Standing Committee of Analysts

The Microbiology of Recreational and Environmental Waters (2016) – Part 11 – Methods for the isolation and enumeration of somatic and F-specific bacteriophages and bacteriophages infecting *Bacteroides fragilis*

Methods for the Examination of Waters and Associated Materials

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Methods for the Examination of Waters and Associated Materials

This booklet contains a method for the concentration of somatic coliphages and F-specific coliphages from water, two methods for their enumeration by plaque assay and one method for the enumeration of *Bacteroides*-infecting bacteriophages by plaque assay

A The concentration of male specific (F+) bacteriophages and somatic coliphages by membrane filtration

B The enumeration of male specific (F+) bacteriophages by plaque formation using an agar overlay technique

C The enumeration of somatic coliphages by plaque formation

D The enumeration of bacteriophages infecting *Bacteroides fragilis* by plaque formation

This bluebook updates, replaces and adds to section 7.15 of the earlier version of The Microbiology of Recreational and Environmental Waters published in 2000.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products. They serve only as illustrative examples of the types of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soils (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Standing Committee of Analysts. At present, there are eight working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical, inorganic and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological, biodegradability and inhibition methods
8 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and strategic committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the SCA web-page (http://standingcommitteeofanalysts.co.uk/) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Robert Carter
Secretary
June 2015

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc. Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety ,"Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

A The concentration of male specific (F+) bacteriophages and somatic coliphages by membrane filtration

A1 Introduction

Bacteriophages are viruses that can only infect and replicate within bacteria. In many cases, these are very specific relationships. Somatic coliphages specifically infect *Escherichia coli* and Male-specific (F+) RNA bacteriophages infect those members of the Enterobacteriaceae which possess F-pili. These groups of bacteriophages have been used as indirect indicators of faecal pollution and to assess the efficiency of wastewater treatment processes. The significance of bacteriophages in recreational and environmental waters is described elsewhere[1] in this series.

The presence and numbers of bacteriophages in water are demonstrated by mixing an aliquot of the sample with the host bacterium in a semi-solid agar overlay and, after incubation, counting areas of bacterial lysis called plaques.

In samples where numbers of bacteriophage are thought to be low, they can be concentrated by an adsorption elution process. This method describes the process for concentrating low numbers of bacteriophages from water samples using adsorption elution through negatively charged membrane filters.

A2 Scope

The method is suitable for the examination of fresh and saline surface waters, swimming pools, spa pools and hydrotherapy pools. It may be applied to secondary wastewater effluents, particularly where disinfection has been used but bacteriophages in untreated wastewater can usually be demonstrated by direct plating.

Users wishing to employ this method should verify its performance under their own laboratory conditions[2, 3].

A3 Definition

In the context of this method, male specific (F+) bacteriophages are bacterial viruses which are capable of entering selected *E. coli* host strains by attachment to the F-pili (also known as sex pili), resulting in infection and replication in the host strain. The result of this infection is lysis of the host cells and the production of visible plaques (clear zones) in a confluent lawn of host bacteria grown under the conditions defined in the method.

Somatic coliphages are bacterial viruses which are capable of infecting selected *E. coli* host strains by attachment to the bacterial cell wall, infection and replication in the host strain. The result of this infection is lysis of the cell and the production of visible plaques (clear zones) in a confluent lawn of host bacteria grown under the conditions defined in the method.

A4 Principle

Samples are filtered through a negatively charged membrane made from cellulose nitrate. In order to adsorb the bacteriophages during filtration the sample is acidified, causing the phages to become positively charged. Adsorbed bacteriophages can then be eluted by passing an organic solution having an alkaline pH through the membrane. This process changes the charge on the bacteriophages again and releases them from the membrane. Bacteriophages

can be concentrated further by reducing the pH of the organic solution below the iso-electric point of the proteins in solution and causing them to flocculate. The bacteriophages and flocculated proteins can be removed by centrifugation, the pellet dissolved in phosphate buffer and used for titration or stored frozen.

A5 Limitations

This method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter and is not suitable for particulate materials such as sand or sediments.

A6 Health and Safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

Male specific (F+) bacteriophages and somatic bacteriophage are non-pathogenic and some strains are very resistant to drying.

Care should be taken when handling high titre cultures to prevent cross-contamination of samples. Cleaning surfaces with a suitable disinfectant (for example a 1% solution of sodium hypochlorite) will help to reduce the risk of cross-contamination.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted) are required. Other items include:

A7.1 Sterile sample bottles of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

A7.2 Compressed air supply (sufficient to provide 3 bar pressure)
or vacuum / peristaltic pump

A7.3 Stainless Steel, 142 or 293 mm diameter, membrane filter holders

A7.4 10 L stainless steel, pressure vessels and connecting hoses

A7.5 142 or 293 mm cellulose nitrate membranes, 0.45 µm porosity

A7.6 125 mm GF/F grade glass fibre pre-filters

A7.7 Boiling water bath

A7.8 pH meter

A7.9 Freezer, -70 ± 10 °C.

A7.10 Cooled centrifuge (7000 x g) with 6 x 500 ml capacity rotor.

A7.11 Sterile 500 ml conical centrifuge tubes

A7.12 Orbital shaker

A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method[(2)]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salts. If the pH of media are not within the stated range, then, before heating, they should be adjusted accordingly.

A8.1 *Hydrochloric acid 1M and 0.2M*

A8.2 *Sodium hydroxide 1M*

A8.3 *3% Beef extract with glycine (0.15M)*

Glycine	2.76 g
Beef extract	30 g
Water	1000 ml

Dissolve the ingredients in the water by gentle warming. The solution after sterilisation should be pH 7.2 ± 0.2. If not adjust with either solutions of A8.1 or A8.2. Distribute into suitable screw-capped bottles in volumes of 300 ml and sterilise by autoclaving at 121 °C for 15 minutes. The solution after sterilisation should be pH 7.2 ± 0.2. If not adjust with either solutions of A8.1 or A8.2. Prepared media may be stored in the dark at room temperature for up to six months.

A8.4 *0.15M Di-sodium hydrogen orthophosphate*

Di-sodium hydrogen phosphate	21.3 g
Water	1000 ml

Dissolve the di-sodium hydrogen phosphate in the water, dispense in 100 ml volumes in suitable containers and sterilise by autoclaving at 121 °C for 15 minutes. Prepared media may be stored in the dark at room temperature for up to six months.

A9 Analytical procedure

A.9.1 *Preparation*

Once collected samples should be stored at 5 ± 3 °C until analysed. The samples should be analysed as soon as is practicably possible but within 48 hours of sampling. This storage period should be verified in the user's laboratory by assessment of stability of cultures of bacteriophages.

Sterilise the pressure vessel and filter housing by autoclaving at 121 °C for 15 minutes. Disinfect the connecting hoses in the same way or by boiling in a water bath for 20 minutes.

A9.2 *Primary concentration*

Adjust the pH of the sample to 3.8 ± 0.1 by addition of 1 M or 0.2 M hydrochloric acid or 1 M sodium hydroxide, mix well and pour it into the sterile pressure vessel. Disinfect the pH probe by immersing in a circulating water bath, heated to 70 ± 2 °C, swirling the probe for 20 ± 10 seconds, before and after each use. Place a cellulose nitrate membrane (and pre-filter if required) into the filter housing and seal the top onto the base. Connect the connecting hoses to the housing and the pressure vessel and filter the sample through the filter using either positive or negative pressure or a peristaltic pump. Ensure after filtration is complete that all the sample from the head space above the filter membrane has been removed. This will be achieved when no further filtrate emerges from the filter outlet.

A9.3 *Elution*

Adjust the pH of the glycine/beef extract buffer to pH 9.25 ± 0.25 using 1M sodium hydroxide and, as necessary, making final adjustments with 1M hydrochloric acid. Using a sterile measuring cylinder pour in 70- 80 ml of 3% beef extract glycine solution into the top of the filter housing. Allow to remain in contact with the membrane for 5 minutes and then connect the pressure tubing to the inlet of the filter.

Place a sterile container under the filter outlet and pump all the glycine/beef extract solution out of the filter housing. The eluted liquid may be assayed immediately or may be stored at 5 ± 3 °C for no longer than 24 hours before assay.

The pressure vessel and filter housing should be washed thoroughly in hot tap water and sterilised as above before using again.

A9.4 *Secondary concentration*

Secondary concentration should be performed immediately after elution of the membrane. Adjust the pH of the eluent to pH 3.3 ± 0.05 by the addition of 1 M hydrochloric acid or 1 M sodium hydroxide. Place the eluent on an orbital shaker and shake for 15 ± 1 minute. Pour the eluent into suitable sterile centrifuge tubes and centrifuge, without breaking, at 7,000 g for 20 minutes at a temperature of 5 ± 3 °C.

Carefully decant the supernatant and add 4.5 ml ± 0.1 ml of 0.15 M disodium hydrogen phosphate solution (A8.4). Shake gently until the concentrated flocculated material has dissolved. Transfer the solution to a suitable container, for example a sterile 7 ml bijou bottle, and store at - 70 °C.

To analyse, remove the concentrate from the freezer, allow to warm to room temperature and proceed according to Part B or Part C depending on the type of bacteriophage being sought.

