (EC) 854/2004 as class B.

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

### Sample RT31A

One batch of oysters was bio-accumulated with *Salmonella* Bristol (NCTC 9853) according to NRL standard procedures. Oysters were stomached and pooled to form one homogeneous sample. Each sample of RT31A comprised a single aliquot of 100ml of stomached shellfish flesh and intravalvular fluid.

### Sample RT31B

Each sample of RT31B comprised 15 randomly selected whole *C. gigas* directly from the harvesting area.

### Sample distribution and examination

Samples were distributed refrigerated on 12th October 2009 to thirty-three 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 as Appendix V or via the CRL website (www.crlcefas.org)

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### 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.

## Results

### Reference results

**Sample RT31A:** Twelve randomly selected sub-samples of RT31A were analysed for *Salmonella* spp. using CRL SOP No 1176 http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_SALMONELLA_17_11_07.pdf applied in a 3 x 3 MPN format (Table 1).

**Note:** Regulation (EC) No. 2073/2005 requires presence/absence testing for *Salmonella* spp. in live bivalve molluscs. Quantitative data is provided for information only.

**Table 1: Reference results – Sample RT31A**

| Sample description | *Salmonella spp.* | No. of replicates giving positive results | Mean MPN *Salmonella* spp. per g |
|---|---|---|---|
| **RT31A** | *Salmonella* spp. detected in 25g | 12 | $3.6 \times 10^5$ |

**Sample RT31B:** Twelve randomly selected sub-samples of RT31B were analysed for *E. coli* using CRL SOP No. 1175 http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_E_coli_04_04_08.pdf (Table 2).

**Table 2: Reference results – Sample RT31B**

| Sample description | *E. coli* MPN/100g | | | |
|---|---|---|---|---|
| | Range | Median | GM | Median ±3*$SD_T$ |
| **RT31B** | $<2.0 \times 10^1$ - $2.3 \times 10^2$ | $2.3 \times 10^2$ | $1.8 \times 10^2$ | $3.8 \times 10^1$ - $1.4 \times 10^3$ |

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

### Participants' results

Performance assessment was performed according to the procedures described in the CRL/HPA EQA shellfish scheme for a single distribution, with minor modifications (Appendix I).

**Sample RT31A**

Participants' results and scores allocated for sample RT31A are shown in Table 3.

**Summary statistics – Sample RT31A**

| | |
|---|---|
| Total participants returning results | 33 |
| Participants reporting expected result | 33 |

**Sample RT31B**

Participants' results and scores allocated for sample RT31B 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 calculation of participants' median.

**Summary statistics – Sample RT31B**

| | |
|---|---|
| Total participants reporting duplicate results for *E. coli* MPN | 33 |
| Number of outlying results | 1 |
| Participants reporting MPN results within the expected range[1] | 30 |
| Participants reporting MPN results outside the expected range for one replicate | 2 |
| Participants reporting MPN results outside the expected range for both replicate | 1 |
| Participants reporting MPN results inconsistent with ISO 7128 (Anon 2007a)[2] | 3 |

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

[2] on this occasion points were not deducted from participants returning results inconsistent with ISO 7128, laboratories were reminded that 5 x 3 MPN tables from ISO 7128 or those provided by the CRL should be used for MPN determination

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

| Lab ID | RT31A *Salmonella* spp. in 25g | | RT31B *E. coli* MPN/100g | | |
|---|---|---|---|---|---|
| | Result | Score | Replicate 1 | Replicate 2 | Score |
| 3* | Present | 2 | 20 | 50 | 12 |
| 7* | Present | 2 | <20 | <20 | 12 |
| 9* | Present | 2 | <20 | 20 | 12 |
| 10* | Present | 2 | 130 | 130 | 6 |
| 13* | Present | 2 | 50 | 20 | 12 |
| 19* | Present | 2 | 1300 | 490 | 2 |
| 21 | Present | 2 | 40 | <20 | 12 |
| 22* | Present | 2 | 70 | 170 | 9 |
| 27* | Present | 2 | <20 | <20 | 12 |
| 30 | Present | 2 | <20 | 20 | 12 |
| 32* | Present | 2 | 20 | <20 | 12 |
| 33* | Present | 2 | <20 | <20 | 12 |
| 35* | Present | 2 | 80 | <20 | 12 |
| 39* | Present | 2 | 20 | 20 | 12 |
| 41* | Present | 2 | 45 | 20 | 12 |
| 43* | Present | 2 | 20 | 20 | 12 |
| 44* | Present | 2 | <20 | <20 | 12 |
| 47* | Present | 2 | <20 | <20 | 12 |
| 54 | Present | 2 | 40 | 50 | 12 |
| 56 | Present | 2 | <20 | 20 | 12 |
| 58 | Present | 2 | 20 | 80 | 12 |
| 68* | Present | 2 | <20 | <20 | 12 |
| 82 | Present | 2 | <20 | <20 | 12 |
| 86 | Present | 2 | <20 | 50 | 12 |
| 90 | Present | 2 | 20 | 80 | 12 |
| 106 | Present | 2 | <20 | <20 | 12 |
| 111 | Present | 2 | 80 | 20 | 12 |
| 119 | Present | 2 | 20 | 20 | 12 |
| 126 | Present | 2 | 80 | 50 | 12 |
| 132 | Present | 2 | <20 | <20 | 12 |
| 144 | Present | 2 | 90 | 40 | 12 |
| 147* | Present | 2 | 20 | 20 | 12 |
| 149 | Present | 2 | <20 | 20 | 12 |

