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

The Centre for Environment, Fisheries & Aquaculture Science
Weymouth Laboratory,
Barrack Road,
The Nothe,
Weymouth,
Dorset DT4 8UB UK
Tel: +44 (0) 1305 206600 Direct: +44 (0) 1305 206619
Fax +44 (0) 1305 206601 Email: fsq@cefas.co.uk
www.crlcefas.org

Report on 3rd FRNA bacteriophage ring trial, 2004-5

CRL ring trial reference: RT8 (FRNA bacteriophage, 2004-5)

CEFAS Weymouth Laboratory,
Barrack Road,
The Nothe,
Weymouth,
Dorset,
DT4 8UB,
UK

Telephone: +44 (0) 1305 206707
Fax: +44 (0) 1305 206601
E-mail: r.e.rangdale@cefas.co.uk
Website: **www.crlcefas.org**

Contents

Title page	1
Contents	2
Introduction	3
Samples and distribution	4
Quality control	4
Participation in FRNA ring trial	5
Reference results	5
Participants results	5
Statistical analyses	5
Results charts	7
Results	7
Participation	7
Reference results	10
Analysis of results	10
Summary	14
Appendices	15

Introduction

The FRNA bacteriophage has been utilised widely as a viral surrogate to model various aspects of bivalve molluscan shellfish (BMS) sanitation. Although currently it is not anticipated that the FRNA enumeration assay will form part of the Community hygiene legislation (SANCO/4198/2001, Rev 12), it is used throughout the EU by a number of NRLs as a complementary assay in the assessment of BMS sanitary quality. The methodology for enumeration of FRNA bacteriophage (ISO 10705-1) is well standardised, and most NRLs have the capacity to undertake the assay. In a few member states, FRNA analysis is also carried out by National Laboratories undertaking BMS testing. In some of those laboratories the ISO 10705-1, as modified for use with bivalve mollusc tissue (MFSSOP0234*i5*), has been incorporated into the suite of ISO 17025 accredited methods. Comparative testing is an integral component of laboratory accreditation, ensures equivalency of test results between laboratories, and can identify and improve the performance of poorly performing laboratories. Outside of the CRL FRNA ring trial scheme, no external quality assessment (EQA) scheme exists for enumeration of FRNA bacteriophage.

At the 3rd meeting of microbiological NRLs in ISS, Rome 2004 resolutions 12 and 13 were agreed with respect to FRNA bacteriophage analyses.

12. Regarding FRNA bacteriophage analysis the NRLs considered that the ring trials had been a useful exercise and demonstrated that for consistent quality of analysis it was important to run the assay on a regular basis. The NRLs therefore agreed to continue the ring trials with 2 further distributions during 2004/5 during NoVember and January. The CRL would invite participation in due course.

13. Further to the above the CRL agreed that it might be possible to include National Testing Laboratories in the bacteriophage ring trials if NRLs considered this important and depending on the numbers of laboratories interested. NRLs agreed to provide the CRL with further information for consideration.

Further to those resolutions, all NRLs were invited to participate in CRL organized FRNA ring trial distributions during NoVember 2004 and January 2005. NRLs were asked to indicate if they required additional material for distribution to National Testing Laboratories.

Samples and distribution

The distribution consisted of two ampoules of freeze-dried 1ml volumes of FRNA bacteriophage MS2 (NCO12487). The ampoules were placed in a centrifuge tube with sufficient absorbent packing to reduce the chances of breakage during transit to the participant's laboratories. Each package contained the relevant transport documentation, information sheets describing procedures for opening, re-hydrating of ampoules and result reporting forms. Samples were distributed by UK Royal Mail airmail priority service at ambient temperature on the 18 October 2004. On receipt by the participating laboratories samples (A and B) were stored at 2-6ºC. Participants were requested to analyse ampoule A between 15 and 19 NoVember 2004 and ampoule B between 10 and 14 January 2005. Participants were asked to prepare a serial $\log_{10}$ dilution series from each ampoule and to analyse 10 replicates of each of a 10^{-7}, 10^{-8} and 10^{-9} dilution. Full instructions on ampoule re-hydration, serial dilutions and example report forms are included as Appendix I.

