FAO Reference Centre for Bivalve Mollusc Sanitation

Generic protocol – Enumeration of F-specific RNA bacteriophage in bivalve molluscan shellfish (based on ISO 10705-1)

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Cefas

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History of Procedure

Issue	Date	Section	Changes
1		All	First issue (replacing protocol originally prepared by the former EURL for monitoring bacteriological and viral contamination of bivalve molluscs)

Contents

1. Introduction

The risks of infectious human diseases acquired from the consumption of bivalve molluscan shellfish (BMS) are internationally recognised. These health hazards are largely due to the phenomenon of filter feeding whereby BMS concentrate and retain bacterial and viral pathogens present in their growing waters. The risks of exposure to infectious agents are compounded by the traditional consumption of raw, or only lightly cooked, BMS.

The F-specific ribonucleic acid bacteriophages (FRNA bacteriophages) are a number of different RNA viruses of the *Fiersviridae* family that are physically and genomically analogous to human enteric viral pathogens such as norovirus. Major FRNA bacteriophage strains include MS2, Qβ and GA viruses. They are abundant in sewage and easily enumerated making them a good prospective indicator of viral contamination in the marine environment. They are particularly useful indicators of viral contamination in shellfish after purification treatment where traditional bacterial faecal indicators such as *Escherichia coli* are removed more readily than the human viruses.

FRNA bacteriophages are capable of infecting bacteria of the *Enterobacteriaceae* family via attachment to the F-pilus. Infection produces visible plaques (empty areas without bacterial growth) on a confluent bacterial lawn grown under appropriate culture conditions, with the infectious process being inhibited by 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 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 (that infect host bacteria via attachment to the cell wall) 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. Scope

This protocol has been produced with reference to ISO 10705-1 with minor modifications to enable testing of BMS samples. It describes a double agar overlay method for the preparation of samples and enumeration of FRNA bacteriophage in shellfish flesh and intravalvular liquid. It includes procedures for the 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 100 g of shellfish flesh and intravalvular fluid.

3. Principle

For enumeration of FRNA bacteriophage an aliquot of *Salmonella* Typhimurium WG49 working culture is mixed with a small volume of molten tryptone-yeast extract-glucose-1% agar medium (TYGA1%). Shellfish homogenate is added, and the mixture is overlaid on a solid TYGA2% plate and allowed to solidify. Agar plates are incubated at 37±1 ˚C for 18±2 hours, resulting in the formation of a confluent lawn of host cells. Where FRNA bacteriophage are present visible plaques (empty areas without bacterial growth) form. Each plaque is derived from one bacteriophage particle. 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 100 g of shellfish.

4. Safety precautions

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before using this protocol. Homogenisation of shucked bivalve molluscs should be performed in a Class II safety cabinet to reduce the risk of infection from aerosol inhalation. Chloroform is acutely toxic, and both this substance and nalidixic acid have potentially carcinogenic and reproductive toxicity effects. Suitable precautions must be taken during the handling of these chemicals. *S.* Typhimurium WG49 is a mutant of low pathogenicity and should be handled in accordance with ACDP category 2 guidelines. Laboratory procedures should conform to the recommendations given in the WHO Laboratory Biosafety Manual Third edition (WHO, 2004) or relevant national legislation or guidelines.

5. Equipment

- Autoclave (or media preparator including an autoclave component)
- Drying cabinet or ventilated oven capable of being maintained between 25±1 °C and 50±1 °C
- Balance capable of weighing to ±0.1 g and reference weights for calibration
- pH-meter, with automatic or manual temperature compensation and having a resolution of 0.01 pH units and accurate to within ±0.1 pH unit at 25 °C. Reference solutions for calibrating the pH meter
- Local Exhaust Ventilation (LEV; for weighing powders during media preparation)
- Class II safety cabinet
- Refrigerators at 3±2 ˚C and 5±3 ˚C
- Freezer at <-20 ˚C
- Thermometer with a resolution of 1 °C or better at the temperature being measured
- Safety Bunsen burner or electric microincinerator
- Pipette filler and graduated pipettes or automatic pipettor and pipette tips of a range of sizes e.g. 1ml and 10ml. For handling mixed shellfish homogenates (1:3 dilution) open-ended pipettes may be necessary due to high concentrations of particulate matter. For handling chloroform, glass pipettes are necessary.
- Pipette, single channel, variable volume, 2 – 20 µl, 20 – 200 µl
- Timer
- Protective gloves – single use
- Safety gloves – for example a chain mail glove (for use with the shucking knife)
- Sterile glass beaker or flask capable of holding 250 ml volumes and sterile glass tubes or screw-topped bottles capable of holding 20 ml volumes (for liquid media)
- Sterile petri dishes (for solid media): diameter 90 mm; height 15-16 mm; vented
- Centrifuge tubes – 50 ml capacity with conical base and chloroform resistant
- Shucking knife, oyster cracker or other suitable equipment for opening bivalve molluscs
- Weighing dish capable of holding the largest size of bivalve mollusc to be tested plus released liquor (intravalvular liquid). Must be capable of being cleaned and sterilized (for use with oyster cracker)
- Food grade plastic bags
- Absorbent paper towel
- Sterile measuring cylinder – 250 ml (a larger cylinder may be needed for some species)
- Blender and 1 litre blending jars or stomacher and stomacher bags
- Sterile container of at least 500 ml capacity (a larger capacity may be required when large bivalve molluscs are to be examined. Examples include *Panopea generosa* (geoducks), *Mya arenaria* (soft shell clams; sand gapers) and some types of razor clams)

