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

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GENERIC STANDARD OPERATING PROCEDURE

ENUMERATION OF MALE-SPECIFIC RNA BACTERIOPHAGES IN BIVALVE MOLLUSCAN SHELLFISH

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INDEX

HISTORY OF PROCEDURE

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1.0 INTRODUCTION

The F-specific RNA bacteriophages (FRNA bacteriophages) are Enterobacteriaceae viruses of the leviviridae family that are physically and genomically analogous to human enteric viral pathogens found in sewage. They are abundant in sewage and easily enumerated making them a good prospective indicator of viral contamination in the marine environment. They have been proposed as indicators of viral contamination in the environment and used to model the behaviour of human pathogenic enteric viruses in shellfish. They are particularly useful indicators of viral contamination in shellfish after purification treatment where traditional bacterial indicators such as *Escherichia coli* are removed more readily than the human viruses. They can be sub-divided into two genus, Levivirus and Allolevivirus, each containing two fully characterised species based upon their genomic organisation and the antigenic specificity of their capsid proteins.

FRNA bacteriophages are capable of infecting F-pili producing bacterial host strains. Infection produces visible plaque on a confluent bacterial lawn grown under appropriate culture conditions, with the infectious process being inhibited in the presence of RNase in the plating media. The accurate and reliable enumeration of FRNA bacteriophages relies on the use of standardised F-pili producing host bacterial cells WG49. WG49 is a strain of *Salmonella typhimurium* that contains a plasmid coding for F-pili production making it a suitable host strain for detecting FRNA bacteriophages. WG49 is particularly useful in this role because it removes potential interference from somatic coliphages found in sewage. Quality control during the production of this host strain is critical to ensure the reliability of results obtained during FRNA bacteriophage testing.

2.0 SCOPE

The procedure has been based with reference to ISO 10705-1 (Anon 1996) with modification. It describes a double agar overlay method for preparation of samples and enumeration of FRNA bacteriophage in shellfish flesh. It includes procedures for production and quality control of *Salmonella typhimurium* WG49 host cells and FRNA bacteriophage (*MS2*) positive control material. The theoretical limit of detection is 30 plaque forming units (pfu) per 100g of shellfish flesh.

3.0 PRINCIPLE

Stock cultures of *Salmonella typhimurium* WG49 are produced by inoculation of a suitable liquid medium and overnight incubation. Working cultures are prepared by subculture of lactose positive colonies in liquid medium. The resulting culture is aliquoted and stored at <-70˚C until use. Quality control procedures are carried out on the working cultures to ensure maintenance of requisite host characteristics. An aliquot of WG49 working culture is mixed with a small volume of molten nutrient medium. Shellfish homogenate is added and the mixture flooded on a solid tryptone-yeast extract-glucose agar 2% plates and allowed to solidify. Agar plates are incubated at 37±1˚C, resulting in the formation of a confluent lawn of host cells. Where bacteriophage are present visible plaques form. Each plaque is derived from one bacteriophage. Where necessary, simultaneous examination of parallel plates with added RNase for confirmation by differential counts is carried out. The results are expressed as the number of plaque forming units (pfu) per 100g of shellfish.

Positive control FRNA bacteriophage is produced by inoculation of an exponentially growing culture of WG49. After incubation chloroform is added to lyse bacterial cells and the suspension centrifuged to remove cell debris. The *MS2* culture is then titrated, diluted to a concentration of 50-300 pfu/ml and aliquots are stored at <-70˚C until use. Control charts are constructed to determine acceptable limits for the control materials and these are used as performance criterion in the enumeration assay.

4.0 SAFETY PRECAUTIONS

Nalidixic acid is a teratogen, suitable precautions must be taken during its handling. Standard microbiology safety precautions should be applied throughout. Risks of cuts and minor physical injury exist when performing this procedure, particularly when using sharp oyster knifes to open shellfish. Appropriate measures to reduce these risks should be taken. Homogenisation of shellfish should be performed in a laminar flow cabinet to reduce the risk of infection from aerosol inhalation. *Salmonella typhimurium* WG49 mutant of low pathogenicity and should be handled in accordance with ACDP category 2 guidelines.