A10 Quality assurance

A bacteriophage (for example MS2) of known titre should be used to seed a water sample as a positive control and be processed with each batch of water samples. Ideally the recovery rate should be equal to or more than 30%. A negative control of 10 litres of tap water should be processed at the end of each batch of samples.

A11 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2016) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. ISO 10705-3:2003 Water quality - Detection and enumeration of bacteriophages - Part 3 – Validation of methods for concentration of bacteriophages from water. Geneva: International Organization for Standardization.

4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

B The enumeration of male specific (F+) bacteriophages by plaque formation using an agar overlay technique

B1 Introduction

Male specific (F+) RNA bacteriophages are infectious for bacteria which possess the F plasmid or sex plasmid originally detected in *Escherichia coli* K12 and absorb to the F pilus (or sex pilus) coded by this plasmid. The F plasmid is transferable to a wide range of Gram-negative bacteria.

The presence of male specific (F+) RNA bacteriophages in a water sample generally indicates pollution by wastewater contaminated by human or animal faeces. Their survival in the environment, removal by widely used water treatment processes and concentration or retention by shellfish widely resembles that of foodborne and waterborne human enteric viruses, for example, enteroviruses, hepatitis A virus and rotavirus. The significance of male specific (F+) RNA bacteriophages in recreational and environmental waters is described elsewhere[(1)] in this series.

B2 Scope

The method is suitable for the examination of fresh and saline surface waters, swimming pools, spa pools and hydrotherapy pools and primary and secondary wastewater effluents. The method is also applicable for sediments and sludges where a pre-concentration step may be required. The method can also be used for the examination of shellfish flesh.

Users wishing to employ this method should verify its performance under their own laboratory conditions[(2)].

B3 Definition

In the context of this method, male specific (F+) bacteriophages are bacterial viruses which are capable of entering selected *E. coli* host strains by attachment to the F-pili (also known as sex pili), resulting in infection and replication in the host strain. The result of this infection is lysis of the host cells and the production of visible plaques (clear zones) in a confluent lawn of host bacteria grown under the conditions defined in the method.

B4 Principle

Escherichia coli has receptor sites for somatic bacteriophages as well as the F-pilus site. A strain of *Salmonella* Typhimurium (WG49) has been genetically modified to contain the F-specific plasmid to prevent somatic coliphages giving false positive results. The plasmid also provides information for the fermentation of lactose and resistance to some antibiotics. This strain can lose the plasmid and, therefore, its susceptibility to male specific (F+) RNA bacteriophages. Quality control of the host strain at various stages within the procedure is, therefore, important.

The host bacterium can be purchased from a culture collection (for example, the National Collection of Type Cultures). The host bacterium is first grown and then frozen to create a reference culture. A portion of this reference culture is subsequently grown and frozen again to create a stock of material for working cultures. The working cultures are used in the assay at a concentration of 10^8 cells per ml. This cell concentration is established by performing a growth curve and making spectrophotometric measurements of absorbance together with a viability

count of the culture using a spread plate method. To perform an assay, the host is grown to an optimum absorbance which correlates to a cell density of 10^8 cells per ml before use.

The sample containing the bacteriophage is mixed with a small volume of semi-solid nutrient medium containing a culture of the host strain and, where necessary, antibiotics. The sample is then poured onto a solid nutrient agar plate, also containing antibiotics, and allowed to set. Plates are incubated under aerobic conditions at 37 ±1 ˚C for 18 ± 2 hours. Areas of clearing in the bacterial lawn (plaques) are counted and the results expressed as plaque forming units (pfu) per unit volume of sample tested.

Loss of the plasmid can be established by demonstration of the failure of colonies to ferment lactose on MacConkey agar, loss of resistance to nalidixic acid and kanamycin and loss of susceptibility to the coliphage MS2.

B5 Limitations

This method is suitable for most types of aqueous samples and, following appropriate concentration techniques, solid and shellfish samples. High numbers of bacteriophage will require serial dilution of the sample and low numbers may require the use of a concentration technique (see Method A).

B6 Health and Safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations(3) and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere(2) in this series. The host strain of *S.* Typhimurium used in this method is of low pathogenicity to man.

Male specific (F+) RNA bacteriophages are non-pathogenic. Some strains are very resistant to drying.

Care should be taken when handling high titre cultures to prevent cross-contamination of samples. Cleaning surfaces with a suitable disinfectant (for example a 1% solution of sodium hypochlorite) will help to reduce the risk of cross-contamination.

Chloroform is a carcinogenic substance. It should be used with care or an alternative method which is equivalent for the removal of host cells may be used, for example membrane filtration providing that the membranes do not adsorb phage particles.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere(2) in this series. Principally, incubators (fan assisted) are required. Other items include:

B7.1 Sterile sample bottles of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

B7.2 Incubators capable of maintaining temperatures of 37.0 ± 1.0 °C and 45.0 ± 1.0 °C.

B7.3 Incubator or water bath capable of maintaining temperatures of 36.0 ± 2.0 °C with a shaking device.

B7.4 Deep freezers capable of maintaining temperatures of - 20.0 ± 5.0 °C and - 70 ± 10 °C. Alternatively, for the latter, a liquid nitrogen storage vessel.

B7.5 Spectrophotometer equipped with a grating or filter for 500 – 650 nm with a maximum bandwidth of 10 nm capable of holding 1 cm cuvettes or the side-arm of a nephelometric flask or a turbidity meter with side-arm fitting.

B7.6 Cuvettes with a 1 cm optical path or nephelometric conical flasks with cylindrical side-arms which fit into the spectrophotometer.

B7.7 Conical flasks of 250 – 300 ml capacity with cotton wool plugs or suitable alternative (for example a 250 ml borosilicate bottle).

B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salts. If the pH of media are not within the stated range, then, before heating, they should be adjusted accordingly.

B8.1 *Tryptone-yeast extract glucose broth (TYGB)*[4]

B8.1.1 *Basal medium*

Trypticase peptone	10 g
Yeast extract	1 g
Sodium chloride	8 g
Water	1 litre

Dissolve the ingredients in the water. Adjust the pH such that after sterilisation it will be pH 7.2 ± 0.2. Distribute into suitable containers in volumes of 200 ml and sterilise by autoclaving at 121 °C for 15 minutes. Prepared media may be stored in the dark at room temperature for up to one month.

B8.1.2 *Calcium-glucose solution*

Calcium chloride dihydrate	3 g
Glucose	10 g
Water	100 ml

Dissolve the ingredients in the water and filter sterilise the calcium glucose solution through a nominal 0.2 µm membrane filter. The filter sterilised solution may be stored in the dark at 5 ± 3 °C for up to six months.

B8.1.3 *Nalidixic acid solution*

Nalidixic acid	250 mg
Sodium hydroxide (1 M solution)	2 ml
Water	8 ml

Dissolve the nalidixic acid in the sodium hydroxide solution, then add the water and mix well. Filter sterilise the nalidixic acid solution through a nominal 0.2 µm membrane filter. The prepared solution may be stored at 5 ± 3 °C for no longer than 8 hours or at - 20 °C for up to 6 months.

Note: If nalidixic acid - sodium salt is purchased the antibiotic can be dissolved directly in the water without the addition of sodium hydroxide.

B8.1.4 *Complete medium*

Basal medium	200 ml
Calcium glucose solution	2 ml
Nalidixic acid solution	2 ml

Aseptically add calcium glucose and antibiotic solutions to the basal medium. If not used immediately, store at between 5 ± 3 °C and use within five days.

B8.2 *Tryptone yeast extract glucose agar (TYGA)*

B8.2.1 *Basal medium*

Trypticase peptone	10 g
Yeast extract	1 g
Sodium chloride	8 g
Agar	12 - 20g
Water	1000 ml

Dissolve the ingredients in the water. Adjust the pH such that after sterilisation it will be pH 7.2 ± 0.2. Distribute into suitable screw-capped containers in volumes of 200 ml and sterilise by autoclaving at 121 °C for 15 minutes. Store in the dark at 5 ± 3 °C for up to one month.

Note: In practice the best results may be achieved by using 2% agar in TYGA.

B8.2.2 *Complete medium*

Basal medium	200 ml
Calcium glucose solution	2 ml
Nalidixic acid solution	2 ml

Melt the basal medium, cool to 50 °C and aseptically add the calcium glucose and antibiotic solution. Mix well and pour into either 9 cm or 14 cm Petri dishes. Poured plates may be kept at a temperature of 5 ± 3 °C for up to 1 month, if protected against dehydration.

B8.3 *Semi-solid tryptone yeast extract glucose agar (ssTYGA)*

Prepare the basal medium (B8.2.1) but use only half the amount of agar i.e. 6 – 10 g. Distribute into suitable containers in 100 ml volumes.

Note: In practice, 1.5% agar has been found to give a satisfactory performance in assays. Lower agar concentrations may result in the overlay detaching from the agar base during incubation.

B8.4 *RNase solution*

RNase	100 mg
Water	100 ml

Dissolve the RNase in the water while heating at 100 °C for 10 minutes. Distribute into suitable containers in 0.5 ml volumes and store at -20 °C for up to 1 year.