- Incubator or recirculating water bath at 37±1 ˚C [1]
- Platinum or sterile disposable loops - 1 µl
- Orbital shaker
- Centrifuge
- Plastic vials
- Spectrophotometer
- Cuvettes
- Microwave oven
- Plastic syringe
- Membrane filter – pore size 0.22 µm

6. Media and reagents [2] [3]

Media stored under refrigeration should be allowed to equilibrate at room temperature before use (ISO 11133). If necessary, dry the surface of plated media before use. For performance testing of media see section 7.

Note: ISO 10705-1 contains further details for the preparation of all media from the basic ingredients. Dehydrated media purchased commercially should be prepared according to the manufacturer's instructions.

- Ethanol
- 0.1% peptone (0.1% P); formula per litre - de-ionised water 1±0.01 litre, peptone bacteriological 1±0.1 g. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121±1 °C for 15 minutes. Store at 5±3 °C in the dark.
- Peptone salt solution (PSS); formula per litre - de-ionised water 1±0.01 litre, peptone 1±0.1 g, sodium chloride 8.5±0.1 g, pH 7±0.2. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121±1 °C for 15 minutes. Store at 5±3 °C in the dark.
- Tryptone-yeast extract-glucose broth (TYGB); formula per litre - de-ionised water 1±0.01 litre, tryptone 10±0.1 g, yeast extract 1±0.1 g, sodium chloride 8±0.1 g, pH 7.2±0.1. Dissolve completely by gentle heating with agitation. Dispense aseptically into bottles or flasks in 50±1 ml or 100±1 ml volumes. Sterilise by autoclaving at 121±1 °C for 15 minutes. Store at 5±3 °C in the dark.
- Tryptone-yeast extract-glucose-1% agar, (TYGA1%); formula per litre - de-ionised water 1±0.01 litre, tryptone 10±0.1 g, yeast extract 1±0.1 g, sodium chloride 8±0.1 g, bacteriological agar (No. 1) 10±0.1 g, pH 7.2±0.1. Dissolve completely by heating with agitation. Dispense aseptically into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day e.g. 200 ml. Sterilise by autoclaving at 121±1 °C for 15 minutes. Store at 5±3 °C in the dark.
- Calcium-glucose solution; formula per 100 ml - de-ionised water 100±1 ml, calcium chloride dihydrate ($CaCl.2H_2O$) 3±0.1 g, glucose 10±0.1 g. Dissolve completely by gentle heating with agitation. Allow to cool to room temperature then sterilise solution using a sterile syringe and a 0.22 µm membrane filter. Store at 5±3 °C in the dark.
- Tryptone-yeast extract-glucose-2% agar, (TYGA2%); formula per litre - de-ionised water

[1] In countries where the ambient temperature exceeds the target incubation temperature, an incubator or recirculating water bath with both heating and cooling units may be required to maintain the required temperature range.
[2] Allow media to equilibrate at room temperature before use.
[3] Formulations provided in this generic protocol are based upon the use of Oxoid Ltd listed products for illustrative purposes, alternative suppliers of media and reagents can be used according to the manufacturers' instructions.

1±0.01 litre, tryptone 10±0.1 g, yeast extract 1±0.1 g, sodium chloride 8±0.1 g, bacteriological agar (No. 1) 20±0.1 g. pH 7.2±0.1. Dissolve completely by heating with agitation. Sterilize by autoclaving at 121±1 °C for 15 minutes. Cool to 45 - 50 °C, then aseptically add 10±0.1 ml of calcium-glucose solution using a sterile syringe and a 0.22 µm filter. Pour 18 ml to 20 ml volumes of molten medium into sterile 90mm Petri dishes and allow to solidify. Store at 5±3 °C in the dark.