5.0 EQUIPMENT

- Waring blender and jars
- Laminar air flow cabinet (Class ll)
- Refrigerator at 3±2˚C
- Sterile glassware
- Shucking knife
- Safety/electric Bunsen system

- Latex gloves
- Safety gloves
- Incubator at 37±1˚C
- Incubator at 44±1˚C
- Loops - sterile, 1µl and10µl
- Pipette - automatic or manual for use with 1ml, 2ml, 5ml, 10ml and 25 ml open/closed ended pipette tips
- Orbital shaker
- Freezer at <-70˚C
- Centrifuge tubes - 50ml conical base and chloroform resistant
- Centrifuge
- Plastic vials
- Spectrophotometer
- Cuvettes
- Waterbath at 45±1˚C
- Microwave oven

6.0 MEDIA AND REAGENTS

- Ethanol.
- 0.1% peptone water (0.1% PW); formula per litre - de-ionised water 1±0.01 litre, peptone bacteriological (Oxoid LP37) 1±0.1g.
- Tryptone-yeast extract-glucose agar 1%, 2% (TYGA1%, TYGA2%); TYGA1% - formula per litre - de-ionised water 1±0.01 litre, tryptone (Oxoid LP42) 10±0.5g, yeast extract (Oxoid LP21) 1±0.01g, sodium chloride 8±0.1g, bacteriological agar No 1 (Oxoid LP11) 10±0.5g. TYGA2% - formula per litre - de-ionised water 1±0.01 litre, tryptone (Oxoid LP42) 10± 0.5g, yeast extract (Oxoid LP21) 1±0.1g, sodium chloride 8±0.1g, bacteriological agar No 1 (Oxoid LP11) 20±0.5g, calcium-glucose solution 10±1.0ml.
- Tryptone-yeast extract-glucose broth (TYGB); formula per litre - de-ionised water 1±0.01L, tryptone (Oxoid LP42) 10±0.5g, yeast extract (Oxoid LP21) 1±0.1g, sodium chloride 8±0.5g.
- Calcium-glucose solution; formula per 100ml - de-ionised water 100±0.01ml, calcium chloride dihydrate ($CaCl.2H_2O$) 3±0.1g, glucose 10±0.1g.
- MacConkey agar (MA); formula per litre - de-ionised water 1.0±0.01 litre, MacConkey agar base (Oxoid CM7) 52±0.5g, pH 7.4±0.2
- Nalidixic acid solution; formula per 100ml - de-ionised water 80±1.0ml, Sodium chloride 20±0.5ml, nalidixic acid 2.5±0.1g.
- RNase; formula per 100ml - de-ionised water 100±1.0ml, RNase 100±1.0mg.
- Antibiotic discs - nalidixic acid 30µg; 6 mm, kanamycin 30µg; 6 mm.
- Chloroform ($CHCl_3$).
- Sterile glycerol.

7.0 MICROBIOLOGICAL REFERENCE MATERIALS

- *Salmonella typhimurium* strain WG49 phage type 3 Nal^r (F' 42 lac:Tn5) - NCTC 12484
- Bacteriophage, *MS2* - NCO12487

8.0 PROCEDURE

8.1 PREPARATION OF WG49 STOCK CULTURES

Rehydrate the contents of a lyophilised ampoule of *Salmonella typhimurium* WG49 strain in 50±0.5ml of TYGB, pre-warmed to 37±1˚C. Incubate at 37±1˚C for 18±2 hours with shaking (100±10rpm). Following incubation add 10±0.2ml of sterile glycerol and mix. Aliquot approximately 1.2ml into plastic vials and store <-70˚C.