Quality control

Prior to the distribution the contents of 2 randomly selected ampoules were re-constituted in 1ml sterile 0.1% peptone water. The FRNA bacteriophage titre was determined using a serial $\log_{10}$ dilution series as described in Appendix I. FRNA bacteriophage was assayed in duplicate as described in MFS SOP0234*i5* (Enumeration of male-specific RNA bacteriophage in molluscan bivalve shellfish; available electronically on www.crlcefas.org). The results are given in Table1.

Table 1. Mean levels (pfuml^{-1}) of MS2 in ampoules used for ring trial distribution 2004-5

Dilution factor	Replicate 1	Replicate 2
10^{-8}	36	49
10^{-9}	6	4
10^{-10}	0	2

Participation in FRNA ring trial

Invitations inviting expressions of interest in the FRNA bacteriophage ring trial were sent to all designated NRLs, candidate countries laboratories (Bulgaria, Turkey and Romania), Norway (Norwegian School of Veterinary Science), U.S. (USFDA Seafood Laboratory) and New Zealand (ESR, Kenepuru Science Centre). Eight NRLs agreed to take part. Denmark NRL and U.K. NRL requested 2 and 4 additional samples for distribution to within MS testing laboratories. In the U.K., 2 from the 4 additional laboratories regularly undertook analysis of BMS the others being water testing labs. Norway and New Zealand also registered with the ring trial. Table 2 summarises the participation in the FRNA ring trial distribution.

Reference results

The CRL performed 34 reference analyses on randomly selected ampoules. The results were obtained using MFS SOP0234*i5* (Enumeration of male-specific RNA bacteriophage in molluscan bivalve shellfish).

Participants results

The participants' results were assessed by comparison with those returned by other participants and with the reference results.

Statistical analyses

Statistical analyses were performed on results submitted for FRNA enumerations reported by each NRL and other testing laboratories using the ISO 10705-1 method. Each reported test result [mean derived from 10 $\log_{10}$ transformed replicate analyses of the 10^{-8} dilution - expressed as equivalent to total FRNA pfuml^{-1} according to accompanying instructions (Appendix I)] was compared to the median value of all participants. The median was used as it is less affected by any outlying results than the mean value. Assessments of NRLs performance were made by comparison with ±2 and ±3 standard deviations based upon the observed variability. The median and standard deviation values for each distribution are shown in Table 3.

In addition, within replicate variation was assessed by determining statistical variation between the 10 replicate analyses of each of the distributions.

Table 2. Participation in the FRNA ring trial distribution.

Member State	Participation in ring trial	Ring trial material sent to these laboratories	Results returned to the CRL
Austria	no	no	no
Belgium & Luxembourg	yes	no	no
Czech Republic	no	no	no
Denmark[1]	yes	yes	yes
Estonia	no	no	no
Finland	yes	yes	yes
France	no	no	no
Germany	no	no	no
Greece	yes	yes	yes
Ireland	no	no	no
Italy	yes	yes	yes
Latvia	no	no	no
Lithuania	no	no	no
Netherlands	no	no	no
Poland	no	no	no
Portugal	yes	no	no
Slovakia	no	no	no
Slovenia	no	no	no
Spain	yes	yes	yes
Sweden	no	no	no
United Kingdom[1]	yes	yes	yes
Candidate country			
Bulgaria	***no***	no	no
Romania	***no***	no	no
Turkey	***no***	no	no
ETFA			
Norway	yes	yes	yes
Third country			
New Zealand	yes	yes	yes
United States	***no***	no	no

[1]requested additional material for National Testing Laboratories, Denmark- 2 additional laboratories, U.K.- 4 additional laboratories

Table 3. Log_{10} FRNA median and standard deviations (±2 and ±3).