- MacConkey agar (MAC); formula per litre - de-ionised water 1±0.01 litre, MacConkey agar base 52±1 g, pH 7.4±0.2. Sterilise by autoclaving at 121±1 °C for 15 minutes. Pour 18 ml to 20 ml volumes of molten medium into sterile 90 mm Petri dishes and allow to solidify. Store at 5±3 °C in the dark.
- Nalidixic acid solution; formula per 10 ml - de-ionised water 8±0.1 ml, sodium hydroxide (1 M) 2±0.1 ml, nalidixic acid 250±1 mg. Sterilise solution using a sterile syringe and a 0.22 µm membrane filter. Store at <-20 °C in the dark.
- RNase solution; formula per 100 ml - de-ionised water 100±1 ml, RNase 100±1 mg. Dissolve completely by boiling for approximately 10 minutes. Dispense into sterile vials in volumes suitable for use for a set of examinations or a usual working day. Store at <-20 °C in the dark.
- Antibiotic discs - nalidixic acid 30 µg; 6 mm, kanamycin 30 µg; 6 mm.
- Chloroform ($CHCl_3$).
- Sterile glycerol.

7. Microbiological reference cultures for media performance testing [4]

The FAORC recommends the use of positive and negative controls throughout the procedure.

- *Salmonella* Typhimurium strain WG49, phage type 3 Nal^r (F' 42 lac::Tn5) - NCTC 12484
- Bacteriophage, MS2 – NCTC 12487 or ATCC 15597-B1.

8. Procedure

8.1. Preparation and quality control of host culture and control material

8.1.1. WG49 culture

8.1.1.1. Preparation of WG49 stock culture

Pre-warm 50±0.1 ml of TYGB in a conical flask at 37±1 ˚C and add 500±5 µl of calcium-glucose solution. Rehydrate a lyophilised ampoule of *S.* Typhimurium WG49 strain using a small volume of TYGB then transfer to the conical flask. Incubate at 37±1 ˚C for 18±2 hours whilst shaking at 100±10 rpm. Following incubation, add 10±0.1 ml of sterile glycerol and mix. Aseptically aliquot approximately 1.2 ml volumes into plastic vials and store <-70

[4] *E. coli* K-12 Hfr (NCTC 12486 or ATCC 23631) is referenced in ISO 10705-1 as an alternative reference culture to *S.* Typhimurium WG49.

˚C.

8.1.1.2. Determining the incubation period for WG49 stock culture

Thaw one vial of WG49 stock culture and use it to inoculate a pre-dried MAC plate, using a 1 µl sterile loop, to obtain well-isolated colonies. Incubate at 37±1 ˚C for 18±2 hours. After incubation, select 5 - 7 lactose-positive colonies (pink colonies) and inoculate a conical flask containing 50±0.1 ml of pre-warmed TYGB and 500±5 µl of calcium-glucose solution. Incubate for 5±1 hours at 37±1 ˚C whilst shaking at 100±10 rpm.

Immediately at the start of the 5 hour incubation pipette 2.5±0.1 ml of TYGB from the flask into a cuvette. This is the time 0 sample. Determine the optical density (O.D.) at 600 nm of the time 0 sample using the spectrophotometer (with uninoculated TYGB as blank), then prepare a serial dilution of the sample to 10^{-6} in 0.1% P and spread 100±1 µl volumes in duplicate of the 10^{-4}, 10^{-5} and 10^{-6} dilutions onto TYGA2% plates. Repeat determination of O.D., serial dilution and inoculation of TYGA2% plates at 30 minute intervals throughout the 5 hour incubation period. Allow the inoculum to absorb fully into the TYGA2% plates for each time point before incubation at 37±1 ˚C for 24±2 hours. After incubation of the plates, 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) at each time point. Compare with the O.D. measurements for each time point of the 5 hour incubation to establish the O.D. corresponding to between 2.5 - 15 x10^8 cfu/ml.

8.1.1.3. Preparation of WG49 working culture

Thaw one vial of WG49 stock culture and use it to inoculate a pre-dried MAC plate, using a 1 µl sterile loop, to obtain well-isolated colonies. Incubate at 37±1 ˚C for 18±2 hours. After incubation, select 3 - 5 lactose-positive colonies (pink colonies) and inoculate a conical flask containing 50±0.1 ml of pre-warmed TYGB and 500±5 µl of calcium-glucose solution. Incubate at 37±1 ˚C whilst shaking at 100±10 rpm. Determine the O.D. at 30 minute intervals as described in 8.1.1.2. When the O.D. reaches the level corresponding to between 2.5 - 15 x10^8 cfu/ml (8.1.1.2) remove the conical flask from the incubator, add 10±0.1 ml of sterile glycerol and mix thoroughly. Aliquot approximately 1.2 ml volumes into plastic vials and store at <-70 ˚C.

8.1.1.4. Determining the incubation period and quality control for WG49 working culture

Note: Repeat this procedure on three separate occasions.

8.1.1.4.1. Determining the incubation period

Thaw one vial of WG49 working culture. Pre-warm 50±0.1 ml of TYGB in a conical flask at 37±1 ˚C and add 500±5 µl of calcium-glucose solution. Inoculate 500±5 µl of WG49 working culture into the pre-warmed TYGB. Incubate the flask for 4±1 hours at 37±1˚C whilst shaking at 100±10 rpm.