B8.5 *Glycerol (sterile)*

Distribute into suitable screw-capped containers in 20 ml volumes and sterilise by autoclaving at 121 °C for 15 minutes. Store in the dark at ambient temperature for up to 6 months.

B8.6 *Other media*

Standard and commercial formulations of other media and reagents used in this method include MacConkey agar, maximum recovery diluent and nutrient agar.

B9 Analytical procedure

B9.1 *Preparation of stock cultures*

A lyophilised reference culture of *Salmonella* Typhimurium should be purchased from a culture collection, for example *S.* Typhimurium strain WG49 NCTC 12484. Rehydrate the host using 1 ml of TYGB. Inoculate the suspension into a 300 ml sterile conical flask containing 50 ml of TYGB. Incubate for 18 ± 2 hours at 37 ± 1 °C whilst gently shaking using an incubator or water bath. Add 10 ml of glycerol (B8.6), mix well and distribute into plastic vials in volumes of approximately 1.2 ml and store at – 70 ± 10 °C or in liquid nitrogen. This represents a reference standard for the laboratory.

Note: In practice, where a shaker or shaking water bath are not available, a magnetic stirrer placed in an incubator and a sterile magnetic following bar placed in the TYGB works equally well.

B9.2 *Preparation of working cultures*

Remove a vial of stock culture (B9.1) and allow to warm to room temperature. Inoculate a plate of MacConkey agar or other lactose containing medium to obtain single colonies. Incubate at 37 ± 1 °C for 18 ± 2 hours. The remaining contents of the vial can be used to inoculate further plates or should be discarded.

Add 50 ml of TYGB to a sterile conical flask or other suitable container and warm to room temperature (faster growth will occur if the broth is warmed to 37 ± 1 °C). Select three to five

lactose positive colonies from the MacConkey agar (see Figure B1) and inoculate these into the flask. Incubate for 5 ± 1 hours at 37 ± 1 °C whilst gently shaking. Add 10 ml of sterile glycerol (B8.6), mix well and distribute into plastic vials in volumes of approximately 1.2 ml and store at -70 ± 10 °C or in liquid nitrogen for a maximum of two years.

B9.3 *Calibration of absorbance measurements for counts of viable bacteria*

Remove a vial of working culture (B9.2) and allow to warm to room temperature. Add 50 ml of TYGB to a nephelometric conical flask and adjust the spectrophotometer or turbidity meter reading to zero on the filled flask side-arm. Alternatively add 50 ml of TYGB to an ordinary conical flask or other suitable container, aseptically transfer a portion to a cuvette and adjust the spectrophotometer reading to zero. Discard the broth from the cuvette.

Inoculate the TYGB with 0.5 ml of working culture and incubate at 37 ± 1 °C with gentle shaking for up to 3 hours. Each 30 minutes, measure the absorbance and remove a 1 ml aliquot for viable counts. The flask should be out of the incubator for as short a time as possible.

Dilute the sample to 10^{-6} using serial ten-fold dilutions in TYGB and count the colony forming units (cfu) in 0.1 ml of the 10^{-4}, 10^{-5} and 10^{-6} dilutions using a standard spread plate technique on TYGA in duplicate. Incubate plates at 37 ± 1 °C for 24 ± 2 hours. Count the total number of colonies in each plate yielding between 30 – 300 colonies and calculate the cfu/ml.

Note: this procedure should be carried out several times (approximately 2 - 3 times) to establish the relationship between the absorbance and the colony count. When sufficient data has been obtained, further work can be carried out based on absorbance alone.

B9.4 *Quality control of the host strain WG49*

Prepare a culture as described in B9.3 or during the turbidity calibration process: At times t = 0 and times t = 3 hours.

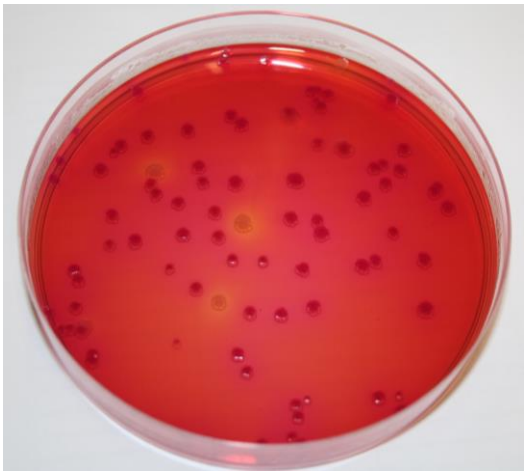
B9.4.1 *Lactose fermentation*

Inoculate two plates of MacConkey agar or other lactose-containing medium with the same dilution series and incubate at 37 ± 1 °C for 24 ± 2 hours. From plates yielding between 30 – 300 colonies, count the number of lactose positive and lactose negative colonies and calculate the number of lactose negative colonies (see Figure B1). The lactose negative colonies should be less than 8 % of the total number.

B9.4.2 *Resistance to nalidixic acid*

Spread 0.2 ml of the 10^{-2} dilution onto a plate of MacConkey agar or alternative medium. Place one disc with nalidixic acid on the plate and incubate at 37 °C for 24 ± 1 hour. There should be no zone of inhibition around the nalidixic acid disc.

Figure B1 Lactose positive (pink) and lactose negative (orange) colonies of *Salmonella* Typhimurium WG49 on MacConkey agar



B9.4.3 *Susceptibility to F-specific bacteriophages*

Thaw four vials of a stock culture of bacteriophage MS2 (see Appendix B1) and plate out 1ml volumes in duplicate on lawns of both *E. coli* K12 (NCTC 10538) and *S.* Typhimurium WG49. After incubation, count the number of plaques for each host and calculate the recovery on WG49 relative to *E. coli* K12. WG49 is acceptable if the recovery is greater than 80 % of the recovery with *E. coli* K12.

B9.5 *Preparation of inoculum cultures*

Remove a vial of working culture of *S.* Typhimurium WG49 (B9.2) and allow to warm to room temperature. Add 50 ml of TYGB to a nephelometric, ordinary conical or other suitable flask, pre-warmed to 37 °C and adjust the spectrophotometer reading to zero. Inoculate 0.5 ml of the working culture into TYGB and incubate at 37 ± 1 °C with gentle shaking. Remove an aliquot every 30 minutes and measure the absorbance. Once the absorbance corresponding to a cell density of 10^8 cfu/ml is reached, cool the suspension in melting ice and use within two hours.

Note: It is essential that the culture is cooled quickly to prevent loss of the F-pili from the cells. In practice, the culture can be kept at ambient temperature and used within 45 minutes before susceptibility to male specific (F+) RNA bacteriophages diminishes.

B9.6 *Standard assay procedure*

Prepare an inoculum culture (B9.5). Melt 100 ml bottles of ssTYGA and place in a water bath or incubator at 45 ± 1 °C. Add 1.0 ml of calcium-glucose and antibiotic solution to each bottle and distribute the medium into suitable containers, for example culture tubes or glass universal containers in 2.5 ml volumes placed at 45 ± 1 °C. Add 1 ml of the original sample or dilutions, and 1 ml of inoculum culture. Examine each aliquot of sample at least in duplicate. Mix by rolling between the palms of both hands to avoid bubbles, and pour onto a TYGA plate making sure that the overlay is distributed evenly. Allow to set and dry the plates by leaving the lid partially open for 10 – 15 minutes. Once dry, cover, invert the plates and incubate at 37 ± 1 °C for 18 ± 2 hours.

Count the number of plaques on each plate within 4 hours after finishing incubation using an oblique light.

Note: The addition of sample and ice-cold host to the overlay may cause the overlay to set. This can be prevented by having a delay between the addition of sample and host to allow the overlay to warm up. Once the sample has been added, the time delay at 45 °C should be no more than 10 minutes.

B9.7 *Samples with low phage counts*

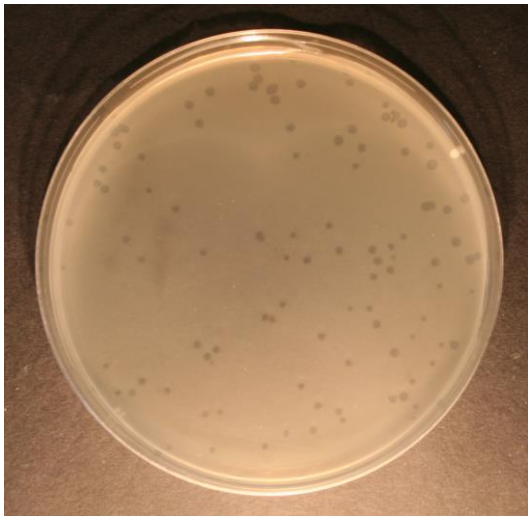
Larger volumes of sample can be analysed by using 14 cm Petri dishes or two 9 cm Petri dishes with 10 ml of overlay and 5 ml of sample.

B9.8 *Reading results*

B9.8.1 *Plaque assay tests*

Count the number of small (typically 2 – 3 mm in diameter) plaques on each plate within 4 hours after finishing incubation using an oblique light (see Figure B2).