8.2 DETERMINING GROWTH RATE / OPTICAL DENSITY FOR WG49 STOCK CULTURE

Thaw one vial of WG49 stock culture and inoculate pre-dried MA plate spreading for single colonies. Incubate at 37±1˚C for 18±2 hours. After incubation, select 5-7 lactose-positive colonies and inoculate the pre-warmed TYGB. Incubate for 5±1hours at 37±1˚C with shaking (100±10rpm). Immediately at the start of incubation pipette approximately 2.5ml of TYGB from the flask into a cuvette. This is the time 0 sample. Determine the optical density (O.D) at 600nm. Serially dilute the time 0 to 10^{-6} in 0.1% PW and spread 100±1µl volumes in duplicate of the 10^{-4}, 10^{-5} and 10^{-6} dilutions onto TYGA2%. Repeat at 30 minutes intervals throughout the incubation period. Allow the inocula to absorb fully into the agar before incubation at 37±1˚C. After incubation count the total number of colonies on each plate yielding 30-300 colonies and

calculate the number of colony forming units per ml (cfu/ml). Establish the optical density corresponding to between 2.5-15x10^8cfu/ml.

8.3 PREPARATION OF WG49 WORKING CULTURE

Thaw one vial of WG49 stock culture and inoculate a MA plate using a 1µl loop, spreading for single colonies. Incubate at 37±1˚C for 18±2 hours. After incubation, select 5-7 lactose-positive colonies and inoculate the pre-warmed TYGB. Incubate for 5±1 hours at 37±1˚C with shaking until an O.D. corresponding to 2.5-15x10^8cfu/ml has been reached. Following incubation add 20±0.5ml of sterile glycerol and mix thoroughly. Aliquot approximately 1.2ml into vials and store at <-70˚C.

8.4 DETERMINING THE INCUBATION PERIOD OF WG49 WORKING CULTURE FOR ASSAY

Thaw one vial of WG49 working culture. Add 500±5µl of calcium-glucose solution to 50.0±0.5ml of TYGB and pre-warm in an incubator at 37±1˚C. Inoculate 500±5µl of WG49 into the pre-warmed TYGB. Immediately pipette approximately 2.5ml of inoculated broth from the flask into a cuvette. This is the time 0 sample. Incubate the flask at 37±1˚C with shaking (100±10rpm). Determine the O.D. at 600nm. Spread 100±1µl volumes in duplicate of the 10^{-4}, 10^{-5} and 10^{-6} dilutions onto TYGA2% and MA plate (see section 8.5). Repeat ever 30 minutes throughout the incubation period. Incubation at 37±1˚C for 24±2 hours. After incubation, count the total number of colonies on each plate yielding 30-300 colonies and calculate the number of cfu/ml. Accept if numbers of cfu are between 7-40×10^7cfu/ml within 4±2 hours. Repeat this procedure on three separate occasions.

From the results obtained above determine an optical density range corresponding to a cell concentration between 7-40x10^7cfu/ml.

8.5 QUALITY CONTROL OF WG49 WORKING CULTURE

8.5.1 Plasmid segregation

Inoculate two MA plates with 100±1µl volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions of WG49 taken at time 0, 180 and 210 minutes. Incubate at 37±1˚C for 24±2 hours. From all MA plates yielding 30-300 colonies count the number of lactose negative and positive colonies. Calculate the percentage of lactose negative colonies. Accept the host strain if lactose negative colonies are <8% of the total.

8.5.2 Nalidixic acid and kanamycin resistance

At each of the times 0, 180 and 210 spread 100±1µl of the 10^{-2} dilution onto two MA plates. Place two nalidixic acid and two kanamycin antibiotic disks on each plate. Incubate at 37±1˚C for 24±2 hours. Following incubation measure the diameter of any zones of inhibition. Accept if inhibition zone around nalidixic acid disk is not present and is <15 mm around the kanamycin disk.

8.5.3 Bacteriophage susceptibility

To assess bacteriophage susceptibility carry out the method described in 8.15 and 8.16 using the WG49 working culture.

8.6 PREPARATION OF MS2 STOCK CULTURE

Add 500±5µl of calcium-glucose solution to 50±0.5ml of prewarmed TYGB. Inoculate with 500±5µl of WG49 working culture and incubate at 37±1˚C for 18±2 hours with shaking (100±10rpm). Following incubation, inoculate the fresh TYGB plus calcium-glucose with 500±5µl of WG49 overnight culture. Incubate at 37±1˚C for 90±10 minutes. Rehydrate an ampoule of *MS2* bacteriophage (NCTC 12487) in 0.1% PW. After incubation, inoculate the 90 minute culture with 1.0±0.1ml of rehydrated MS2. Incubate at 37±1˚C for 5±1 hours.