Immediately at the start of the 4 hour incubation pipette 2.5±0.1 ml of TYGB from the flask into a cuvette. This is the time 0 sample. Determine the O.D. at 600 nm, prepare a serial dilution and inoculate TYGA2% plates as described in 8.1.1.2. Repeat determination of O.D., serial dilution and inoculation of TYGA2% plates at 30 minute intervals throughout the 4 hour incubation period. Allow the inoculum to absorb fully

into the TYGA2% plates for each time point before incubation at 37±1 ˚C for 24±2 hours. After incubation of the plates, 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) at each time point. Accept the WG49 working culture batch if the number of cfu reach between 7 - 40 x10^7 cfu/ml by the end of the 4 hour incubation. Separately compare the plate counts with the O.D. measurements for each time point to establish the O.D. corresponding to between 7 - 40 x10^7 cfu/ml.

8.1.1.4.2. Plasmid segregation

Using the same dilution series prepared in 8.1.1.4.1, inoculate two MAC plates with 100±1 µl volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions of WG49 working culture taken at time 0, 180 and 210 minutes. Incubate at 37±1 ˚C for 24±2 hours. Following incubation, select all MAC plates that yield 30 - 300 colonies and count the number of lactose negative (orange) and positive (pink) colonies. Calculate the percentage of lactose negative colonies.

Accept the WG49 working culture batch if lactose negative colonies are <8% of the total.

8.1.1.4.3. Nalidixic acid and kanamycin resistance

Using the same dilution series prepared in 8.1.1.4.1, inoculate two MAC plates with 100±1 µl volumes of the 10^{-2} dilution of WG49 working culture taken at time 0, 180 and 210 minutes. Place two nalidixic acid and two kanamycin antibiotic discs on each plate and incubate at 37±1 ˚C for 24±2 hours. Following incubation, measure the diameter of any zones of inhibition present on the MAC plates.

Accept the WG49 working culture batch if the inhibition zone around the nalidixic acid disc is not present and the inhibition zone around the kanamycin disc is <15 mm (total diameter).

8.1.1.5. Determination of bacteriophage susceptibility for WG49 working culture

Note: Repeat this procedure on three separate occasions.

To assess bacteriophage susceptibility, follow steps 8.7 to 8.10 of this protocol using the WG49 working culture batch. Once the WG49 working culture O.D. falls between the range identified in 8.1.1.4, test an MS2 control vial. Accept the WG49 working culture batch if the MS2 control levels fall within the acceptable MS2 range (warning limits) described in 8.1.2.5.

Once all QC checks have been performed, accept the batch of WG49 working culture for routine work if a pass has been recorded for all QC criteria described in sections 8.1.1.4 and 8.1.1.5. If the WG49 working culture has failed any part of the QC, the batch must be discarded and a new batch prepared.

8.1.2. MS2 control

8.1.2.1. Preparation of MS2 stock culture

Thaw one vial of WG49 working culture. Pre-warm 50±0.1 ml of TYGB in a conical flask at 37±1 ˚C and add 500±5 µl of calcium-glucose solution. Inoculate 500±5 µl of WG49 working culture into the pre-warmed TYGB. Incubate the flask for 18±2 hours at 37±1˚C whilst shaking at 100±10 rpm. Following incubation, inoculate a new 50±0.1 ml volume of TYGB (pre-warmed in a conical flask) with 500±5 µl of calcium-glucose solution and add 500±5 µl of the WG49 overnight culture. Incubate at 37±1 ˚C for 90±10 minutes. Rehydrate an ampoule of MS2 bacteriophage in 0.1% P. Following incubation, inoculate the 90 minutes WG49 culture with 1±0.1 ml of rehydrated MS2. Incubate at 37±1 ˚C for 5±1 hour.

8.1.2.2. Harvesting of MS2 stock culture

Note: The following steps should be carried out in a laboratory other than that used for routine sample analysis. Use disposable glass pipettes for transfer of chloroform and supernatants that may contain residual chloroform.

Following incubation, add 5±0.1 ml of chloroform and mix thoroughly. Refrigerate at 3±2 ˚C for 18±2 hours. Following refrigeration, carefully transfer the supernatant (aqueous phase) into a chloroform resistant centrifuge tube and centrifuge at 3000±200 x *g* for 20±5 minutes. Carefully pipette the supernatant into a sterile 100 ml bottle and add glycerol equal to 5% of the total volume. Store at 3±2 ˚C until titration testing has taken place.