Figure B2 Male specific (F+) RNA bacteriophage plaques in host *Salmonella* Typhimurium WG49 on ssTYGA overlaid with TYGA caused by coliphage MS2



B9.8.2 *Confirmation test*

It is important to confirm that plaques are produced by RNA phages. This is done by incorporating RNase (B8.5) into the ssTYGA. The N_{RNase} count is the proportion of the total count that do not produce plaques on RNase supplemented ssTYGA. Confirmatory tests should at least be carried out

a) when examining new sampling points;

b) regularly at fixed sampling points when N_{RNase}/N (see C10.1) is usually less than 10 %;

c) always at fixed sampling points when N_{RNase}/N is usually greater that 10 %;

d) if large (typically approximately 6 mm in diameter), circular, clear plaques with smooth edges (probably somatic *Salmonella* phages) are regularly seen.

On rare occasions, RNA phages may not be inhibited by RNase at 40 µg/ml and it may be necessary to increase the RNase concentration to 400 µg/ml.

B10 Calculations

From the number of plaques counted calculate the number of plaque-forming units (pfu) of bacteriophage in 1 ml of the sample as follows:

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

Where:

C_{pfu} is the confirmed count of male specific (F+) RNA bacteriophages per ml

N is the total number of plaques counted on WG 49 plates according to B9.6, B9.7 or B9.8

N_{RNase} is the total number of plaques counted with RNase according to B9.8.2

n is the number of replicates

F is the dilution or concentration factor

B11 Expression of results

Presumptive and confirmed male specific (F+) RNA bacteriophages are expressed in plaque forming units (pfu) per volume of sample. For most samples the volume is typically 1 ml.

B12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *Salmonella* Typhimurium WG49 and a suitable male specific (F+) RNA bacteriophage, for example MS2, see Appendix B1). Petri dishes should be incubated for 18 ± 3 hours at 37 °C. Further details are given elsewhere[(2)] in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2016) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. ISO 10705-1:1995 Water quality - Detection and enumeration of bacteriophages - Part 1 – Enumeration of F-specific RNA bacteriophages. Geneva: International Organization for Standardization.

Appendix B1 Method for the preparation of MS2 stock suspensions

A stock of bacteriophage MS2 should be prepared in order to assess the susceptibility of *Salmonella* Typhimurium to the phage. Ideally this should be done in a room or area separate from where sample analysis is done. This is because MS2 is a highly resistant phage and working cultures can become readily contaminated.

A lyophilised reference culture of bacteriophage MS2 should be purchased from a culture collection, for example NCTC 12487 together with the appropriate host *E. coli* K12 Hfr. Inoculate a 300 ml conical flask containing 50 ml of TYGB with 0.5 ml of a culture of *E. coli* K-12. Incubate for 18 ± 2 hours at 37 ± 1 ˚C whilst gently shaking using an incubator or water bath. Inoculate a fresh flask of TYGB with 0.5 ml of the overnight culture of *E. coli* K12 and incubate as above for 90 ± 10 minutes. Rehydrate the phage using 1 ml of TYGB and add the resultant suspension to the flask. Incubate for a further 5 ± 1 hour.

Add 5 ml of chloroform to the flask and mix thoroughly. Store in a refrigerator at 5 ± 3 °C for 18 hours. Carefully remove the supernatant and centrifuge at 3,000 x g for 20 minutes. Carefully pipette the supernatant into a 100 ml bottle.

To titrate the stock suspension, dilute to 10^{-10} in MRD. Test each dilution in duplicate using the method described in B9.6. Adjust the titre of the stock suspension to 10^{7} pfu/ml, add glycerol to a final concentration of 5%, dispense into plastic vials in volumes of approximately 1.2 ml and store at – 70 ± 10 ˚C or in liquid nitrogen.

C The enumeration of somatic coliphages by plaque formation

C1 Introduction

Somatic coliphages in water indicate contamination by human or animal faeces or by wastewater containing such material. Their survival in water resembles that of human enteric viruses more closely than commonly used faecal indicator bacteria. The natural host for coliphages is *Escherichia coli* but other closely related species which may be found in pristine waters may permit the multiplication of somatic coliphages in these environments. They provide a rapid and simple method of determining faecal pollution in environmental waters.

Somatic coliphages are classified into a number of different groups depending on their morphology and type of genome. They attach to lipopolysaccharide or protein receptors in the cell wall and may lyse the host cell within 20 – 30 minutes of infection. They produce plaques of widely differing size and morphology.

The significance of coliphages in recreational and environmental waters is described elsewhere(1) in this series.

C2 Scope

The method is suitable for the examination of fresh and saline surface waters, swimming pools, spa pools and hydrotherapy pools and primary and secondary wastewater effluents. The method is also applicable for sediments and sludges where a pre-concentration step may be required.

Users wishing to employ this method should verify its performance under their own laboratory conditions(2).

C3 Definition

In the context of this method, somatic coliphages are bacterial viruses which are capable of infecting selected *E. coli* host strains by attachment to the bacterial cell wall, infection and replication in the host strain. The result of this infection is lysis of the cell and the production of visible plaques (clear zones) in a confluent lawn of host bacteria grown under the conditions defined in the method.

C4 Principle

The host bacterium can be purchased from a culture collection (for example, the American Type Culture Collection). The host bacterium is first grown and then frozen to create a stock culture. This is subsequently grown and frozen to create working cultures. The working cultures are used in the assay at a concentration of 10^8 cells per ml. This cell concentration is established by performing a growth curve and making spectrophotometric measurements of absorbance together with a viability count of the culture using a spread plate method. To perform an assay, the host is grown to an optimum absorbance which correlates to a cell density of 10^8 cells per ml before use.

The sample containing the bacteriophage is mixed with a small volume of semi-solid nutrient medium containing a culture of the host strain and, where necessary, antibiotics. The sample is then poured onto a solid nutrient agar plate, also containing antibiotics, and allowed to set. Plates are incubated under aerobic conditions at 36 ± 1 °C for 18 ± 2 hours. Areas of clearing in

the bacterial lawn (plaques) are counted and the results expressed as plaque forming units (pfu) per unit volume of sample tested.

C5 Limitations

This method is suitable for most types of aqueous samples and, following appropriate concentration techniques, solid and shellfish samples. High numbers of bacteriophage will require serial dilution of the sample and low numbers may require the use of a concentration technique (see Method A).

C6 Health and Safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

Somatic bacteriophages are non-pathogenic. Some strains are very resistant to drying.

Care should be taken when handling high titre cultures to prevent cross-contamination of samples. Cleaning surfaces with a suitable disinfectant (for example a 1% solution of sodium hypochlorite) will help to reduce the risk of cross-contamination.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, incubators (fan assisted) are required. Other items include:

C7.1 Sterile sample bottles of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

C7.2 Incubators capable of maintaining temperatures of 36.0 ± 2.0 °C and 45.0 ± 1.0 °C.

C7.3 Incubator or water bath capable of maintaining temperatures of 36.0 ± 2.0 °C with a shaking device.

C7.4 Deep freezers capable of maintaining temperatures of - 20.0 ± 5.0 °C and - 70 ± 10 °C. Alternatively, for the latter, a liquid nitrogen storage vessel.

C7.5 Spectrophotometer equipped with a grating or filter for 500 – 650 nm with a maximum bandwidth of 10 nm capable of holding 1 cm cuvettes or the side-arm of a nephelometric flask.

C7.6 Cuvettes with a 1 cm optical path or nephelometric conical flasks with cylindrical side-arms which fit into the spectrophotometer.

C7.7 Conical flasks of 250 – 300 ml capacity with cotton wool plugs or suitable alternative (for example a 250 ml borosilicate bottle).

C8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salts. If the pH of media are not within the stated range, then, before heating, they should be adjusted accordingly.

C.8.1 *Modified Scholtens' broth (MSB)[4]*

Peptone	10 g
Yeast extract	3 g
Meat extract	12 g
Sodium chloride	3 g
Sodium carbonate solution (150 g/l)	5 ml
Magnesium chloride solution (100 g of $MgCl_2$ $6H_20$ in 50 ml water)	0.3 ml
Water	1 litre

Dissolve the ingredients in the water. Adjust the pH such that after sterilisation it is 7.2 ± 0.2. Dispense in volumes of 200 ml in suitable screw-capped containers and sterilise at 121 °C for 15 minutes. The sterile media can be stored at a temperature of 5 ± 3 °C for up to one month.

C8.2 *Modified Scholtens' agar (MSA)*

C8.2.1 *Basal medium*

Peptone	10 g
Yeast extract	3 g
Meat extract	12 g
Sodium chloride	3 g
Sodium carbonate solution (150 g/l)	5 ml
Magnesium chloride solution (100 g of $MgCl_2$ $6H_20$ in 50 ml water)	0.3 ml
Agar	10 – 20 g
Water	1 litre

Dissolve the ingredients in the water. Adjust the pH such that after sterilisation it is 7.2 ± 0.2. Dispense in volumes of 200 ml in suitable screw-capped containers and sterilise at 121 °C for 15 minutes. The sterile media can be stored at a temperature of 5 ± 3 °C for up to one month.

C8.2.2 *Calcium chloride solution*

Calcium chloride dihydrate	14.6 g
Water	1 litre

Dissolve the calcium chloride in the water whilst heating gently. Filter sterilise the calcium chloride solution through a nominal 0.2 µm membrane filter. Store at 5 ± 3 °C for up to one month.