* Designated NRL's

**Table 4: Participants results RT31B**

| | *E. coli* MPN/100g | | | |
|---|---|---|---|---|
| | Range | Median | GM | Median±3*$SD_T$ |
| **Participants results** | <2.0 x $10^1$-1.3 x $10^3$ | 2.0 x $10^1$ | 2.4 x $10^1$ | 3.0 – 1.2 x $10^2$ |

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

**General comments**
Thirty-three laboratories (18 NRL and 15 other laboratories) returned results for this distribution. Sixty-one percent of samples arrived within 24 hr of dispatch. Eighteen laboratories analysed the samples on the day of arrival. Of the remaining 15 laboratories, 11 analysed on the following day (i.e. within 48-72 hr of dispatch). One laboratory analysed the sample 5 days after receipt into the laboratory. The maximum internal temperature of samples recorded by participants on arrival did not exceed 10°C. Temperature loggers showed an in transit temperature, range of 2.5 - 7.5°C, temperature data for participants are given in Appendix VI. Thirteen samples failed to reach their destination laboratory within the 24-hours recommended in the microbiological monitoring of bivalve mollusc harvesting areas, a guide to good practice: technical application (Anon 2007b). Two samples arrived outside of 48 hours. Notwithstanding this laboratories analysed the samples on arrival, the results did not appear to have been affected by the extended transport time.

**Sample RT31A**
All laboratories returned expected results for RT31A, this is however unsurprising as levels of *Salmonella* spp. in the bio-accumulated oysters were exceptionally high. It is noted that levels <100cfu/25g would be required to fully challenge the methodology.

Eighteen laboratories used the EU specified reference method for detection of *Salmonella* spp (ISO 6579). Three laboratories referenced NMKL 71: *Salmonella* detection in foods. Two laboratories referenced Vidas, Elisa method and BAX (PCR method) to detect for *Salmonella* analysis. **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). Alternative methods should be validated against this reference according to ISO 16140 Microbiology of food and animal feeding stuffs – protocol for validation of alternative methods (Anon 2005) before use in official control testing.

**Sample RT31B**
Thirty laboratories returned both replicate *E. coli* MPN/100g results between ±3 SD of the participants' median for RT31B. Laboratory 10 returned two replicate results above +3 SD but below +5 SD of the participants' median. Laboratory 22 returned one replicate result between +3 and +5 SD of the participants' median. Laboratory 19 returned both replicates outside 5 SD of the participants' median. It is recommended that laboratory 19 critically examine its procedures for analysis of bivalve shellfish for *E. coli* and if necessary consult the CRL for assistance.

Laboratories 21, 41 and 144 reported one or both MPN value(s) that were not consistent with 5 x 3 MPN tables in ISO 7218, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations (Anon 2007) or 5 x 3 MPN tables derived from this standard and previously supplied to NRLs by the CRL. Laboratories are reminded that that 5 x 3 MPN tables from ISO 7128 or those provided by the CRL should be used for MPN determination (Appendix VII). On this occasion points were not deducted from participants returning inconsistent results in future PT distributions points will be deducted for failure to use specified tables and laboratories should update their procedures accordingly.

Twenty-two laboratories cited ISO TS 16649-3 (Anon 2005) or a derivative of this as their laboratory method for enumeration of *E. coli* in sample RT31B. Laboratories are reminded that 5 x 3 MPN tables in Donovan *et al* (1998) and those contained in ISO 7251 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 5 x 3 MPN Tables in ISO 7218 or those provided by the CRL should be used.**

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

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

Anon 2005a ISO TS 16649-3:2005. 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.

Anon 2005a ISO 16140:2005. Microbiology of food and animal feeding stuffs - protocol for validation of alternative methods. Geneva, Switzerland.