	Median	Standard deviation (sd)	±2sd	±3sd
Reference results	9.79	0.14	0.28	0.42
Participants results ampoule A	9.72	0.33	0.66	0.99
Participants results ampoule B	9.77	0.33	0.66	0.99

Results charts

Log_{10} transformed reported mean values (PFU/ml) for NRLs and testing laboratory, and reference results were plotted on the same charts (Figures 1-2). The results charts also indicate the median value for all participants and the values for ±2 and ±3 standard deviations. The reference results were omitted from these calculations.

Results

Participation

Eight NRLs registered to participate in the FRNA ring trial (Table 1), six returned results. NRLs Portugal and Belgium/Luxembourg initially expressed an interest in the scheme but withdrew citing lack of resources and no wish to proceed respectively as reasons. In addition, laboratories in Norway and New Zealand and 7 testing laboratories (4 UK laboratories and 2 Danish) also returned at least one set of results. Results reported for ampoules A and B are detailed in Tables 4 and 5.

Table 4. Results reported for ampoule A analysed between 15 - 19 NoVember 2005

			Replicate									
ID No.	**Vial ID**	**Mean PFU/ml**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
025	A	$4.6x10^{9}$	42	40	44	47	45	41	52	40	55	58
010	A	$7.25x10^{9}$	62	72	81	77	68	77	74	70	65	74
017	A	$3.97x10^{9}$	36	35	40	44	33	44	43	43	42	37
022	A	$1.7x10^{10}$	152	153	152	160	156	145	120	140	123	156
036	A	$3.75x10^{9}$	46	32	40	42	33	30	44	43	38	27
024	A	$6.5x10^{9}$	56	54	42	49	79	62	74	71	68	95
027	A	$1.2x10^{10}$	120	123	116	*	*	*	*	*	*	*
021	A	$3.3x10^{9}$	28	35	34	42	38	33	23	28	36	35
001	A	$6.2x10^{9}$	61	68	62	54	46	74	69	81	49	58
049	A	$8.1x10^{9}$	85	94	69	84	89	76	84	59	81	90
037	A	$1.2x10^{9}$	19	10	11	10	10	13	6	14	11	17
005†	A	$3.24x10^{10}$	320	300	300	310	230	380	300	520	210	370
031	A	$4.1x10^{9}$	52	48	41	37	31	45	48	36	39	31
031.1	A	$3.6x10^{9}$	35	38	34	41	30	28	36	26	42	48
072†	A	$1.0x10^{10}$	111	97	76	100	107	102	132	106	81	94

† non BMS testing laboratories
nt- not tested
nr- non returned
* replicate results missing

Table 5. Results reported for ampoule B analysed between 10 - 14 January 2005

			Replicate									
ID No.	**Vial ID**	**Mean PFU/ml**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
025	B	$5.8x10^9$	42	43	57	70	65	60	60	59	76	51
010	B	$8.3x10^9$	81	65	93	80	86	73	93	90	81	84
017	B	$1.2x10^{10}$	122	123	121	137	127	137	139	122	120	135
022	B	$1.3x10^{10}$	145	150	126	136	150	140	122	125	115	116
036	B	$5.6x10^9$	50	45	54	54	56	74	60	63	52	44
027	B	$1.1x10^{10}$	113	110	110	*	*	*	*	*	*	*
024	B	$3.4x10^9$	65	41	51	34	13	36	25	8	50	17
021	B	$2.7x10^9$	29	26	27	25	24	20	22	39	31	26
001	B	$1.3x10^{10}$	108	109	124	124	126	127	143	143	146	149
049	B	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
037	B	$2.6x10^9$	30	22	27	25	32	39	31	19	18	19
005†	B	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
031	B	$1.5x10^9$	16	19	15	16	13	12	17	16	12	17
031.1	B	$2.3x10^9$	18	26	24	30	23	17	29	15	25	27
072†	B	$1.0x10^{10}$	104	91	104	108	97	97	95	104	98	115

† non BMS testing laboratories
nt- not tested
nr- non returned
* replicate results missing

Reference results

The log_{10} transformed median of the reference results for ampoules A and B was calculated as 9.79 ($6.2x10^9$ PFU/ml).