8.1.2.3. Titration of MS2 stock culture

Prepare a serial log dilution of the MS2 stock culture preparation to 10^{-10} in 0.1% P. Test each dilution from 10^{-5} to 10^{-10} in duplicate to enumerate FRNA bacteriophage in the stock culture by following steps 8.7 to 8.10 of this protocol. After preparation of the dilutions, stir the MS2 stock culture for approximately 10 minutes using a magnetic stirrer and distribute in 1.2±0.1 ml volumes into plastic vials and store at <-70 ˚C.

8.1.2.4. Preparation of MS2 working culture

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

Thaw one vial of MS2 stock culture and dilute using 0.1% P to obtain a final concentration of 30 - 300 pfu/ml based on the results obtained in 8.1.2.3. Stir for approximately 10 minutes using a magnetic stirrer and aliquot in 5±0.1 ml volumes into sterile bijoux and store at <-70 ˚C.

8.1.2.5. Determination of MS2 control limits

Thaw twenty aliquots of MS2 working culture and test following steps 8.7 to 8.10 of this protocol. Repeat on 5 separate occasions to obtain approximately 100 replicate results.

Log_{10} transform data from all replicates and calculate the mean value and standard deviation (SD). Construct control charts incorporating warning and action limits.

Warning and action limits are defined as:

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

Accept the batch of MS2 working culture if no less than 95% of the results fall within the warning limits (Mean $\pm$ 2 $\times$ SD).

8.2. Sample transport and receipt

Samples must be placed in an intact food-grade plastic bag, or equivalent, and properly packed in a cool box with ice packs – packed in this manner they should reach a temperature between 0 ˚C and 10 ˚C within 4 hours and then maintain this for at least 24 hours [5]. The specific cool box, ice pack and transport condition combination should be validated to ensure that this can be achieved (https://www.cefas.co.uk/nrl/information-centre/nrl-laboratory-protocols/). For samples where less than 4 hours have elapsed between collection from the growing area and receipt at the laboratory, internal air temperature (or between-shellfish sample temperature) should be less than the temperature recorded at the time of sampling. Samples from harvesting areas should be rinsed, but not immersed, and drained at time of sampling. Samples should be regarded as unsatisfactory if on receipt at the laboratory the sample is frozen, the container is leaking, the shellfish are covered in mud or immersed in water or mud/sand.

The sample transport criteria given here are extracted from ISO 6887-3. The use of alternate sample transport criteria may be acceptable, where verification studies have been undertaken and the results of those studies demonstrate that there is no significant effect on the quality of the test results. For samples being taken in support of a growing area sanitation programme, it is recommended that verification studies supporting the use of sample transport criteria outside of the ranges given in ISO 6887-3 are approved by the responsible authority. (See Section 4.3.6 and Annex 13 of the FAO/WHO technical guidance (https://www.fao.org/3/cb5072en/cb5072en.pdf).

8.3. Sample storage

Upon receipt in the laboratory the temperature of the samples should be recorded. Samples should be examined immediately - if storage in the laboratory is necessary then samples should be stored at 3±2 ˚C and should be processed within 24 hours of collection. If, due to logistical problems, microbiological analysis of samples taken in support of a growing area programme cannot be started within 24 hours of sample collection, a verification study should be undertaken to show that extended storage does not affect the microbiological content of the sample.

8.4. Sample selection

Choose shellfish that are alive according to the following criteria:

- Reaction or movement of exposed flesh after touching using a sterile shucking knife
- Shellfish open and close of their own accord
- 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 depending on the species (Appendix 1). More shellfish can be used, if necessary, to produce the required volumes for each analysis.

[5] A temperature data logger may be used to monitor the sample temperature during transit. The data logger must be held in a central position within the cool box and not allowed to come into contact with the ice packs. If the logger has not been activated or is found to be in contact with the ice packs on arrival at the laboratory, then the temperature of the shellfish sample itself should be measured using an appropriately calibrated temperature probe.

8.5. Sample preparation

Any 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 [6].

8.5.1. Preparation of bivalve molluscs using the oyster cracker

Sterilise the blade of the oyster cracker before use (see Figure 1 and Figure 2). Place a single animal in a weighing dish on the platform underneath the blade. Lower the lever so that the blade engages the hinge of the shellfish, then fully pull the lever down so that the blade separates the shells. Using a sterilised shucking knife cut the muscle and scrape the flesh of both shells into a sterilised container. Transfer any liquor (intravalvular liquid) collected in the weighing boat into the sterilised container. Repeat with the other animals in the sample.

