Standing Committee of Analysts

The Microbiology of Recreational and Environmental Waters (2016) – Part 13 – Methods for the isolation and enumeration of microbial tracers

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 four methods for the isolation and enumeration of microbial tracers.

A The enumeration of *Bacillus atrophaeus (B. globigii*) spores by a membrane filtration method.

B The enumeration of *Enterobacter cloacae* bacteriophage by a semi-solid agar overlay method.

C The enumeration of *Escherichia coli* bacteriophage MS2 by a semi-solid agar overlay method.

D The enumeration of *Serratia marcescens* bacteriophage by a semi-solid agar overlay method.

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 (https://www.gov.uk/government/publications/standing-committee-of-analysts-sca-blue-books) 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.

Mark Gale
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 enumeration of *Bacillus atrophaeus* (*B. globigii*) spores by a membrane filtration technique

A1 Introduction

Microbial tracers have been used to study the movement of water, retention times and dispersion characteristics in rivers and marine environments, and can provide information on the investigation of sources of pollution. Ideal microbial tracers are absent from environmental samples, can be produced in high numbers and have good survival characteristics. In addition, there is a simple and inexpensive method for their assay. A number of tracers have been developed for environmental use and include spores of *Bacillus atrophaeus* (*B. globigii* or *B. subtilis* var. *niger*)[(1)].

Bacillus atrophaeus spores are relatively resistant in the environment and can be useful in the marine environment where tidal cycles may be studied. Large numbers of spores can be added to bodies of water to compensate for spores being adsorbed to particulate material or being inactivated, for example, by strong sunlight. Details of the uses of this tracer are given elsewhere in this series[(2)].

A2 Scope

This method is suitable for the detection of *B. atrophaeus* from all types of water including river waters, estuarine and seawaters, untreated and treated wastewater, and recreational waters. The volume of sample, or diluted sample, to be analysed will depend upon the concentration of tracer spores in the sample. Generally, 100 ml of sample is used for *B. atrophaeus* determinations but where the concentration of tracers is known to be high, dilution may be necessary.

A3 Definition

The *Bacillus* genus contains a wide range of aerobic, spore-bearing, Gram-positive bacteria. *Bacillus atrophaeus* spores are heat-resistant and produce characteristic orange colonies on culture.

A4 Principle

Bacillus atrophaeus can be isolated from samples by membrane filtration and cultured on either tryptone glucose mannitol broth or tryptone glucose mannitol agar. Colonies are easily recognised by the production of a bright orange pigment and can be easily counted after incubation at 30 °C for 48 hours. Samples are pasteurised before filtration to reduce background contamination and enhance spore germination.

A5 Limitations

The method is suitable for most types of aqueous samples except those with high turbidities which tend to block the membrane filter. This will limit the volume of sample that can be filtered. Accumulated deposit on the membrane filter may mask or inhibit the growth of *B. atrophaeus* colonies. Where high numbers of organisms may be expected serial ten-fold dilutions should be made to obtain a countable number of colonies. The maximum number of colonies that should be counted from a single membrane filter is approximately 100. Counts can be obtained from membranes containing more than 100 colonies, providing that isolated colonies are present and

that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as estimated counts.

A6 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[4] in this series.

Some members of the *Bacillus* genus are known to be pathogenic. *Bacillus cereus*, *B. subtilis* and *B. licheniformis* can cause gastro-enteritis. They may also cause wound infections and septicaemia. *B. atrophaeus* may germinate in certain foods and may cause food poisoning. The spores can pass through water treatment and are resistant to chlorination. The use of *B. atrophaeus* as a tracer in rivers where water is abstracted for drinking or ground water tracing, or marine waters where shellfish may be contaminated is not recommended. It is, however, unlikely to germinate in the environment, and for marine tracing is unlikely to represent a risk to public health.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [4] in this series. Principally appropriate membrane filtration apparatus and incubators (fan assisted, static temperature) are required. Other items include:

A7.1 Sterile sample bottles of appropriate volume (for example 120 ml), made of suitable material, should be used.

A7.2 Heated water bath capable of maintaining a temperature of 63 ± 3 °C.

A7.3 Incubator capable of maintaining a temperature of 30 ± 1.0 °C.

A7.4 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

A7.5 Sterile, membrane filters, white, gridded, 47 mm diameter, cellulose-based 0.45 µm nominal pore size.

A7.6 Incubation pads, 47 mm diameter, sterile.

A7.7 Smooth-tipped forceps.

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[4]. 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 *Tryptone glucose mannitol broth*[1]

Tryptone	20.0 g
Sodium chloride	5.0 g
Glucose mannitol solution	100 ml
Water	900 ml

Suspend the ingredients (except the glucose mannitol solution) in the water, dissolve by stirring and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution in volumes of 90 ml, or multiples of 90 ml, into suitable containers and sterilize by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the partial medium should be checked to confirm a pH of 6.8 ± 0.2.

Prepare a solution of glucose and mannitol, each at a concentration of 10 % m/v, and filter-sterilize using a 0.2 µm pore size membrane filter. Aseptically dispense the solution into sterile containers in 10 ml volumes, store in the range of 5 ± 3 °C and use within one month.

To prepare the complete medium, aseptically add 10 ml of glucose mannitol solution to 90 ml of partial medium. The complete medium should be used on the day of preparation.