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

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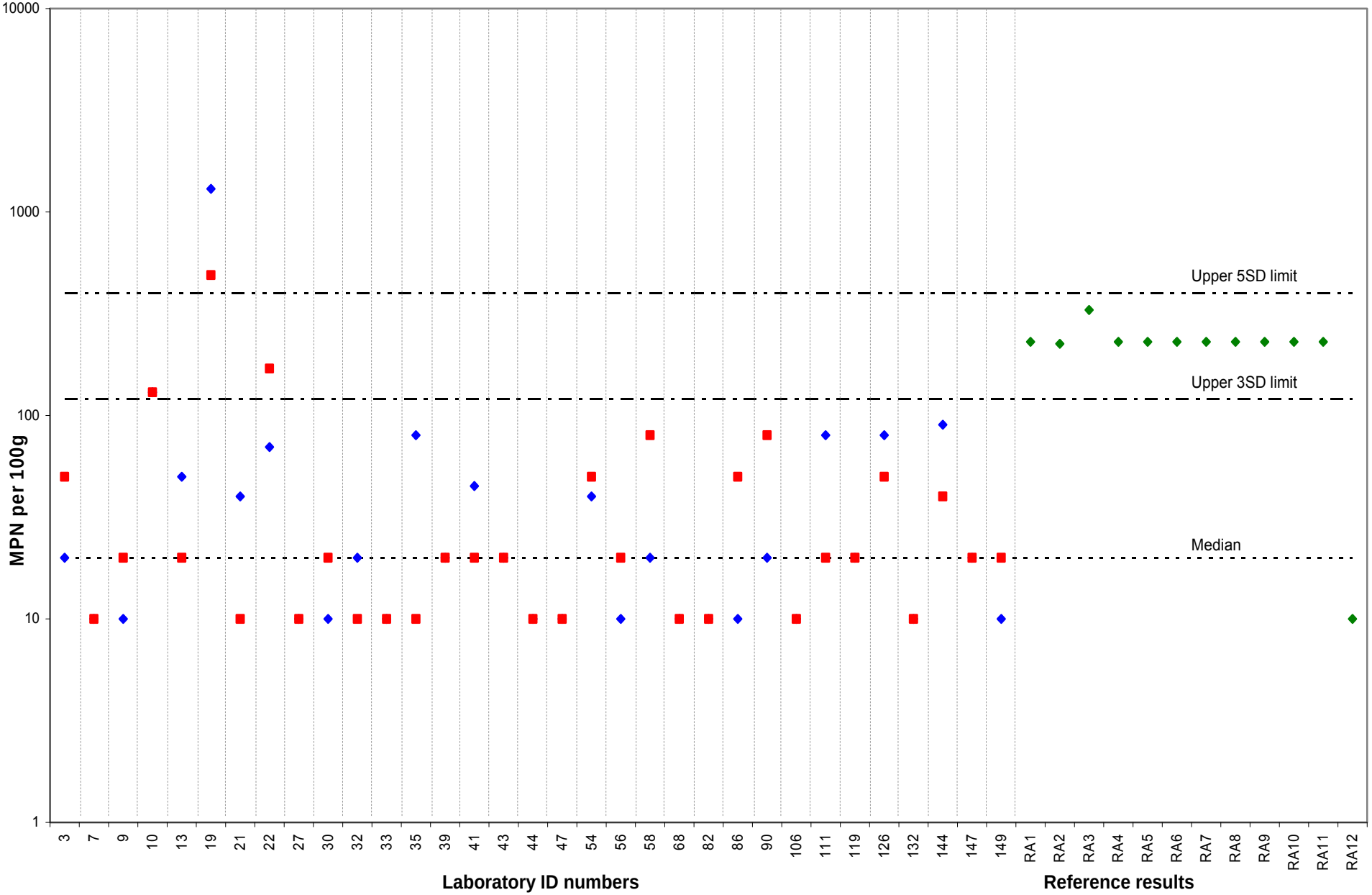
## Results chart - Sample RT31B



**Appendix I:**

***E.coli* MPN scoring**

| Result | Score 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[1] |
| Or | |
| Tube combination inconsistent with MPN reported (both replicates) | 5[1] |
| 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 |

[1]on this occasion points were not deducted from participants returning results inconsistent with ISO 7128, laboratories were reminded that 5 x 3 MPN tables from ISO 7128 or those provided by the NRL should be used for MPN determination

***Salmonella* spp scoring**

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

## Appendix II

**Protocol for management of underperformance in comparative testing and/or lack of collaboration of National Reference Laboratories (NRLs) with Community reference laboratories (CRLs) activities**

According to article 32 of Regulation (EC) 882/2004[1], Community Reference Laboratories (CRLs) shall be responsible for coordinating application by the NRLs of analytical methods, in particular by organising comparative testing and by ensuring an appropriate follow-up of such comparative testing.

Article 33 of the Regulation establishes that NRLs shall collaborate with the CRLs in their area of competence.

The NRLs are a key tool for the proper implementation of official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, therefore their performance is of outmost importance.

Appropriate actions must be taken if the results of comparative tests reveal underperformance or if NRLs fail to collaborate properly with the corresponding designated CRL.

The following two-step protocol is suggested in case of

(a) underperformance (i.e. failure in proficiency test)

(b) lack of collaboration by the NRLs with the CRL

**Phase 1**

(a) Underperformance (i.e. failure in proficiency test)

- CRL should contact the NRL and provide assistance trying to identify the origin of the bad result. On the spot visits and training could be foreseen if necessary.

- Repetition of the comparative test if feasible (e.g. within 3 months) and close assessment of the results by the CRL

Confidentiality should be kept during this phase in order to ensure good co-operation from the NRL. The results of the proficiency test and the codes of the laboratories are included in the report transmitted to the Commission. Apart from that there is no need to further involvement of the Commission until the results of the following comparative test are available and re-assessed.

[1] OJ L 165, 30.4.2004, p. 1, corrected by OJ L 191, 28.5.2004, p. 1. Regulation as last amended by Commission Regulation (EC) No 776/2006 (OJ L 136, 24.5.2006, p. 3)

(b) Lack of collaboration by the NRLs with the CRL:

- CRL should contact the NRL if lack of collaboration with CRL activities. CRL should ask the NRL for the reasons of no participation to a proficiency test or a workshop. The justification provided by the NRL should be included in the report submitted to the Commission

**Phase 2**

(c) Underperformance (i.e. fail in proficiency test)

- If the results of the following comparative test still reveal underperformance of the NRL or the collaboration of the NRL is not adequate, the Commission shall be informed officially by the CRL including a report of the main findings and corrective actions to improve the situation.