Analysis of results

Results charts for ampoules A and B are shown in Figures 1 and 2. No NRL reported results that were more than 2 standard deviations higher or lower than the participant's median value. Participant's results (10 replicate analyses at 10^{-8} dilution) are also displayed graphically as boxplots[‡] for ampoule A (Figure 3) and ampoule B (Figure 4). Amongst NRL laboratories for both ampoules A and B within replicate variation was not considerable and no outlying results were identified. One-way analysis of variance of log_{10} - transformed data indicated no significant differences between results. One NRL laboratory returned only three of the requested ten replicates for each ampoule. One non-NRL laboratory reported significantly higher results in excess of 2 standard deviations of the participant's median value for ampoule A. This laboratory did not return results for ampoule B.

Figure 1. Results chart: Ampoule A showing median, ± 2 and ±3 standard deviation (SD) limits

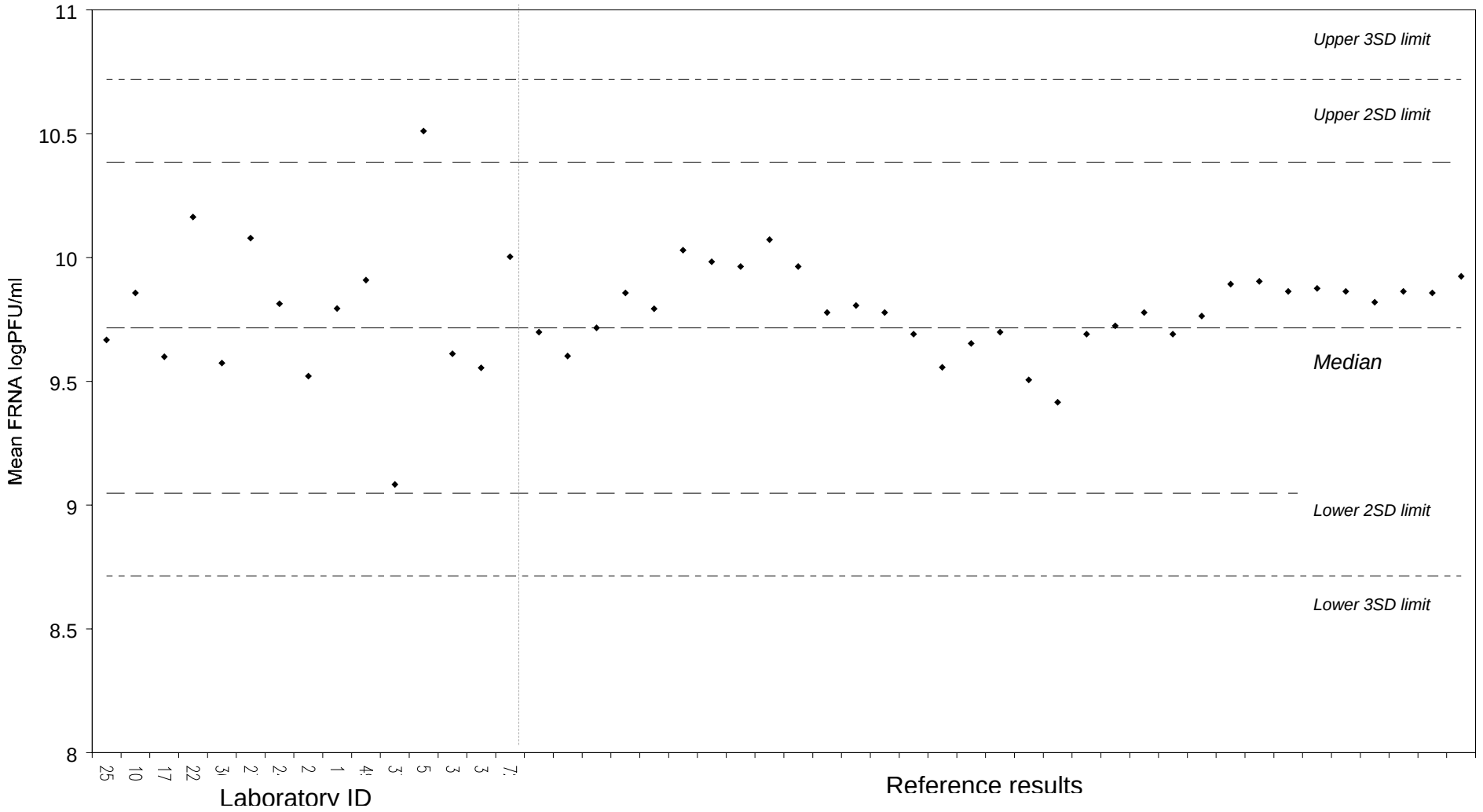


Figure 2. Results chart: Ampoule B showing median, ±2 and ±3 standard deviation (SD) limits