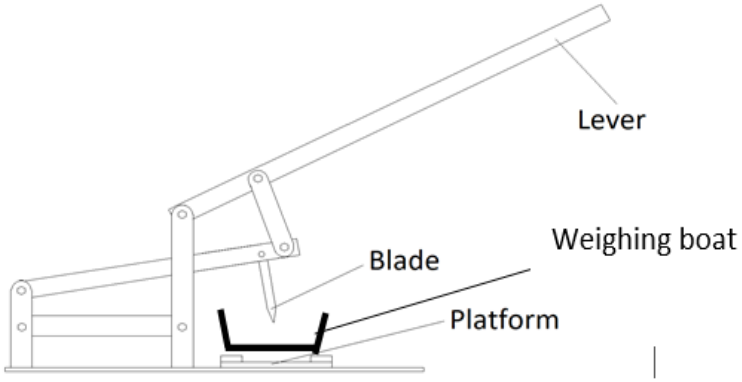

Figure 1: Oyster cracker apparatus

Figure 2: Opening an oyster using an oyster cracker

8.5.2. Preparation of bivalve molluscs using a shucking knife

Open all selected shellfish as described below using a sterilised shucking knife or equivalent and empty flesh and liquor into a sterile container. If sterilised by heating 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. For other species of bivalve molluscs a suitable method based on the methods for oysters and clams, or mussels and cockles (below) should be used.

8.5.2.1. 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 a sterilised container. 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 sterilised container.

8.5.2.2. Mussels and cockles

[6] Alternative suitable equipment can be used to open bivalve shellfish.

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 sterilised container then cut the muscle between the shells and scrape the contents into the sterilised container.

8.6. Dilution and homogenisation

Weigh the sterilised container and calculate the weight of the contents by subtracting the weight of the pre-weighed container to the nearest gram.

Note: ISO 6887-3 allows the use of PSS for the preparation of the primary dilution. FAORC advice is to use 0.1% P instead at this stage to avoid the addition of extra NaCl.

Note: Complete either sections 8.6.1 or 8.6.2 to obtain the primary suspension.

8.6.1. Homogenisation in a blender

Measure 2 ml of sterile 0.1% P per 1 g of shellfish (±2 ml) using a measuring cylinder. Place the contents of the sterilised container into a 1 litre blender jar [7] with the 0.1% P from the measuring cylinder and homogenise at high speed for approximately 1 minute (4 bursts of 15 seconds with at least 5 seconds between bursts) in a Class II safety cabinet. Decant the contents (mixed shellfish homogenate (1:3 dilution)) back into the sterilised container.

Note: It may be necessary to use an open-ended pipette to transfer mixed shellfish homogenate due to high concentrations of particulate matter.

Note: Homogenates prepared in section 8.5 of the FAORC *E. coli* protocol can be used in this protocol.

8.6.2. Homogenisation in a stomacher [8]

Place the contents of the sterilised container into at least three stomacher bags (e.g. place three stomacher bags inside each other to avoid small pieces of shell from puncturing the bags). Remove excess air from the bags and operate the stomacher for 2 - 3 minutes. Transfer 50±0.1 g of homogenised shellfish into another set of 3 stomacher bags, add 100±0.1 ml of 0.1% P and homogenise for a further 2 - 3 minutes. After homogenisation decant the contents (mixed shellfish homogenate (1:3 dilution)) back into the sterilised container.

8.6.3. Preparation of further dilutions

Following homogenisation (8.6.1 or 8.6.2), centrifuge 30 - 50 ml of homogenate at 2000±200 x *g* at room temperature for 5 minutes. Make decimal dilutions (e.g. 1:30, 1:300 etc.) of the supernatant as required in 0.1% P.

Note: The dilution prepared will depend on the expected level of faecal contamination of the sample. If the sample is likely to have low levels of phage, prepare 10 replicate plates using the 1:3 sample.

Note: Homogenates may be stored 3±2 ˚C for up to 48 hours for FRNA bacteriophage analysis.

[7] If shellfish are particularly small, it may be necessary to use a smaller blender to achieve a consistent homogenate.
[8] The FAORC recommends using a blender and not a stomacher when preparing clam and cockle samples.

8.7. Preparation of the host culture

Thaw one vial of WG49 working culture. Pre-warm 50±0.1 ml of TYGB in a conical flask at 37±1 ˚C and add 500±5 µl of calcium-glucose solution. Inoculate 500±5 µl of WG49 working culture into the pre-warmed TYGB. Incubate the flask at 37±1˚C whilst shaking at 100±10 rpm.

Every 30 minutes during the incubation, determine the O.D. at 600 nm as described in 8.1.1.2. Once the O.D. reaches a level corresponding to 7 - 40 $x10^7$ cfu/ml as determined in 8.1.1.4.1, remove the host culture from the incubator and quickly cool on melting ice. Use within 2 hours.

8.8. Preparation of agar overlay

Note: carry out all stages in a waterbath set at 45±2 ˚C. Ensure the water level of the water bath covers the media in each test tube to prevent agar from solidifying.