- The Commission shall inform the competent authority and require that appropriate actions are taken.

(d) Continuous lack of collaboration by the NRLs with the CRL:

- In case of repetitiveness of the lack of response of the NRL, the Commission shall be informed officially by the CRL and the Commission shall inform the competent authority and require that appropriate actions are taken.

**Appendix III**

**Guidance on performance assessment in proficiency testing and follow-up activities**
Version 1.1 December 2009

*Introduction*
Article 33 of Regulation (EC) No 882/2004 of the European Parliament and of the Council on Official Controls performed to ensure the verification of compliance with feed and food law (Anon 2004) sets out the remit of European National Reference laboratories (NRLs). This article specifies that where appropriate, NRLs should organise comparative tests, also known as proficiency testing (PT), between official laboratories and ensure an appropriate follow-up of such comparative testing.

All laboratories undertaking official controls on live bivalve molluscs should participate in a relevant PT scheme organised by their NRL or another designated programme (e.g. those organised by the CRL). Proficiency testing enables both an independent assessment of laboratory performance and comparative performance assessments with other participants. The frequency of such participation should be at least biannual to enable identification of poor performance over a realistic timescale. Laboratory performance should be monitored by the NRL on a regular basis. Poor performance should be investigated and reasons for failures identified. Laboratories that continually or persistently fail in proficiency tests may be suspended from official control testing by the relevant authorities.

Frequently PT schemes utilise statistical approaches to assess participant's performance and assign acceptability criteria. The following document describes an approach to assessing performance in comparative testing based upon allocation of numerical scores. Examples of follow-up procedures and suggested courses of action in the event of continual or persistence poor performance are provided.

*The use of scoring*
Allocation of scores in PT schemes enables measurement of performance based on empirical data. The advantages of the use of scoring in proficiency testing are listed below.

- Scoring systems are used to help assess participants' results in PT schemes. Allocation of scores helps participants', and other entities (CRL, NRL, Accreditation bodies), assess their performance.
- Scores can be used to assess performance in a single distribution (or sample) and to monitor ongoing performance over time with assessments on cumulative scores over a specified timeframe or number of distributions.
- Scores help scheme organisers recognise those participants' who experience problems and thus enable provision of additional help, advice and support.
- Scores are usually allocated following statistical analysis of participants' results. It is important that scoring procedures are reviewed frequently to ensure continued fitness for purpose.

*Monitoring of laboratory performance*
Laboratory performance should be monitored frequently and according to a defined schedule. Where poor performance is noted certain procedures should be instigated. When scoring systems are utilised failures may be identified by participants' scores that fall outside of defined performance criteria. Such occurrences should trigger follow-up activities by NRLs, all PT failures should be examined by the NRL. Follow-up procedures should be fit for purpose and regularly reviewed.

In the first instance it is recommended that the laboratory experiencing a failure in a proficiency test should be contacted and reasons for failure identified. This will enable the laboratory to conduct an investigation under their quality procedures into the nature of the failure and if available repeat the test.

The NRL should undertake proactive checks covering OCL performance in PT on at least an annual basis

Example follow-up procedures
Follow-up procedures can include:

- Examination of methodology in use, through for example, scrutiny of the laboratories standard operating procedures and result interpretation/reporting protocols.
- For culture based methods in microbiology e.g. ISO TS 16649-3 and EN/ISO 6579, quality control information of media should be scrutinized to ensure that media is performing adequately.
- Equipment records for equipment used in the procedures (e.g. incubators, measuring instruments, refrigerators) should be checked to ensure appropriate calibration, maintenance and performance.
- Staff training records should be examined to ensure that staff are adequately trained; familiar with procedures and that ongoing checks of staff competence are in place.
- Clerical procedures should be scrutinized to ensure that sample receipt, sample labeling, laboratory numbering and supporting clerical procedures are in place. It is worthy of note that frequently failures in proficiency testing can stem from failure to return results within a specified time frame. Laboratory systems should be in place to ensure that results are reported accurately and on time.
- Accreditation records should be checked to ensure that staff adhere the laboratory quality policy at all times.
- The use of, type and relevance of internal quality controls should be examined.
- Laboratory quality procedures for reacting to internal/external quality control failures.
- Onsite observation of practices in the poorly performing laboratories.

***Corrective Actions***

**If a laboratory continues to fail in a proficiency test (or series of tests), or fails to provide adequate justification for the responsible authorities should be notified.**

Continued failure in PT may result in the formal removal of the laboratory from official control testing.

*References*
Anon 2004. European Communities 2004. Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Off. J. Eur. Communities L 165, 30.4.04 : 1-141.