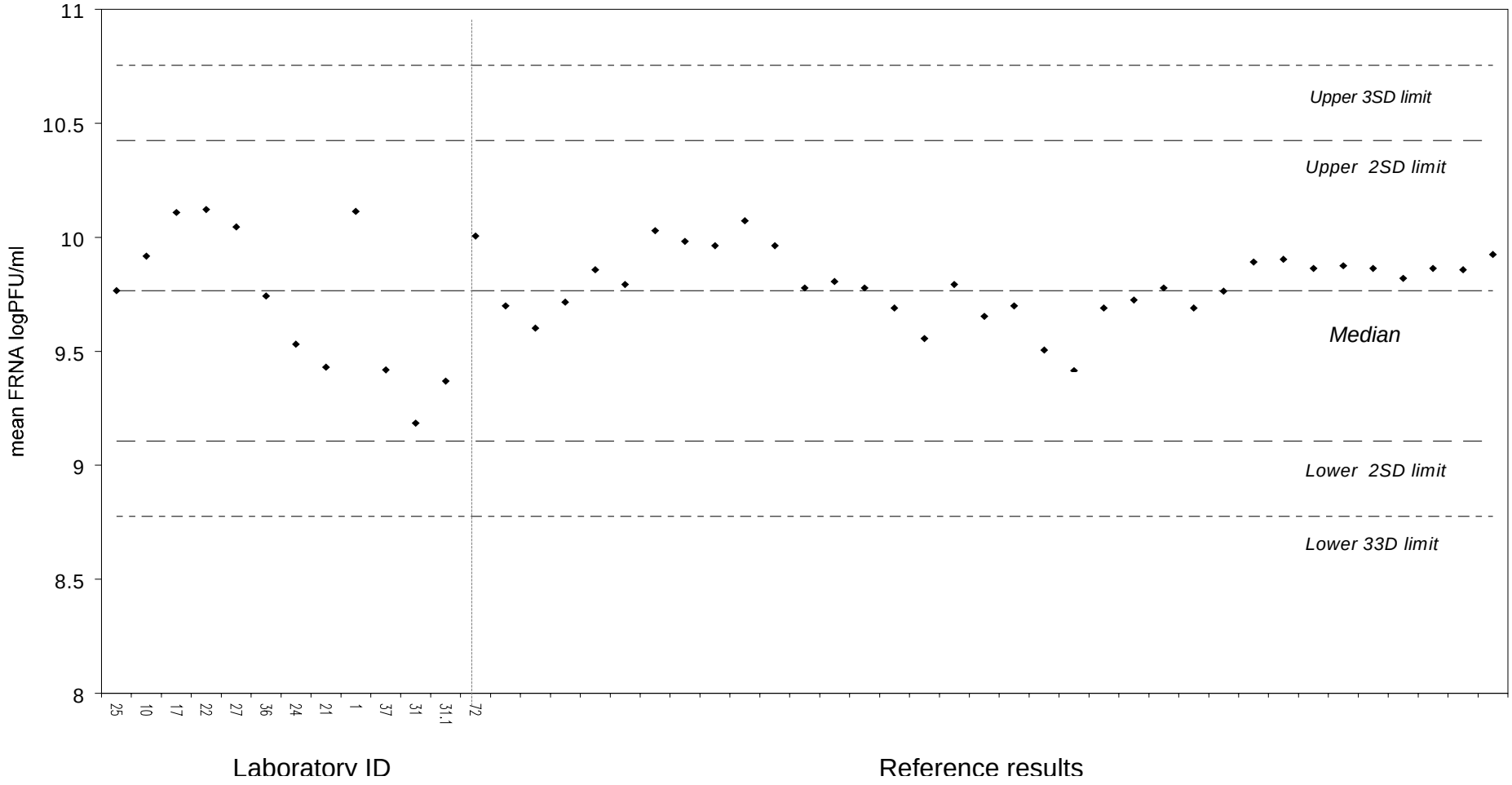


Figure 3. Boxplot showing the distribution of FRNA bacteriophage levels at 10^{-8} PFU/ml recorded by participants for ampoule A.

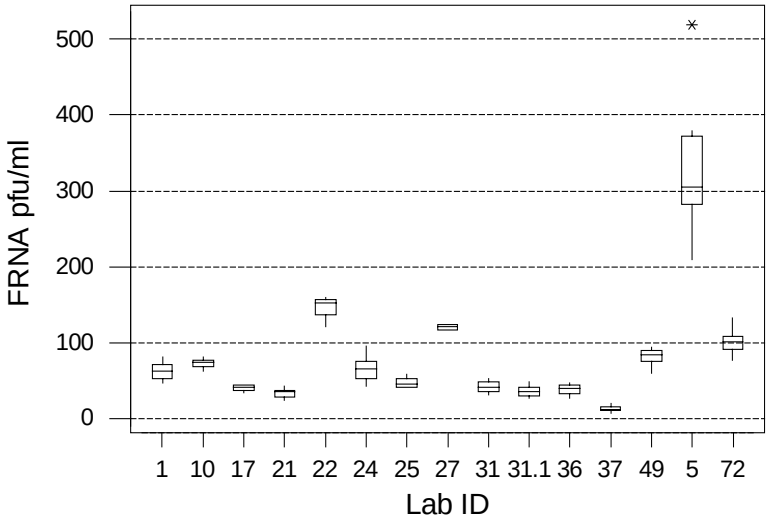