8.7 HARVESTING MS2

NOTE: The following steps should be carried out in a laboratory other than that used for routine sample analysis.

Following incubation, add 5.0±0.1ml of chloroform and mix thoroughly. Refrigerate at 3±2˚C for 18±2 hours. Carefully aspirate the supernatant and centrifuge at 3000±200 x *g* for 20±5 minutes. Carefully pipette the supernatant into a 100ml bottle. To the supernatant add glycerol equal to 5% of the total volume. Distribute in 1±0.1ml volumes into plastic vials and store at <-70˚C.

8.8 TITRATION

Make serial log dilutions of the *MS2* stock culture preparation to 10^{-10} in 0.1% PW. Test each dilution in duplicate for the presence of FRNA bacteriophage according to the method described in 8.15 and 8.16.

Adjust the volume of stock solution added to give a final *MS2* concentration of 10^7pfu/ml.

8.9 PREPARATION OF MS2 WORKING CULTURE

NOTE: The following steps should be carried out in a laboratory other than that used for routine sample analysis.

Repeat procedures for the preparation of *MS2* stock culture, substituting the addition of rehydrated reference material with inoculation with 500±5µl of stock *MS2*, to produce a working culture. Titrate the working culture and adjust to a final concentration of 50-200pfu/ml using 0.1% PW. Do not add glycerol. Aliquot in 5±0.1ml volumes and store at <-70˚C.

8.10 DETERMINATION OF MS2 CONTROL LIMITS

Thaw a vial of MS2 working culture and assay for FRNA bacteriophage level by the method described in 8.15 and 8.16. Repeat on twenty times on ten separate occasions. From the observations, the control limits for the MS2 can be determined. Data is $\log_{10}$ transformed and the mean value and standard deviation (sd) calculated. Construct control charts incorporating warning and action limits.

Warning and action limits are defined as:

- warning limits: Mean ± 2sd
- action limit: Mean ± 3sd

8.11 SAMPLE STORAGE

Upon receipt in the laboratory the temperature of the samples should be recorded. Samples should be examined immediately or stored at 3±2˚C for no more than 24 hours until examination. Samples for analysis for FRNA bacteriophage can be frozen if necessary.

8.12 SAMPLE SELECTION

Choose shellfish that are alive according to the following points:

- If any flesh is exposed and reacts to touch using a sterile shucking knife with movement of any -kind.
- If the shellfish are open and then close of their own accord.
- If a tap on the shell causes closing or movement.
- Tightly closed shellfish.

Discard all dead shellfish and those with obvious signs of damage. Select the appropriate number of shellfish depending on the species (Appendix 1). More shellfish can be used, if necessary, to produce the required volumes for each analysis.

8.13 SAMPLE PREPARATION

Mud and sediment adhering to the shellfish should be removed prior to opening the shellfish by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be re-immersed in water as this may cause them to open. Open all selected shellfish as described below with a flame sterilised shucking knife and empty meat and liquor into a beaker. To flame sterilise the shucking knife place the knife in the beaker of ethanol and sterilise using an electric Bunsen system. Allow the knife to cool before using. When opening shellfish ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

8.13.1. Oysters and Clams

Insert the knife between the two shells towards the hinge end of the animal. Push the knife further into the animal and prise open the upper shell, allowing any liquor to drain into the beaker. Push the blade through the animal and sever the muscle attachments by sliding across the animal. Remove the upper shell and scrape the contents of the lower shell into the beaker.

8.13.2. Mussels and Cockles

Insert the knife in between the shells of the animal and separate the shells with a twisting motion of the knife. Collect the liquor from the animal in the beaker. Cut the muscle between the shells and scrape the contents into the beaker.

8.14 DILUTION AND HOMOGENISATION

Weigh the beaker and calculate the weight of the contents by subtracting the weight of the pre-weighed beaker to the nearest gram. Add 2ml of 0.1% PW per 1g of shellfish using a measuring cylinder and measure to ±2ml. Place contents of beaker into a 1 litre blender jar* and homogenise at high speed for approximately 1 minute (4 bursts of 15 seconds with at least 5 seconds between bursts) in a class two microbiological laminar flow cabinet. Decant the contents back into the labelled beaker whilst still in the

laminar flow cabinet. Homogenates may be stored 3±2˚C for up to 48 hours for FRNA bacteriophage analysis.