**Appendix IV:**

**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 NRL on request.

**Appendix V**

## Community Reference Laboratory (CRL) Proficiency Testing Schemes

### Instruction sheet for shellfish samples

**Supplementary information for whole live bivalve molluscan shellfish proficiency testing schemes**

**Samples:**

- Individual samples in this proficiency testing (PT) scheme each comprise a single species of live bivalve molluscs.
- Commission Regulation EC (No) 2073/2005 on microbiological criteria stipulates the use of a pooled sample of a minimum number of 10 individual animals for enumeration of *Escherichia coli* in live bivalve molluscs. This is to reduce the potential for individual animal-to-animal variation in microbiological content to bias the sample result.
- Samples provided as part of this PT scheme comprise at least 10 animals in the case of smaller species additional animals are provided to ensure that sufficient flesh and intravalvular fluid is provided for the analyses. The PT sample should comprise at least the following numbers of individuals:

| | |
|---|---|
| • Oysters (*Crassostrea gigas* and *Ostrea edulis*) | 12 - 18 |
| • Mussels (*Mytilus* spp.) | 18 - 35 |
| • Hard shell clams (*Mercenaria mercenaria*) | 12 - 18 |
| • Manila clams (*Tapes philippinarum*) | 18 - 35 |
| • King scallops (*Pecten maximus*) | 12 - 18 |
| • Queen scallops (*Aequipecten opercularis*) | 18 - 35 |
| • Cockles (*Cerastoderma edule*) | 35 - 55 |
| • Razor clams (*Ensis* spp.) | 12 - 18 |
| • Palourdes (*Tapes decussatus*) | 18 – 35 |

- **Sample temperature**-
  - The internal air temperature should be recorded immediately on arrival. The temperature should not exceed 8°C.
  - The sample temperature should be recorded, the sample temperature should be between 1-8°C, if sample temperature exceeds 8°C or is below 1°C **please contact the CRL immediately**. **Samples should not be frozen**.
- Where possible samples should be processed within 24 hours of dispatch, if not processed immediately samples should be stored at 3±2°C.
- Before commencement of analysis shellfish should be examined visually, only animals that are alive according to any of the following criteria should be chosen:
  - Any exposed flesh should react to touch.
  - Shellfish should open and close of their own accord.
  - They should respond to percussion.
  - Tightly closed shellfish.
- Dead or damaged shellfish should be discarded.
- **Samples should not be re-immersed in water.**
- **If the samples are considered unsatisfactory please contact the CRL immediately.**

**Methods:**

- Samples should be processed in duplicate according to your routine procedures.
- The number of dilutions required for the sample is indicated on the results form, to avoid making insufficient dilutions please refer to the form prior to initiating the test.
- The reference method for the enumeration of *E. coli* in live bivalve molluscs is 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.
- The reference method for detection of *Salmonella* spp. in live bivalve molluscs is ISO 6579:2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

**Reporting:**

- Results should be reported on the form supplied by the CRL.
- Both the MPN *E. coli* per 100g and the numerical combination used to determine the MPN should be recorded on the form.
- MPNs should be determined using 5 x 3 MPN tables provided in ISO 7218:2007. ISO 7218:2007 Microbiology of food and animal feeding stuffs - General recommendations and guidance for microbiological examinations.
- For examination for *Salmonella* spp. result should be recorded as presence or absence in 25g.
- The laboratory ID number should be clearly recorded on the form.
- Results should be returned by the date stipulated on the base of the form.
- Results can be faxed, emailed or posted, please ensure to return the form to the nominated named member of the CRL indicated on the base of the form.
- **Results received after the deadline cannot be included in the proficiency testing report.**

**Useful references**:

- 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. Geneva, Switzerland.
- The CRL standard operating procedure based upon the above standard can be accessed via the CRL website http://www.nrlcefas.org/InformationCentre/docs/NRL_SOP_E_coli_31_03_08.pdf
- Anon. 2002. ISO 6579:2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva, Switzerland.
- The CRL standard operating procedure based upon the above standard can be accessed via the CRL website. http://www.nrlcefas.org/InformationCentre/docs/NRL_SOP_SALMONELLA_17_11_07.pdf
- ISO 7218:2007 Microbiology of food and animal feeding stuffs - General recommendations and guidance for microbiological examinations. Geneva, Switzerland.
- http://www.crlcefas.org/InformationCentre/docs/854_h3oregulation.pdf
- http://www.crlcefas.org/InformationCentre/docs/882_Official_food_feed_controls.pdf
- http://www.crlcefas.org/InformationCentre/docs/20732005_microcriteria.pdf

**Useful contacts:**

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## Appendix VI:

### Sample arrival and temperature

| Lab ID | Date arrived | Time of arrival | Temp. logger (°C) | Internal air (°C) | Sample (°C) | Storage (°C) |
|---|---|---|---|---|---|---|
| 3 | 13/10/2009 | 10:00 | 3 - 6 | 11.1 | 7.4 | - |
| 7 | 13/10/2009 | 14:10 | 4.5 - 7.5 | 8.0 | 7 | 2 - 4 |
| 9 | 13/10/2009 | 16:50 | - | 7.5˚ | 5 | 4 - 8 |
| 10 | 13/10/2009 | 09:30 | 3 - 6 | - | - | - |
| 13 | 13/10/2009 | 11:50 | 3.5 - 6 | 6.5 | 5 | 3.5 |
| 19 | 14/10/2009[1] | 14:15 | 6.5 | 0.8 | 0.8 | 4 |
| 21 | 13/10/2009[1] | 14:30 | 5 - 7.5 | 11 | 6.2 | - |
| 22 | 15/10/2009 | 11:15 | 6 | 6.8 | 9.8 | - |
| 27 | 13/10/2009 | 12:10 | 5 - 7 | 8 | 7 | 3±2 |
| 30 | 13/10/2009 | 13:00 | 4 - 6 | 3.4 | 4.9 | 2.5 |
| 32 | 13/10/2009 | 09:30 | 4 - 6.5 | 12 | 10 | 4 |
| 33 | 13/10/2009 | 14:15 | 3 - 5 | 5.8 | 4.3 | 3.5 |
| 35 | 13/10/2009 | 12:32 | 5 - 6.5 | 8.5 | 3.9 | |
| 39 | 13/10/2009 | 11:30 | 4.5 - 6 | 8 | 8 | 42 |
| 41 | 14/10/2009 | 11:10 | 5.5 - 7 | 5.3 | 5.6 | 4±2 |
| 43 | 14/10/2009[1] | 08:30 | 2.5 - 6.5 | 7.0 | 5 | - |
| 44 | 14/10/2009 | 15:40 | 5 - 7.5 | 7.5 | 5.1 | - |
| 47 | 14/10/2009 | 14:45 | 4.5 - 6 | 6.4 | 5.5 | 1.4 |
| 54 | 14/10/2009 | 13:00 | 4.5 - 6 | 8 | 5 | 4 |
| 56 | 13/10/2009 | 14:30 | 3.5 - 6.5 | 6.9 | 3.4 | 3 |
| 58 | 13/10/2009 | 10:30 | 4 - 6 | 5.9 | 4.1 | - |
| 68 | 14/10/2009 | 12:00 | 5.5 | 5 | 8 | 4 |
| 82 | 13/10/2009[1] | 11:35 | - | 3.9 | 7.5 | 2±2 |
| 86 | 13/10/2009 | 12:50 | <8.5 | 6.5 | 5.4 | 4.4 |
| 90 | 16/10/2009 | 10:12 | 4 - 7.5 | 14.3 | 10.5 | 3 |
| 106 | 14/10/2009[1] | 13:26 | | 2.0 | 2 | 2 |
| 111 | 13/10/2009 | | 4.5 - 7 | - | - | 2 |
| 119 | 14/10/2009 | 13:00 | 4.5 - 6 | 3 | 3 | 4 |
| 126 | 13/10/2009 | 11:00 | 2.5 - 6 | 9.1-0.2-8.9 | 3.8-0.2-3.6 | 4.2 |
| 132 | 14/10/2009 | 12:00 | 4 - 5.5 | 5.0 | 5 | 4 |
| 144 | 13/10/2009 | 13:50 | 4 - 5.5 | 5.0 | 4.7 | 4 |
| 147 | 13/10/3009 | 15:46 | 4 - 6 | 12˚C | - | 4 |
| 149 | 14/10/2009 | 12:05 | 4.5 - 7 | 10.8˚C | 9.9 | 2.4 |

## Appendix VII

### *E.COLI* MOST PROBABLE NUMBER (MPN) TABLES

Cefas Standard Operating Procedure – Enumeration of *Escherichia coli* in molluscan shellfish http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_E_coli_04_04_08.pdf Adapted from: ISO 7218:2007.

**Table 1**: Most probable number of organisms: table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g.

| 1g | 0.1g | 0.01g | *MPN/100g* | Category |
|---|---|---|---|---|
| 0 | 0 | 0 | <20 | |
| 0 | 1 | 0 | 20 | 2 |
| 1 | 0 | 0 | 20 | 1 |
| 1 | 0 | 1 | 40 | 2 |
| 1 | 1 | 0 | 40 | 1 |
| 2 | 0 | 0 | 50 | 1 |
| 2 | 0 | 1 | 70 | 2 |
| 2 | 1 | 0 | 70 | 1 |
| 2 | 1 | 1 | 90 | 2 |
| 2 | 2 | 0 | 90 | 1 |
| 3 | 0 | 0 | 80 | 1 |
| 3 | 0 | 1 | 110 | 1 |
| 3 | 1 | 0 | 110 | 1 |
| 3 | 1 | 1 | 140 | 2 |
| 3 | 2 | 0 | 140 | 1 |
| 3 | 2 | 1 | 170 | 2 |
| 3 | 3 | 0 | 170 | 2 |
| 4 | 0 | 0 | 130 | 1 |
| 4 | 0 | 1 | 170 | 1 |
| 4 | 1 | 0 | 170 | 1 |
| 4 | 1 | 1 | 210 | 1 |
| 4 | 2 | 0 | 220 | 1 |
| 5 | 0 | 0 | 230 | 1 |
| 4 | 2 | 1 | 260 | 2 |
| 4 | 3 | 0 | 270 | 1 |
| 4 | 3 | 1 | 330 | 2 |
| 4 | 4 | 0 | 340 | 2 |
| 5 | 0 | 1 | 310 | 1 |
| 5 | 1 | 0 | 330 | 1 |
| 5 | 1 | 1 | 460 | 1 |
| 5 | 1 | 2 | 630 | 2 |
| 5 | 2 | 0 | 490 | 1 |
| 5 | 2 | 1 | 700 | 1 |
| 5 | 2 | 2 | 940 | 2 |
| 5 | 3 | 0 | 790 | 1 |
| 5 | 3 | 1 | 1100 | 1 |
| 5 | 3 | 2 | 1400 | 1 |
| 5 | 4 | 0 | 1300 | 1 |
| 5 | 4 | 1 | 1700 | 1 |
| 5 | 4 | 2 | 2200 | 1 |
| 5 | 4 | 3 | 2800 | 2 |
| 5 | 4 | 4 | 3500 | 2 |
| 5 | 5 | 0 | 2400 | 1 |
| 5 | 5 | 1 | 3500 | 1 |
| 5 | 5 | 2 | 5400 | 1 |
| 5 | 5 | 3 | 9200 | 1 |
| 5 | 5 | 4 | 16000 | 1 |
| 5 | 5 | 5 | >18000 | |