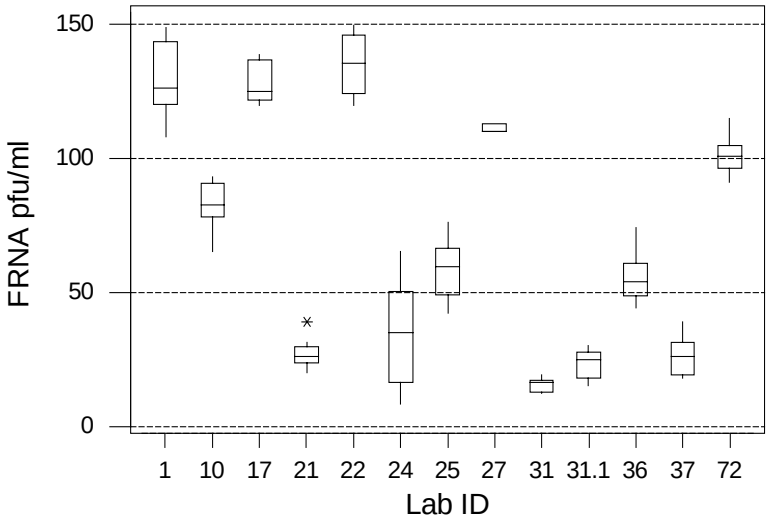


Figure 4. Boxplot showing the distribution of FRNA bacteriophage levels at 10^{-8} PFU/ml recorded by participants for ampoule B.