C8.2.3 *Complete medium*

Basal medium	200 ml
Calcium chloride solution	1.2 ml

Melt the basal medium, cool to 45 – 50 °C and aseptically add the calcium chloride solution. Mix and pour the medium into Petri dishes and allow the agar to solidify. Poured Petri dishes may be stored at 5 ± 3 °C for up to one month if protected against dehydration.

C8.3 *Semi-solid modified Scholtens' agar (ssMSA)*

Prepare the basal medium (C8.2.1) but use only half the amount of agar i.e. 6 – 10 g.

Note: the concentration of agar will vary depending on the gel strength. A concentration should be chosen which gives the highest plaque count but also restricts plaque size to prevent confluence.

Note: triphenyltetrazolium chloride solution (1 ml of a solution of 1 g in 100 ml 96 % ethanol per 100 ml of ssMSA) may be added to the overlay to enhance contrast for counting plaques.

C8.4 *Nalidixic acid solution*

Nalidixic acid	250 mg
Sodium hydroxide (1 M solution)	2 ml
Water	8 ml

Dissolve the nalidixic acid in the sodium hydroxide solution, then add the water and mix well. Filter sterilise the nalidixic acid solution through a nominal 0.2 µm membrane filter. The prepared solution may be stored at 5 ± 3 °C for no longer than 8 hours or at - 20 °C for up to 6 months.

Note: Purchase of nalidixic acid - sodium salt means that the antibiotic can be dissolved directly in the water without the addition of sodium hydroxide.

C8.5 *Glycerol (sterile)*

Distribute into suitable containers in 20 ml volumes and sterilise by autoclaving at 121 °C for 15 minutes. Store in the dark at ambient temperature for no longer than 6 months.

C8.6 *Other media and reagents*

Standard and commercial formulations of other media and reagents used in this method include MacConkey agar, maximum recovery diluent nutrient agar and chloroform.

C9 Analytical procedure

Note: For culturing the host strain, gentle shaking will increase the growth rate and ensure that all cells are actively growing. Inoculum cultures should be repeatedly shaken by hand if a shaker is not available.

Once collected samples should be stored at 5 ± 3 °C until analysed. The samples should be analysed as soon as is practicably possible but within 48 hours of sampling. This storage period should be verified in the user's laboratory by assessment of stability of cultures of bacteriophages.

C9.1 *Preparation of stock cultures*

A lyophilised reference culture of *E. coli* should be purchased from a culture collection, for example *E. coli* strain C ATCC 13706. For polluted environmental waters and wastewater, the nalidixic acid resistant mutant *E. coli* strain CN ATCC 700078 should be used. Rehydrate the host using 1 ml of MSB. Inoculate the suspension into a 300 ml conical flask containing 50 ml of MSB. Incubate for 20 ± 4 hours at 36 ± 2 °C whilst gently shaking using an incubator or water bath. Add 10 ml of glycerol (C8.5), mix well and distribute into plastic vials in volumes of approximately 0.5 ml and store at - 70 ± 10 °C or in liquid nitrogen.

C.9.2 *Preparation of working cultures*

Remove a vial of stock culture (C9.1) and allow to warm to room temperature. Inoculate a plate of MacConkey agar or other lactose containing medium to obtain single colonies. Incubate at 36 ± 2 °C for 20 ± 4 hours. The remaining contents of the vial can be used to inoculate further plates or should be discarded.

Add 50 ml of MSB to a conical flask and warm to room temperature (faster growth will occur if the broth is warmed to 36 ± 2 °C). Select three to five lactose positive (pink) colonies from the MacConkey agar and inoculate these into the flask. Incubate for 5 ± 1 hours at 36 ± 2 °C whilst gently shaking. Add 10 ml of sterile glycerol (C8.5), mix well and distribute into plastic vials in volumes of approximately 0.5 ml and store at - 70 °C or in liquid nitrogen for no longer than two years.

C9.3 *Calibration of absorbance measurements for counts of viable bacteria*

Remove a vial of working culture (C9.2) and allow to warm to room temperature. Add 50 ml of MSB to a nephelometric conical flask and adjust the spectrophotometer reading to zero on the filled flask side-arm. Alternatively add 50 ml of MSB to an ordinary conical flask, aseptically transfer a portion to a cuvette and adjust the spectrophotometer reading to zero. Discard the broth.

Inoculate the MSB with 0.5 ml of working culture and incubate at 36 ± 2 °C with gentle shaking for up to 3.5 hours. Each 30 minutes, measure the absorbance and remove a 1 ml aliquot for viable counts. The flask should be out of the incubator for as short a time as possible.

Dilute the sample to 10^{-7} using serial ten-fold dilutions and count the colony forming units (cfu) in 1 ml of the 10^{-5}, 10^{-6} and 10^{-7} dilutions using a standard pour plate technique in nutrient agar or MSA in duplicate. Membrane filtration may also be used to count the colonies. Count the total number of colonies in each plate yielding between 30 – 300 colonies and calculate the cfu/ml.

Note: this procedure should be carried out approximately 2 - 3 times to establish the relationship between the absorbance and the colony count. When sufficient data has been obtained, further work can be carried out based on absorbance alone.

The optimum cell density for assay is approximately 10^8 cells per ml. If this cell density is not reached within 3.5 hours, the working culture inoculum can be increased to 1 ml.

C9.4 *Preparation of inoculum cultures*

Remove a vial of working culture (C9.2) and allow to warm to room temperature. Add 50 ml of MSB to a nephelometric or ordinary conical flask pre-warmed to 37 °C and adjust the spectrophotometer reading to zero. Inoculate 0.5 ml of the working culture into MSB and incubate at 36 ± 2 °C with gentle shaking. Remove an aliquot every 30 minutes and measure the absorbance. Once the absorbance corresponding to a cell density of 10^8 cfu/ml is reached, cool the suspension by plunging the flask into a bucket/beaker of melting ice and use within the day.

Note: An alternative but less controlled way of preparing the inoculum culture is to incubate for 3 ± 1 hours with gentle shaking but not using spectrophotometric measurements. The inoculum can be the working culture or several colonies from an agar plate or slope.

C9.5 *Standard assay procedure*

Prepare an inoculum culture (C9.4). Melt 50 ml bottles of ssMSA and place in a water bath or incubator at 45 ± 1 °C. Add 300 μl of calcium chloride solution (C8.2.2) and distribute the medium into culture tubes in 2.5 ml volumes placed at 45 ± 1 °C. Add 1 ml of the original sample or dilutions, and 1 ml of inoculum culture. Examine each aliquot at least in duplicate. Mix, avoiding bubbles, and pour onto an MSA plate making sure that the overlay is distributed evenly. Allow to set and dry the plates by leaving the lid partially open for 10 – 15 minutes. Once dry, cover, invert the plates and incubate at 36 ± 2 °C for 18 ± 2 hours. Count the number of plaques on each plate within 4 hours after finishing incubation using an oblique light.

Note: Plates may be read after 6 hours incubation. This is useful if a high contaminating bacterial background is suspected. Plates should be returned to the incubator to complete incubation.

Where a large number of samples are anticipated, several flasks may be inoculated. The contents of these flasks should be combined into one flask for the assay and mixed well.

If required, a freshly prepared triphenyltetrazolium chloride solution can be added to the overlays to enhance contrast for counting plaques.

The addition of sample and ice-cold host to the overlay may cause the overlay to set. This can be prevented by having a delay between the addition of sample and host to allow the overlay to warm up. Once the sample has been added, the time delay at 45 °C should be no more than 10 minutes.

C.9.6 *Samples with high background bacterial flora*

Proceed according to C9.5. Add nalidixic acid solution to the ssMSA to give a final concentration of 250 μg/ml and use *E. coli* CN as the host.

C9.7 *Samples with low phage counts*

Larger volumes of sample can be analysed by using 14 cm Petri dishes or two 9 cm Petri dishes with 10 ml of overlay and 5 ml of sample.

C9.8 *Presence absence test*

A presence absence test in 1 ml of sample (or dilutions) can be done as follows:

Remove a working culture (C9.2) and to warm up to room temperature. Add 25 ml of MSB, 150 μl of calcium chloride solution and 0.25 ml of working culture to a conical flask. Incubate at 36 ± 2 °C for 3 hours with gentle shaking. Add 1 ml of sample and continue incubating for 18 ± 2 hours.

Decontaminate the resulting bacteriophage suspension, to inactivate any residual bacteria, by adding 0.4 ml of chloroform to 1 ml of culture, mixing well and centrifuging at 3,000 g for 5 minutes.

Prepare an agar overlay on an MSA plate including the host but with no sample (C9.5). Place one drop of the decontaminated culture onto the agar plate, dry, invert the plates and incubate at 36 ± 2 °C for 18 hours. Examine inoculated plates for clear zones in the spotted areas. Several spots can be used on one inoculated plate.

Note: the presence absence test can be used as an MPN test by inoculating different volumes of sample into MSB. In addition, the sample volume can be increased by using double strength MSB.

C9.9 *Reading results*

C9.9.1 *Plaque assay tests*

Count the number of plaques on each plate within 4 hours after finishing incubation using an oblique light.