**Table 2:** Most probable number of organisms: table for multiple tube methods using 5 × 0.1g, 5 × 0.01g, 5 × 0.001 g.

| 0.1g | 0.01g | 0.001g | MPN/100g | Category |
|---|---|---|---|---|
| 0 | 0 | 0 | <200 | |
| 0 | 1 | 0 | 200 | 2 |
| 1 | 0 | 0 | 200 | 1 |
| 1 | 0 | 1 | 400 | 2 |
| 1 | 1 | 0 | 400 | 1 |
| 2 | 0 | 0 | 500 | 1 |
| 2 | 0 | 1 | 700 | 2 |
| 2 | 1 | 0 | 700 | 1 |
| 2 | 1 | 1 | 900 | 2 |
| 2 | 2 | 0 | 900 | 1 |
| 3 | 0 | 0 | 800 | 1 |
| 3 | 0 | 1 | 1100 | 1 |
| 3 | 1 | 0 | 1100 | 1 |
| 3 | 1 | 1 | 1400 | 2 |
| 3 | 2 | 0 | 1400 | 1 |
| 3 | 2 | 1 | 1700 | 2 |
| 3 | 3 | 0 | 1700 | 2 |
| 4 | 0 | 0 | 1300 | 1 |
| 4 | 0 | 1 | 1700 | 1 |
| 4 | 1 | 0 | 1700 | 1 |
| 4 | 1 | 1 | 2100 | 1 |
| 4 | 2 | 0 | 2200 | 1 |
| 5 | 0 | 0 | 2300 | 1 |
| 4 | 2 | 1 | 2600 | 2 |
| 4 | 3 | 0 | 2700 | 1 |
| 4 | 3 | 1 | 3300 | 2 |
| 4 | 4 | 0 | 3400 | 2 |
| 5 | 0 | 1 | 3100 | 1 |
| 5 | 1 | 0 | 3300 | 1 |
| 5 | 1 | 1 | 4600 | 1 |
| 5 | 1 | 2 | 6300 | 2 |
| 5 | 2 | 0 | 4900 | 1 |
| 5 | 2 | 1 | 7000 | 1 |
| 5 | 2 | 2 | 9400 | 2 |
| 5 | 3 | 0 | 7900 | 1 |
| 5 | 3 | 1 | 11000 | 1 |
| 5 | 3 | 2 | 14000 | 1 |
| 5 | 4 | 0 | 13000 | 1 |
| 5 | 4 | 1 | 17000 | 1 |
| 5 | 4 | 2 | 22000 | 1 |
| 5 | 4 | 3 | 28000 | 2 |
| 5 | 4 | 4 | 35000 | 2 |
| 5 | 5 | 0 | 24000 | 1 |
| 5 | 5 | 1 | 35000 | 1 |
| 5 | 5 | 2 | 54000 | 1 |
| 5 | 5 | 3 | 92000 | 1 |
| 5 | 5 | 4 | 160000 | 1 |
| 5 | 5 | 5 | >180000 | |

**Table 3**: Most probable number of organisms: table for multiple tube methods using 5 × 0.01g, 5 × 0.001g, 5 × 0.0001g.

| 0.01g | 0.001g | 0.0001g | MPN/100g | Category |
|---|---|---|---|---|
| 0 | 0 | 0 | <2000 | |
| 0 | 1 | 0 | 2000 | 2 |
| 1 | 0 | 0 | 2000 | 1 |
| 1 | 0 | 1 | 4000 | 2 |
| 1 | 1 | 0 | 4000 | 1 |
| 2 | 0 | 0 | 5000 | 1 |
| 2 | 0 | 1 | 7000 | 2 |
| 2 | 1 | 0 | 7000 | 1 |
| 2 | 1 | 1 | 9000 | 2 |
| 2 | 2 | 0 | 9000 | 1 |
| 3 | 0 | 0 | 8000 | 1 |
| 3 | 0 | 1 | 11000 | 1 |
| 3 | 1 | 0 | 11000 | 1 |
| 3 | 1 | 1 | 14000 | 2 |
| 3 | 2 | 0 | 14000 | 1 |
| 3 | 2 | 1 | 17000 | 2 |
| 3 | 3 | 0 | 17000 | 2 |
| 4 | 0 | 0 | 13000 | 1 |
| 4 | 0 | 1 | 17000 | 1 |
| 4 | 1 | 0 | 17000 | 1 |
| 4 | 1 | 1 | 21000 | 1 |
| 4 | 2 | 0 | 22000 | 1 |
| 5 | 0 | 0 | 23000 | 1 |
| 4 | 2 | 1 | 26000 | 2 |
| 4 | 3 | 0 | 27000 | 1 |
| 4 | 3 | 1 | 33000 | 2 |
| 4 | 4 | 0 | 34000 | 2 |
| 5 | 0 | 1 | 31000 | 1 |
| 5 | 1 | 0 | 33000 | 1 |
| 5 | 1 | 1 | 46000 | 1 |
| 5 | 1 | 2 | 63000 | 2 |
| 5 | 2 | 0 | 49000 | 1 |
| 5 | 2 | 1 | 70000 | 1 |
| 5 | 2 | 2 | 94000 | 2 |
| 5 | 3 | 0 | 79000 | 1 |
| 5 | 3 | 1 | 110000 | 1 |
| 5 | 3 | 2 | 140000 | 1 |
| 5 | 4 | 0 | 130000 | 1 |
| 5 | 4 | 1 | 170000 | 1 |
| 5 | 4 | 2 | 220000 | 1 |
| 5 | 4 | 3 | 280000 | 2 |
| 5 | 4 | 4 | 350000 | 2 |
| 5 | 5 | 0 | 240000 | 1 |
| 5 | 5 | 1 | 350000 | 1 |
| 5 | 5 | 2 | 540000 | 1 |
| 5 | 5 | 3 | 920000 | 1 |
| 5 | 5 | 4 | 1600000 | 1 |
| 5 | 5 | 5 | >1800000 | |