‡Each boxplot consists of a box, whiskers, and outliers. The line across the centre of the box indicates the median value for each participant's set of ten replicate results, the bottom of the box is at the first quartile (Q1) and the top is at the third quartile (Q3). The whiskers are the lines that extend from the top

and bottom of the box to the adjacent values. The adjacent values are the lowest and highest observations that are still inside the region defined by as - Lower Limit - Q1 - 1.5 (Q3 - Q1), Upper Limit - Q3 + 1.5 (Q3 - Q1) Outliers are points outside of the lower and upper limits and are plotted with asterisks (*).

Summary

Uptake of the third FRNA ring trial 2004-5 was not as extensive as that of the 2003 ring trial, with just six MS NRLs and one EFTA NRL returning results compared with eleven in 2003. This was probably due to the removal of the FRNA bacteriophage process criterion from the Community hygiene legislation (SANCO/4198/2001, Rev 12). The distribution was extended to MS in country testing laboratories for the first time although again the uptake was limited with just two NRLs requesting additional material. Not withstanding this the results of this two-part ring trial demonstrate that participating NRLs can run the FRNA bacteriophage assay reproducibly and consistently. All NRL laboratories obtained results within the expected range, generally grouped closely both with other participant's and with the reference results generated by the CRL. The existence of standard methods (ISO 10705-1) and the training exercises previously performed have helped to achieve this good performance.

This ring trial scheme now in its third year is the only FRNA bacteriophage proficiency testing programme available to laboratories. As such it provides those institutes regularly carrying out the assay on bivalve mollusc and water matrices with EQA in support of intra-laboratory quality systems and external accrediting procedures.

Appendix I – Ampoule re-hydration, serial dilutions and example report forms

Methodology for opening, preparation and dilution of FRNA bacteriophage ampoules

Opening of ampoules (wear protective gloves throughout the procedure.)

1. Care should be taken in opening the ampoule as the contents are in a vacuum.

2. Make a file mark on the ampoule near the middle of the cotton wool plug and either use a diamond pen to cut the glass or apply a red-hot glass rod to crack the glass.

3. Allow time for air, filtered by the plug, to seep into the ampoule and then gently remove the pointed top part. (If the pointed top part is snapped off suddenly the plug will be drawn to one end and may release fine particles of dried organisms into the air).

4. The plug may be impregnated with dried culture and should be regarded as dangerous to handle and removed with forceps.

Preparation of Samples

5. Flame the open end of the tube and rehydrate the contents of the ampoule in 1±0.1 ml of 0.1% peptone water.

6. Mix the contents carefully to avoid frothing or creating aerosols and leave the contents to rehydrate for a few minutes.

Dilution of Sample: serial dilute the sample as follows

7. Make the dilutions using 30ml sterile universals or equivalent; fill the first universal with 4.5ml of 0.1% peptone water. Fill eight further universals with 18.0ml of 0.1% peptone water. Label the universals consecutively 10^{-1} through to 10^{-9}. ***NB*** **All measurements must be exact.**

8. Transfer 500µl from the ampoule, using a 100µl pipette or equivalent, into 4.5ml of 0.1% peptone water (tube labelled 10^{-1}), vortex (or shake) for 30±10 sec to mix the sample. Then using a 2.0ml pipette or equivalent, transfer 2.0ml of the 10^{-1} sample and add to the next universal containing 18.0ml of 0.1% peptone water (10^{-2}), mix well and repeat down the series until all dilutions to 10^{-9} have been carried out.

9. Keep the dilutions out of direct sunlight, to avoid any risk of damaging the viral particles in the suspensions.

10. The last three of the nine universals (10^{-7}, 10^{-8} and 10^{-9}) contain the dilutions to be assayed. Ten replicate 1ml aliquots should be assayed for each dilution.

11. Parallel positive (MS2 NCO12487) and negative (0.1% peptone water) controls must be used throughout in triplicate.

12. Expression of Results

Results should be expressed according to the following equation.

$$C_{pfu} = \frac{N \times F}{}$$

C_{pfu} is the confirmed number of FRNA bacteriophages, expressed as pfu in one mL of undiluted sample,
where
N is the total number of plaques counted
F is the dilution factor
n is the number of replicates