Melt the required volume of TYGA1% bottles in a microwave and allow to cool before placing in the pre-warmed waterbath for a minimum of 1 hour. Add 1±0.1 ml of calcium-glucose solution per 100 ml of TYGA1%. If high levels of background bacteria are expected add 400±2 µl of nalidixic acid solution per 100 ml to the bottle of TYGA1%. Aliquot 2.5±0.1 ml of TYGA1% (with added calcium-glucose solution and nalidixic acid if required) into 10 plastic bijoux for each sample.

For the controls - Prepare a further 4 bijoux for the controls (2 x MS2 and 2 x 0.1% P).

Note: If necessary, when large clear plaques (somatic coliphage) are expected in the sample at more than 5% of the total plaque count, prepare in parallel a second set of 10 plastic bijoux and add 100±1 µl RNase solution.

8.9. FRNA bacteriophage assay

Add 1±0.1 ml of host culture prepared in 8.7 to each bijoux prepared in 8.8. Add 1±0.1 ml of sample prepared in 8.6 to each of the 10 bijoux without RNase prepared for it (plus the 10 bijoux with RNase if used) and mix the contents by inversion before pouring the contents of each bijou over the surface of a TYGA2% plate. Distribute evenly by circular movements.

For the controls – Inoculate two bijoux with 1±0.1 ml of thawed MS2 working culture (positive control) and 2 bijoux with 1±0.1 ml of 0.1% P.

Allow the TYGA2% plates to solidify at room temperature for approximately 20 minutes before incubating inverted at 37±1 ˚C for 18±4 hours.

8.10. Calculation of FRNA bacteriophage

Following incubation, plates should be counted (Figure 3), within 4 hours. Control plates should be checked; negative control plates should be negative and positive control levels should be between the action limits described in 8.1.2.5.

If control results are acceptable, count all pfu on each plate (excluding those plaques exhibiting typical DNA phage morphology, i.e. plaques of approximately 6mm diameter with a clear lysis zone in the centre).

Note: If plates cannot be read within 4 hours, store at 3±2 ˚C for up to 48 hrs.

Note: Where multiple dilutions have been tested for a sample, select the dilution where plates contain between 30 - 300 plaques.

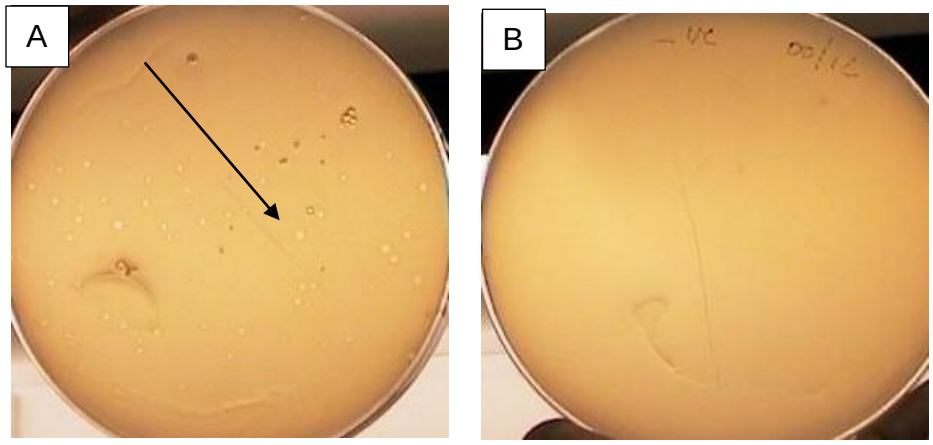

Figure 3: Image A shows a positive control plate containing MS2 plaques (shown by the arrow). Image B shows a negative control plate with a continual bacterial lawn and no plaques present.

Calculate the pfu per 100g of shellfish flesh, according to the following equation:

$$\left[\frac{N-N_{RNase}}{10}\right] \times F \times 100$$

where

N is the total number of plaques counted on all plates without RNase

N_{RNase} is the total number of plaques counted on plates with RNase (if used – see 8.8).

F is the dilution factor (e.g. if the count is made using the 1:3 dilution, F shall be 3 – see 8.6)

8.11. Reporting results

Results should be reported as pfu per 100 g of flesh and intravalvular liquid. If a sample result is negative at the 1:3 dilution, the result is expressed as <30 pfu/100 g shellfish flesh.

9. 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 inter-comparability exercises, which would highlight any uncertainties within the test methods.

The inclusion of the measurement of uncertainty (MU) or confidence limits (CL) for the result is not required but it is recommended that laboratories determine, as part of their quality procedure, the MU for the reported results and provide this information on request.

10. Quality control

Quality control systems offer a laboratory a mechanism to control preventable inaccuracies within a procedure.

10.1. Internal Quality Controls

Sterility controls should be set up for each container of diluent (0.1% P or PSS) that is used, at the beginning and end of each set of samples that is examined.