Note: Plates may be read after 6 hours incubation. This is useful if a high contaminating bacterial background is suspected. Plates should be returned to the incubator to complete incubation.

C9.9.2 *Presence absence tests*

After incubation, examine the plates for clear zones where the enrichment cultures were spotted. These are evidence of the presence of somatic coliphages in the original sample.

C9.9.3 *Confirmation*

Occasionally, artefacts may resemble plaques, for example, bubbles in the medium or the agar overlay partially setting during plating. Where there is doubt about the presence of a plaque, this can be confirmed by picking the plaque with a straight wire into an ssMSA containing the host, plating and incubating as above. Confluent lysis should be evident after incubation.

C10 Calculations

C10.1 *Plaque count assay*

From the number of plaques counted calculate the number of plaque-forming units (pfu) of bacteriophage in 1 ml of the sample as follows:

$$X = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2)}$$

where

X is the number of plaque forming units per ml;

N is the total number of plaques counted on the plates;

n_1, n_2 are the number of replicates counted for dilutions F_1, F_2 respectively;

V_1, V_2 are the test volumes used with F_1, F_2 respectively;

F_1, F_2 are the dilution or concentration factors used for the test portions V_1, V_2 respectively.

If only one dilution is counted, the formula may be simplified to

$$X = \frac{N}{nVF}$$

C10.2 *Presence absence test*

Express the results as bacteriophage detected, or not detected, in the volume of sample tested. Where an MPN system is used, the results can be calculated from probability tables published elsewhere in this series[5].

C11 Expression of results

C.11.1*Plaque count assay*

Express the results as plaque forming units (pfu) per ml of sample examined. If no plaques are observed, express the results as not detected in the volume of sample examined.

C.11.2*Presence absence procedure*

Express the results as bacteriophage detected, or not detected, in the volume of sample tested.

C12 Quality assurance

C12.1 *Plaque count assay*

For each series of samples, examine a blank control using sterile diluent as the sample to ensure that the inoculum culture is not infected with bacteriophage. In addition, examine a reference standard preparation of bacteriophage B56-3 (ϕx174). This should be prepared as follows:

Inoculate 25 ml of MSB in a conical flask and inoculate with the host *E. coli* or a naladixic acid resistant strain. Incubate at 36 ± 2 °C for 20 ± 4 hours. Add 25 ml of MSB to a second flask and inoculate with 0.25 ml of the host culture. Incubate as above for 90 minutes. Add ϕx174 to give an approximate concentration of 10^7 pfu/ml and incubate for 4 - 5 hours. Add 2.5 ml of chloroform, shake well and store at 5 ± 3 °C overnight. Centrifuge the aqueous phase at 3,000 g for 5 minutes, without breaking. Remove the supernatant and store at 5 ± 3 °C.

Prepare a decimal dilution series and plate out as in C9.5. Store the dilutions at 5 ± 3 °C overnight. Count the numbers of plaques after incubation and calculate the pfu/ml of the original suspension. Prepare 100 ml to 1,000 ml of a suspension with an approximate concentration of 100 pfu/ml. Add 5 % glycerol, mix well, dispense in volumes of 2.4 ml in plastic vials and store at - 70 ± 10 °C. This forms the positive control. An alternative, or additional, control can be a natural sample, of known positive character, diluted to give 100 pfu/ml and stored as above.

C12.2 *Presence absence test*

Prepare a stock suspension as above but containing approximately 5 pfu/ml. Incorporate one positive and one negative control with each batch of samples tested. Additionally, add one positive control to a duplicate actual sample as a spiked positive demonstrating no inhibitory affect in the sample.

C13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2016) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. ISO 10705-2:2000 Water quality - Detection and enumeration of bacteriophages - Part 2 – Enumeration of somatic coliphages. Geneva: International Organization for Standardization.

5. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2016) - Part 3 - Methods for the isolation and enumeration of *Escherichia coli* (including *E. coli* O157). *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

D The enumeration of bacteriophages infecting *Bacteroides fragilis* by plaque formation

D1 Introduction

Bacteroides fragilis is one of the most common bacteria in the gastro-intestinal system of humans and animals. It is an anaerobic bacterium. Bacteriophage specific to *B. fragilis* are indicative of human or animal faecal contamination and can be found in wastewater and abattoir effluents in concentrations which are greater than those of somatic and F-specific coliphages. They do not multiply in the environment and are resistant to natural inactivation. They are considered to be a better indicator of human enteric viruses than the bacterial faecal indicators. The significance of *Bacteroides*-infecting bacteriophages in recreational and environmental waters is described elsewhere(1) in this series.

Bacteriophages attach to the host cell wall and lyse the host within 30 to 40 minutes under optimal conditions. They produce clear plaques in assay plates which do not vary much in size and morphology.

D2 Scope

The method is suitable for the examination of surface waters both fresh and saline, swimming pools, spa and hydrotherapy pools and primary and secondary wastewater effluents. The method is also applicable for sediments and sludges where a pre-concentration step may be required.

Users wishing to employ this method should verify its performance under their own laboratory conditions(2).

D3 Definitions

In the context of this method, bacteriophages infecting *B. fragilis* are capable of infecting selected *B. fragilis* host strains by attachment to the bacterial cell wall, infection and replication. The result of this infection is lysis of the cell and the production of visible plaques (clear zones) in a confluent lawn of host bacteria grown under the conditions defined in the method.

D4 Principle

The sample containing the bacteriophage is mixed with a small volume of semi-solid nutrient medium containing a culture of the host strain. The sample is then poured onto a solid nutrient agar plate and allowed to set. Plates are incubated under anaerobic conditions at 36 ± 2 °C for 21 ± 3 hours. Areas of clearing in the bacterial lawn (plaques) are counted and the results expressed as plaque forming units (pfu) per unit volume of sample tested.

The method requires the establishment of stock and working cultures of the host and the determination of cell numbers in the working suspension for inoculation of the semi-solid nutrient medium.

D5 Limitations

This method is suitable for most types of aqueous samples and, following appropriate concentration techniques, solid and shellfish samples. High numbers of bacteriophage will

require serial dilution of the sample and low numbers may require the use of a concentration technique.

D6 Health and Safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series. The host strain of *B. fragilis* used in this method is non-pathogenic to man and animals.

Bacteriophages infecting *B. fragilis* are also non-pathogenic and some strains are very resistant to drying.

Care should be taken when handling high titre cultures to prevent cross-contamination of samples and the host strain.

Chloroform is a carcinogenic substance.

D7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, static) are required. Other items include:

D7.1 Sterile sample bottles of appropriate volume, made of suitable material, should be used. For swimming pools, spa and hydrotherapy pools the containers must not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

D7.2 Incubators capable of maintaining temperatures of 36.0 ± 2.0 °C and 45.0 ± 1.0 °C.

D7.3 Incubator or water bath capable of maintaining temperatures of 36.0 ± 2.0 °C with a shaking device.

D7.4 Deep freezer capable of maintaining temperatures of - 20.0 ± 5.0 °C and –70 ± 10 °C or liquid nitrogen storage vessel.

D7.5 Spectrophotometer equipped with a grating or filter for 500 – 650 nm with a maximum bandwidth of 10 nm capable of holding 1 cm cuvettes or the side-arm of a nephelometric flask.

D7.6 Cuvettes with a 1 cm optical path

D7.7 Anaerobic jars, or similar equipment, and anaerobic gas-generating system together with anaerobiosis indicators.

D7.8 Hungate glass tubes having a butyl rubber stopper and screw cap or suitable alternative tubes which can fit in the spectrophotometer.

D8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. Commercial formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in this method[(2)]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated, chemical constituents should be added as the anhydrous salts. If the pH of the medium is not within the stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

D8.1 *Bacteroides phage recovery medium broth (BPRMB)*

D8.1.1 *Basal medium*

Meat peptone	10 g
Casein peptone	10 g
Yeast extract	2 g
Sodium chloride	5 g
L-cysteine monohydrate	0.5 g
Glucose	1.8 g
Magnesium sulphate heptahydrate	0.12 g
Calcium chloride solution	1 ml
Water	1 litre

Dissolve the ingredients in hot water. Sterilise at 121 °C for 15 minutes. The sterile media can be stored at a temperature between 5 ± 3 °C for up to one month.

D8.1.2 *Calcium chloride solution*

Calcium chloride dihydrate	5 g
Water	100 ml

Dissolve the calcium chloride in the water whilst heating gently. Filter sterilise the calcium chloride solution through a nominal 0.2 µm membrane filter. Store at 5 ± 3 °C for up to one month.

D8.1.3 *Haemin solution*

Haemin	100 mg
Sodium hydroxide (1 M solution)	0.5 ml
Water	99.5 ml

Dissolve the haemin and sodium hydroxide in the water by stirring for a minimum of 30 minutes. Sterilise by filtration or autoclaving at 121 °C for 15 minutes. Store at room temperature for no longer than 6 months.

D8.1.4 *Di-sodium carbonate solution*

Sodium carbonate	10.6 g
Water	100 ml

Dissolve the sodium carbonate in the water. Filter sterilise the di-sodium carbonate solution through a nominal 0.2 µm membrane filter. Store at room temperature for up to six months.