**Cefas Standard Operating Procedure – Enumeration of *Escherichia coli* in molluscan shellfish** http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_E_coli_04_04_08.pdf 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.

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

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## Material Safety Data Sheets (MSDS)

***1. Description:*** naturally or artificially contaminated live bivalve shellfish

***2. IATA Characterisation:*** – In the context of proficiency testing live bivalve shellfish are designated BIOLOGICAL SUBSTANCES, Division 6.2, Infectious substances Category B, UN3373.

***Name*:**

| | |
|---|---|
| Oysters (*Crassostrea gigas* and *Ostrea edulis*) | ☒ |
| Mussels (*Mytilus* spp.) | ☐ |
| Hard shell clams (*Mercenaria mercenaria*) | ☐ |
| Manila clams (*Tapes philippinarum*) | ☐ |
| King scallops (*Pecten maximus*) | ☐ |
| Queen scallops (*Aequipecten opercularis*) | ☐ |
| Cockles (*Cerastoderma edule*) | ☐ |
| Razor clams (*Ensis* spp.) | ☐ |
| Palourdes (*Tapes decussatus*) | ☐ |

***Synonyms*:** Bivalve molluscan shellfish, bivalve molluscs, common or Latin names (see above)

***3. Hazard***

Live bivalve shellfish used in proficiency testing maybe naturally contaminated with a range of potentially pathogenic microorganisms and indicator organisms at the harvesting area or artificially bio-accumulated or spiked in the laboratory. If contamination occurs naturally in UK harvesting areas it is highly unlikely that contaminants will be other than ACDP Hazard Group 2 organisms present in human sewage or animal faeces. These may include *E. coli,* FRNA bacteriophage*,* norovirus, *Salmonella* spp., *Vibrio* spp. and rarely hepatitis A. Hazard Group 2 organisms may cause human disease and may be hazardous to persons working in the laboratory. Good microbiological practice should be observed to reduce risk to laboratory staff.

Artificially contaminated bivalve shellfish will contain microorganisms in addition to those potentially present naturally. The National Reference Laboratory uses standardized approaches to bio-accumulation of bivalve molluscs under controlled conditions for the following Hazard Group 2 organisms:

- **Non-pathogenic *Escherichia coli*** - strains of *E. coli* used in bio-accumulation are non-enterotoxigenic, non-enteropathogenic, non-enteroinvasive, non-enterohaemorrhagic and non-enteroaggregative.
- ***Salmonella* spp.** - excluding *Salmonella* Typhi and Paratyphi.
- **Norovirus -** genetically characterised genotypes of genogroup I and II human norovirus from faecal material.
- **Hepatitis A virus** - strain pHM175 43c (HM-175) vaccine strain HM 175 strain contains mutations involved in culture adaptation which enable it to grow well in culture and which attenuate its human pathogenicity.

The above are considered low risk for laboratory personnel, aerosol exposure has not been demonstrated.

**Storage and handling**

It is advised that samples of live bivalve shellfish are processed immediately, under certain circumstances however they maybe stored at 3±2°C for short periods. Samples must be processed in a laboratory environment that is suitable for handling microorganisms categorized as ACDP Hazard Group 2 or equivalent. Staff handling live bivalve shellfish should have undergone appropriate training to include handling infectious biological substances and opening and homogenization of shellfish.

Precautions to prevent accidental injury when opening (shucking) shellfish should be taken. Accidental injury through stabbing with shucking knifes or puncture wounds from shells are common. The use of personnel protective equipment including protective specialist gloves (Kevlar or chain mail), or semi-automated shucking machine (e.g. Florida Cracker), and wooden or plastic blocks to hold animals is recommended. Good personal hygiene and thorough washing of hands is required.

***Disposal:*** Decontaminate before disposal by autoclaving or equivalent.

**Note.** The information and recommendations contained in this Materials Safety Data Sheet are complied from sources understood to be reliable, however we accept no responsibility for the accuracy, or reliability or for any loss of injury resulting from the use of this information. Equally emerging hazards may not be covered in this document.

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