Positive and negative controls should be included with each set of examinations (that is, those processed at approximately the same time). The control strains listed in Section 7 may be used for this purpose. Controls should be inoculated at the same time as the samples. An uninoculated tube or bottle and/or plate should also be incubated with each set as a sterility control. Where more than one batch of medium has been used for the sample examinations, positive, negative and sterility controls should be included for each batch.

11. References

Regulations, guidance documents and international standards may be superseded, revised or updated over time. The user of this protocol should make sure that they refer to the most recent version.

ISO 6887-3:2017+Amd1:2020. Microbiology of the food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 3: Specific rules for the preparation of fish and fishery products. International Standards Organisation, Geneva.

ISO 10705-1:1995. Water Quality – Detection and Enumeration of Bacteriophage Part 1: Enumeration of F-Specific RNA bacteriophages – Part 1: Enumeration of F-specific RNA bacteriophages. International Standards Organisation, Geneva.

ISO 11133:2014+Amd1:2018+Amd2:2020. Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media. International Standards Organisation, Geneva.

WHO, 2004. Laboratory biosafety manual – third edition. Geneva: World Health Organization. 178pp. http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

12. Appendices

12.1. Appendix 1: Numbers of shellfish recommended for FRNA Bacteriophage analysis

ISO 6887-3 requires that a representative test sample contains a minimum of 10 animals, with a minimum weight of shellfish flesh and liquor of 50 g. The following numbers of animals are recommended for inclusion in the homogenisation step to ensure the required minimum weight is obtained (the recommended number of animals for collection at the sampling stage is 10% greater to allow for morbidity in a proportion of animals on receipt at the laboratory):

Type	Common name [9]	Scientific name [10]	Sample size [11]
Scallops	Mediterranean scallop	*Pecten jacobaeus*	10 - 12
	King (Great Atlantic) scallop	*Pecten maximus*	10 - 12
	Queen scallop	*Aequipecten (Chlamys) opercularis*	15 - 30
	Variegated scallop	*Mimachlamys (Chlamys) varia*	10 - 18
Oysters	Pacific oyster	*Magallana (Crassostrea) gigas*	10 - 18
	Portuguese oyster	*Magallana (Crassostrea) angulata*	10 - 18
	European flat oyster	*Ostrea edulis*	10 - 18
Mussels	Blue or common mussel	*Mytilus edulis*	15 - 30
	Mediterranean mussel	*Mytilus galloprovincialis*	10 - 30
	Northern horse mussel	*Modiolus modiolus*	10 - 12
	Bearded horse mussel	*Modiolus barbatus*	15 - 30
Ark clams	Ark clam	*Barbatia barbata*	15 - 25
	Noah's ark shell	*Arca noae*	15 - 30
Clams	Smooth clam	*Callista chione*	10 - 30
	Striped venus clam	*Chamelea gallina*	40 - 80
	Rayed artemis	*Dosinia exoleta*	18 - 35
	Hard clam	*Mercenaria mercenaria*	12 - 18
	Palourde (Grooved carpet shell)	*Ruditapes (Tapes/Venerupis) decussatus*	18 - 35
	Manila clam	*Ruditapes (Tapes) philippinarum*	18 - 35
	Banded carpet shell	*Polititapes (Venerupis) rhomboides*	20 - 25
	Pullet carpet shell	*Venerupis corrugata (senegalensis)*	20 - 25
	Warty venus clam	*Venus verrucosa*	15 - 30
	Atlantic surf clam (Thick trough shell)	*Spisula solida*	30 - 50
	Cut trough shell	*Spisula subtruncata*	70 - 90
	Bean clam	*Donax spp.*	30 - 50
	Wedge shell clam	*Donax trunculus*	40 - 80
	Common cockle	*Cerastoderma edule*	30 - 50
	Soft shell clam (Sand gaper)	*Mya arenaria*	10 - 12
	Pacific geoduck	*Panopea generosa*	10 - 12
	Sword razor	*Ensis ensis*	10 - 12

[9] Species of relevance provided by European NRLs.
[10] Scientific names are those used by the World Register of Marine Species at the time of writing, however commonly used alternatives for genus or species names are shown in brackets.
[11] For species not given in the table, sufficient shellfish should be opened to achieve a 50g weight of flesh and liquor, with the provision that a minimum of ten animals should be used in all cases. In general, the more shellfish that are included in the initial homogenate, the less the final result will be influenced by the inherent animal-to-animal variation.

Razor clams and geoducks	Razor shell	*Ensis magnus (arcuatus)*	10 - 12
	Atlantic razor clam (Jacknife clam)	*Ensis leei*	10 - 12
	Pod razor	*Ensis siliqua*	10 - 12
	Grooved razor shell	*Solen marginatus*	10 - 12

Note: Laboratories are encouraged to submit suggestions on minimum sample sizes for other species to the FAORC. Please note that these recommendations must conform to the minimum requirement of 10 animals given in ISO 6887-3.

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