D8.1.5 *Kanamycin monosulphate solution*

Kanamycin monosulphate	1.25 g
Water	10 ml

Dissolve the kanamycin sulphate in the water. Filter sterilise the kanamycin sulphate solution through a nominal 0.2 µm membrane filter. Store at 5 ± 3 °C for no longer than 8 hours or at -20 °C for no longer than 6 months.

Note: Many supplies of kanamycin monosulphate contain less than 100 % active kanamycin. Adjust the weight such that 1.25 g of active kanamycin monosulphate is used.

D8.1.6 *Nalidixic acid solution*

Nalidixic acid	250 mg
Sodium hydroxide (1 M solution)	2 ml
Water	8 ml

Dissolve the nalidixic acid in the sodium hydroxide solution, then add the water and mix well. Filter sterilise the nalidixic acid solution through a nominal 0.2 µm membrane filter. The prepared solution may be stored at 5 ± 3 °C for no longer than 8 hours or at - 20 °C for up to 6 months.

Note: Purchase of nalidixic acid - sodium salt means that the antibiotic can be dissolved directly in the water without the addition of sodium hydroxide.

D8.1.7 *Complete broth*

Basal medium	1 litre
Haemin solution	10 ml
Disodium carbonate solution	25 ml
Kanamycin monosulphate solution	1 ml
Nalidixic acid solution	4 ml

Aseptically add the additives to the basal broth and mix by gentle shaking. Adjust the pH to 6.8 ± 0.2 by aseptically adding 2.5 ml of hydrochloric acid (10 M). Use immediately.

D8.2 *Bacteroides phage recovery medium agar (BPRMA)*

D8.2.1 *Basal agar*

Basal broth (D8.1)	1 litre
Agar	15 g

Mix the broth and agar and dissolve by heating. Distribute into containers in suitable volumes and sterilise by autoclaving at 121 °C for 15 minutes.

D8.2.2 *Complete agar*

Basal agar (molten at 45 - 50 °C)	1 litre
Haemin solution	10 ml
Disodium carbonate solution	25 ml
Kanamycin monosulphate solution	1 ml
Nalidixic acid solution	4 ml

Note: Where heavy background contamination is suspected (for example, sewage effluent), the kanamycin monosulphate solution should be increased to 3 ml.

Aseptically add the additives to the molten agar and mix. Adjust the pH to 6.8 ± 0.2 by aseptically adding 2.5 ml of hydrochloric acid (10 M) (in a fume cabinet). Pour the medium into Petri dishes and allow to set. Poured plates may be kept at a temperature of 5 ± 3 °C for up to 1 month, if protected against dehydration.

D8.3 *Semi-solid Bacteroides phage recovery medium agar (ssBPRMA)*

D8.3.1 *Basal semi-solid agar*

Basal broth	1 litre
Agar	7.5 g

Dissolve the agar in the broth and dispense in volumes of 50 ml into suitable containers. Sterilise by autoclaving at 121 °C for 15 minutes. Allow the agar to solidify and store at 5 ± 3 °C for up to one month.

Note: The concentration of agar will vary depending on the gel strength. A concentration should be chosen which gives the highest plaque count but also restricts plaque size to prevent confluence.

D8.3.2 *Complete medium*

Basal semi-solid agar	50 ml
Haemin solution	0.5 ml
Sodium carbonate solution	1.25 ml
Kanamycin monosulphate solution	50 µl
Nalidixic acid solution	200 µl

Note: Where heavy background contamination is suspected, the kanamycin monosulphate solution should be increased to 150 µl.

Melt the agar and cool to 45 - 50 °C. Aseptically add the haemin solution, disodium carbonate and antibiotics.

D8.4 *Cryoprotector (carrier) BSA + sucrose*

Bovine serum albumin, fraction 5	10 g
Sucrose	20 g
Distilled, deionised or similar grade water	100 ml

Dissolve the ingredients in the water by stirring for approximately 1 hour. Filter sterilise the resultant solution through a nominal 0.2 µm membrane filter and use immediately.

Note: Filtration can be difficult.

D8.5 *Glycerol*

Sterilise in 20 ml volumes by autoclaving at 121 °C for 15 minutes. Store in the dark for up to one year.

D8.6 *Peptone saline solution (maximum recovery diluent)*

Peptone	1 g
Sodium chloride	8.5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2 (at 45 ± 3 °C) such that after sterilisation it will be 7.2 ± 0.2. Dispense in suitable containers and sterilise by autoclaving at 121 °C for 15 minutes. Store in the dark at room temperature for up to three months.

D8.7 *Double strength BPRMB (dsBPRMB)*

D8.7.1 *Basal double strength broth*

Meat peptone	20 g
Casein peptone	20 g
Yeast extract	4 g
Sodium chloride	10 g
L-cysteine monohydrate	1 g
Glucose	3.6 g
Magnesium sulphate heptahydrate	0.24 g
Calcium chloride solution (D8.1.2)	2 ml
Water	1 litre

Dissolve the ingredients in the water and dispense into suitable containers. Sterilise by autoclaving at 121 °C for 15 minutes. Store at 5 ± 3 °C and use within 1 week if the bottle has been opened or within 1 month if unopened.

D8.7.2 *Complete double-strength broth*

Basal double-strength broth	1 litre
Haemin solution	20 ml
Disodium carbonate solution	50 ml
Kanamycin monosulphate solution	2 ml
Nalidixic acid solution	8 ml

Note: Where heavy background contamination is suspected, the kanamycin monosulphate solution should be increased to 6 ml.

Add the ingredients to the basal medium and adjust the pH to 6.8 ± 0.2. Use immediately

D8.8 *Triphenyltetrazolium chloride (TTC) solution*

Triphenyltetrazolium chloride	1 g
Ethanol (96%)	100 ml

Dissolve the TTC in the ethanol. For use add 1 ml to 100 ml of ssBPRMA.

D9 Analytical procedure

The analytical procedure is split into three sections detailing the preparation of stock cultures, working cultures and the test procedure. Although *B. fragilis* is a strict anaerobe, and incubation of cultures on solid media must be carried out under anaerobic conditions, incubation of broth cultures can be carried out without the use of anaerobic jars or cabinets providing tubes are completely filled and sealed with a screw cap.

Once collected sample samples should be stored at 5 ± 3 °C until analysed. The samples should be analysed as soon as is practicably possible but within 48 hours of sampling. This storage period should be verified in the user's laboratory by assessment of stability of cultures of bacteriophages.

D9.1 *Preparation of stock cultures*

A lyophilised reference culture of *B. fragilis* should be purchased from a culture collection, for example *B. fragilis* RYC2056 ATCC 700786. Rehydrate the host using 1 ml of BPRMB. Inoculate the suspension into 10 ml of BPRMB in a 10 ml screw-capped tube and incubate at 36 ± 2 °C for 21 ± 3 hours. Inoculate the broth onto BPRMA and incubate anaerobically at 36 ± 2 °C for 44 ± 4 hours.

Inoculate the entire growth of culture from the BPRMA into 10 ml of BPRMB in a 10 ml screw-topped glass tube and incubate anaerobically overnight at 36 ± 2 °C.

Mix the overnight culture with an equal volume of cryoprotector (D8.4) and mix well avoiding forming bubbles. Distribute in screw-capped vials, preferably glass, in volumes of approximately 1 ml and store at -70 ± 10 °C or in liquid nitrogen for up to 5 years.

Note: This is the first passage of the host strain and should be used as a reference in the laboratory. The purity of the culture should be checked by Gram stain, absence of growth in aerobic conditions and sensitivity to a reference bacteriophage. The stock culture is used to prepare working cultures which should also be stored at -70 °C. The working cultures should be in the logarithmic phase of growth and have a standard cell density determined by absorbance measurements.

D9.2 *Preparation of working cultures*

Remove one vial of the stock culture and allow to warm to room temperature. Inoculate onto a plate of BPRMA and incubate under anaerobic conditions at 36 ± 2 °C for 44 ± 4 hours. Inoculate the cell material from the plate into 10 ml of pre-warmed BPRMB in a screw-capped glass tube. If dense growth occurs, inoculate 1/8 of the growth onto a BPRMA plate and if poor growth occurs inoculate 1/2 of the growth onto the plate. Incubate the BPRMB culture anaerobically at 36 ± 2 °C for 21 ± 3 hours.

Add BPRMB to a 10 ml tube and warm to at least room temperature (faster growth will occur if the broth is warmed to 36 ± 2 °C). Transfer an aliquot of the BPRMB culture from above without shaking the tube and taking the aliquot from the middle of the tube and add it to the pre-warmed BPRMB in a ratio of 1.5:10(v/v). Make sure that the inoculated tube is completely filled and incubate the tube at 36 ± 2 °C to reach approximately 2 x 10^9 cells per ml.

Mix equal volumes of the working culture and cryoprotector (D8.4), mix well avoiding forming bubbles. Distribute in screw-capped vials, preferably glass, in volumes of approximately 1.5 ml, store at -70 ± 10 °C and use within 12 months. These are now the working cultures.

Note: Ensure that the culture does not reach the stationary phase of growth before mixing it with the cryoprotector. Absorbance stabilisation will indicate the end of the log phase which may last for 5 - 8 hours.