Note : *If shellfish are particularly small or only small numbers are available it may be necessary to use a smaller blender to achieve a consistent homogenate.

Centrifuge 30-50±2ml of homogenate at 2000±200 x *g* at room temperature for 5 minutes. Make decimal dilutions of the supernatant as required in 0.1% PW. The dilutions will depend on the expected levels of faecal contamination of the sample. If the sample is likely to have low levels of phage then 10 replicate plates using the neat sample must be analysed. Where less than 10 plates are used in the neat dilution series the nominal limit of detection must be adjusted accordingly.

8.15 AGAR OVERLAY PREPARATION

NOTE: The following procedures should be carried out at 45±1˚C in a waterbath.

Melt bottles of TYGA1% and cool to 45±2˚C for a minimum of 1 hour. Add 1±0.1ml of calcium-glucose solution per 100±2ml of TYGA1%. If high levels of background bacteria are expected add 400±2µl of nalidixic acid solution. Aliquot 2.5±0.1ml of TYGA1% into 10 plastic bijoux for each sample. If necessary a parallel series of plates with addition of 100±1µl RNase solution should be set up.

8.16 ASSAY

After incubation of the host culture, immediately add 1±0.1ml of host to all bijoux. If the host culture cannot be used immediately it ca be placed on ice for up to 2 hours. Add 1±0.1ml of the sample and mix contents by inversion. Pour the prepared bijoux contents over the surface of a TYGA2% plate and distribute evenly by circular movements. Use positive (MS2) and negative (0.1% PW) controls throughout.

Allow plates to solidify at room temperature for approximately 20 minutes and incubate inverted at 37±1˚C for 18±4 hours. Following incubation, count all pfu on each plate within 4 hours, excluding those plaques exhibiting typical DNA phage morphology, i.e. plaques of approximately 6mm diameter with a clear lysis zone in the centre.

9.0 EXPRESSION OF RESULTS

Results are expressed as pfu per 100g of shellfish flesh, according to the following equation.

$$C_{pfu} = \left(\frac{N - N_{RNase}}{n} \times F \right)$$

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

If a sample result is negative, and 10 replicates have been carried out in the neat series, the result is expressed as <30pfu/100g shellfish flesh. The positive controls must fall within the limits specified in the *MS2* control charts.

10.0 UNCERTAINTY OF TEST RESULTS

Uncertainty inherent in any test method, i.e. instruments, media, analyst performance etc can be assessed by the repeatability and reproducibility of test results. These should be monitored through control tests analysed alongside sample tests, through in-house comparability testing between analysts and through external intercomparison exercises, which would highlight any uncertainties within the test methods.

11.0 REFERENCES

Anon 1999. ISO 6887-1:1999. 'Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions'.

Anon 1996. ISO 10705-1:1995. Water Quality – 'Detection and Enumeration of Bacteriophage Part 1: Enumeration of F-Specific RNA bacteriophages'.

12.0 APPENDICES

12.1 APPENDIX 1: SUB SAMPLE SIZES OF SHELLFISH REQUIRED FOR FRNA BACTERIOPHAGE ANALYSIS

The following sub-sample sizes are recommended for inclusion in the homogenisation step:

- King scallops (*Pecten maximus*) 10-12
- Horse mussels (*Modiolus modiolus*) 10-12
- Sand Gapers (*Mya arenaria*) 10-12
- Razor clams (*Ensis* spp.) 10-12
- Oysters (C*rassostrea gigas* and *Ostrea edulis*) 12-18
- Hard clams (*Mercenaria mercenaria*) 12-18
- Queen scallops (*Aequipecten opercularis*) 15-30
- Mussels (*Mytilus* spp.) 15-30
- Manila clams (*Tapes philippinarum*) 18-35
- Palourdes (*Tapes decussatus*) 18-35
- Cockles (*Cardium edule*) 30-50
- Thick trough shells (*Spisula solida*) 30-50