D9.3 *Calibration of absorbance measurements for counts of viable bacteria*

Remove a vial of a working culture from the freezer and allow to equilibrate to room temperature. Add BPRMB to a 10 ml capped tube for anaerobic culture and warm to room temperature (faster growth will occur if the BPRMB is warmed to 36 ± 2 °C).

D9.3.1 *Spectrophotometric measurements*

Using the blank tube of uninoculated medium, set the spectrophotometer to read zero. Add the working stock to the BPRMB in a ratio of 1:10 making sure that the tube is completely filled. Incubate at 36 ± 2 °C. Every 30 minutes, remove the tube from the incubator and read and record the absorbance.

D9.3.2 *Viable cell counts*

Melt 50 ml of ssBPRMA, cool to 45 ± 1 °C and add the haemin, disodium carbonate and antibiotics, adjust the pH to 6.8 ± 0.25 and dispense in 2.5 ml volumes into capped culture tubes. Maintain these at 45 ± 1 °C.

Immediately after taking the spectrophotometric reading, remove an aliquot of 0.3 ml from the tube. This may best be done by using a hypodermic needle and syringe. Return the culture to the incubator. Prepare serial 10-fold dilutions of the culture up to 10^{-8} in freshly autoclaved and cooled diluent. Add 1 ml volumes of the 10^{-6}, 10^{-7} and 10^{-8} dilutions in duplicate to the melted and dispensed ssBPRMA, mix gently and pour onto a plate of BPRMA in a 90 mm Petri dish. Allow the overlay to set and incubate the plates under anaerobic conditions at 36 ± 2 °C for 44 ± 4 hours. Count each plate yielding between 30 and 300 colonies and calculate the number of viable cells per ml.

Note: this procedure should be carried out several times (approximately 4 - 5 times) to establish the relationship between the absorbance and the colony count. When sufficient data has been obtained, further work can be carried out based on absorbance alone. The optimum cell density for assay is approximately 2 x 10^8 cells per ml.

D9.4 *Preparation of inoculum cultures*

Remove a vial of working culture from the freezer and allow to equilibrate to room temperature. Add BPRMB to a tube for anaerobic culture and warm to room temperature (faster growth will occur if the BPRMB is warmed to 36 ± 2 °C). Using the blank tube of unicoculated medium, set

the spectrophotometer to read zero. Add the working stock to the BPRMB in a ratio of 1:10 making sure that the tube is completely filled. Incubate at 36 ± 2 °C. After 2 hours incubation, remove the tube and measure the absorbance every 30 minutes. Once the absorbance corresponding to a cell density of 10^8 cfu/ml is reached, cool the suspension by plunging the tube into a bucket/beaker of melting ice and use within the day. The culture should be used for assay within 6 hours.

Note: If a cell density of approximately 2 x 10^8 cells per ml is not reached in 3 hours the ratio of inoculum of working stock culture to BPRMB can be increased to 1.5:10.

D9.5 *Standard assay procedure*

Prepare an inoculum as described in D9.4. Allow the sample to equilibrate to room temperature. Melt 50 ml of ssBPRMA, cool to 45 ±1 °C and add the haemin, di-sodium carbonate and antibiotics, adjust the pH to 6.8 ± 0.2 and dispense in 2.5 ml volumes into capped culture tubes. Maintain these at 45 ± 1 °C.

Prepare serial 10 fold dilutions of the sample if required. Add 1 ml of sample and 1 ml of inoculum to the ssBPRMA, mix carefully and pour over the surface of a BPMRFA plate. Allow to set and incubate anaerobically at 36 ± 2 °C for 21 ± 3 hours.

Note: Triphenyltetrazolium chloride solution may be added to the overlay to enhance contrast for counting plaques. If this is used, the plates should be kept aerobically for 1 - 2 hours before counting.

The addition of ice-cold culture to the overlay may cause the overlay to set. To avoid this, the inoculum can be warmed to room temperature immediately before use.

Samples likely to contain a high contaminating background flora can be decontaminated by membrane filtration through a sterile, low protein-binding 0.2 μm membrane, for example, a polyvinylidene difluoride membrane. Alternatively, the kanamycin sulphate concentration can be increased from 100 μg to 300 μg per ml.

D9.6 *Presence absence test*

A presence absence test can be performed by adding 100 ml of sample to 100 ml of double strength medium and 30 ml of the inoculum culture to provide an enrichment. It can also be used as a most probable number method by adding different volumes of sample to an equal volume of double strength medium.

It is important that the bottles used are completely filled and samples are incubated at 36 ± 2 °C for 21 ± 3 hours. To minimise the toxic effect of oxygen in the sample, it can be gassed for 5 minutes with nitrogen at the rate of 5 litres per minute. Alternatively, a reducing solution, for example, sodium sulphide at a final concentration of 0.04%, can be added to the sample. The addition of resazurin at a concentration of 0.5 ml per 100 ml of sample (from a stock solution of 0.025%) will help to demonstrate anaerobiosis by a colour change from blue/pink to a straw colour.

ssPRMA overlay is prepared as described in D9.5. The host is added, the mixture plated onto BPRMA and the overlay allowed to set. The enrichment culture is decontaminated by taking 1 ml of the culture, adding 0.4 ml of chloroform, mixing well and centrifuging at 3,000 x g for 5

minutes. The supernatant is dropped onto the agar overlay using a Pasteur pipette. Incubate plates anaerobically at 36 ± 2 °C for 21 ± 3 hours.

D9.7 *Reading results*

D9.7.1 *Plaque assay tests*

After incubation, count the plaques on each plate. Where possible, select plates with more than 30 well-separated plaques. When plates cannot be counted immediately, they may be stored at 5 ± 3 °C for no longer than 48 hours.

D9.7.2 *Presence absence tests*

After incubation, examine the plates for clear zones where the enrichment cultures were spotted. These are evidence of the presence of *B. fragilis* bacteriophage in the original sample.

D9.7.3 *Confirmation*

Occasionally, artefacts may resemble plaques, for example, bubbles in the medium or the agar overlay partially setting. Where there is doubt about the presence of a plaque, this can be confirmed by picking the plaque with a straight wire into an ssBPRMA containing the host, plating and incubating as above. Confluent lysis should be evident after incubation.

D10 Calculations

D10.1 *Plaque count assay*

From the number of plaques counted calculate the number of plaque-forming units (pfu) of bacteriophage in 1 ml of the sample as follows:

$$X = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2)}$$

where

X is the number of plaque forming units per ml;

N is the total number of plaques counted on the plates;

n_1, n_2 are the number of replicates counted for dilutions F_1, F_2 respectively;

V_1, V_2 are the test volumes used with F_1, F_2 respectively;

F_1, F_2 are the dilution or concentration factors used for the test portions V_1, V_2 respectively.

If only one dilution is counted, the formula may be simplified to

$$X = \frac{N}{nVF}$$

D10.2 *Presence absence test*

Express the results as bacteriophage detected, or not detected, in the volume of sample tested. Where an MPN system is used, the results can be calculated from probability tables published elsewhere in this series[5].

D11 Expression of results

D.11.1*Plaque count assay*

Express the results as plaque forming units (pfu) per ml of sample examined. If no plaques are observed, express the results as not detected in the volume of sample examined.

D.11.2*Presence absence procedure*

Express the results as bacteriophage (not) detected in the volume of sample tested.

D12 Quality assurance

D12.1 *Plaque count assay*

For each series of samples, examine a blank control using sterile diluent as the sample to ensure that the inoculum culture is not infected with bacteriophage. In addition, examine a reference standard preparation of bacteriophage B56-3. This should be prepared as follows:

Incubate a culture of *Bacteroides fragilis* until there are approximately 2 x 10^8 cells/ml. Add B56-3 from a stock suspension at approximately 10^8 cells/ml i.e. a ratio of one bacterial cell to one phage particle. Incubate overnight at 36 ± 2 °C and filter through a 0.2 µm filter to remove the bacterial cells. The filtrate should be stored at 5 ± 3 °C. Dilute the suspension down to 100 pfu/ml in 100 ml of suspension. Add 5 % (volume) of glycerol, dispense in 2.4 ml aliquots and store at - 70 °C. Thaw the vials out and plate according to the procedure described in D9.5. An environmental sample, for example wastewater, can be used as an additional control. These should be filtered and stored as described above.

D12.2 *Presence absence procedure*

With each series of samples examine a blank of sterile diluent and a reference standard as described in D12.1. This should have a phage concentration of 5 – 10 pfu/ml. The examination of an enrichment containing the real sample might also be considered to demonstrate whether there are any interfering effects from the sample.

D13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2016) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency. (currently in preparation).

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency. (currently undergoing revision).

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. ISO 10705-4:2001 Water quality - Detection and enumeration of bacteriophages - Part 4 – Enumeration of bacteriophages infecting *Bacteroides fragilis*. Geneva: International Organization for Standardization.

5. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2016) - Part 3 - Methods for the isolation and enumeration of *Escherichia coli* (including *E. coli* O157). *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

Address for correspondence

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Secretary
Standing Committee of Analysts
Environment Agency (National Laboratory Service)
NLS Nottingham
Meadow Lane
Nottingham
NG2 3HN
(http://www.gov.uk/environment-agency)

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