The medium may be used in an agar form, tryptone glucose mannitol agar, by dissolving bacteriological agar (usually 10 - 15 g) before autoclaving. The medium should be cooled to approximately 50 °C before adding the filter sterilised glucose mannitol solution, mixing well and pouring into Petri dishes. Once the agar has set Petri dishes containing the agar medium can be stored at 5 ± 3 °C for up to one month, protected against dehydration.

A8.2 *Other media*

Standard and commercial formulations of other media and reagents used in this method may include quarter-strength Ringer's solution or maximum recovery diluent.

A9 Analytical procedure

A9.1 *Sample preparation*

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 7 days of sampling. This acceptable storage period should be verified in the user's laboratory.

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, ideally be between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on at least one of the membrane filters is likely to fall within this range. For surface and sea waters filter 100 ml of the sample. Where counts are likely to be above 100 on a membrane, filter smaller volumes, or prepare an appropriate dilution series of the sample with quarter-strength Ringer's solution or maximum recovery diluent before filtration.

A9.2 *Sample processing*

Heat the undiluted sample to 63 ± 3 °C and maintain at this temperature for 30 minutes. After pasteurisation, cool the sample rapidly to room temperature in cold water.

Dispense the tryptone glucose mannitol broth onto sterile 47 mm incubation pads contained in sterile Petri dishes. Allow the pads to soak for a minimum of 15 minutes and drain off the excess medium. Drained pads should be used within 1 hour. Alternatively prepare tryptone glucose mannitol agar plates in sterile 50 – 60 mm Petri dishes.

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. Any remaining sample should be stored at temperatures between 5 ± 3 °C until the analysis is completed. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the spores over the entire surface of the membrane filter during the filtration process. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered. Remove the funnel and transfer the membrane filter carefully to a Petri dish containing a pad soaked in tryptone glucose mannitol broth or to tryptone glucose mannitol agar. Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

Pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume, or highest dilution of sample, is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, after filtration of each sample disinfect the funnel by immersing it in boiling water for at least five minutes. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

Where broth medium is used the dishes should be placed in a sealed container to prevent drying out of the medium. The Petri dishes are inverted and incubated at 30 °C for 44 ± 4 hours.

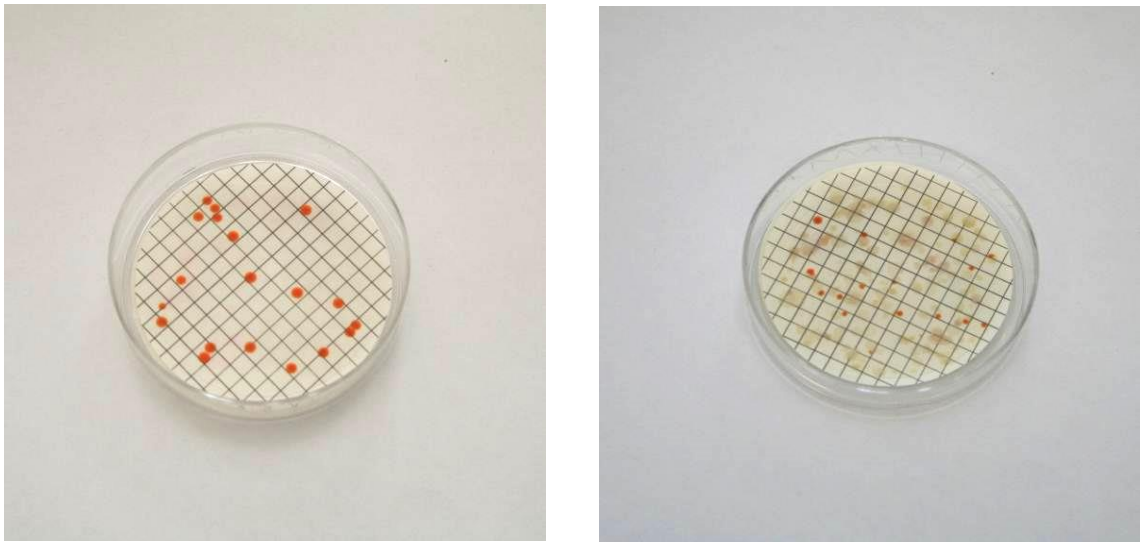
The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

A9.3 *Reading of results*

After incubation, count colonies that are 0.5 - 2 mm in diameter, rough in appearance

and orange in colour. Large numbers of colonies on membrane filters may restrict the size of individual colonies and a hand lens may be required. The membranes may be examined after 24 hours and, where necessary, suitable dilutions can be prepared (for re-testing) of any sample where the corresponding filter shows a large number of colonies that would make subsequent counting difficult to undertake.

Figure A1 Colonies of *B. atrophaeus* growing on tryptone glucose mannitol broth



A9.4 *Confirmation tests*

Confirmation of colonies is not necessary due to the characteristic colony colour and morphology provided there are no other background characteristic colonies.

A10 Calculations

The number of *Bacillus atrophaeus* colonies is generally quoted as the number of colonies per 100 ml. Calculate the count as follows:

$$\text{Count/100 ml} = \frac{\text{Number of colonies counted x DF x 100}}{\text{Volume of sample filtered (ml)}}$$

Where DF is dilution factor if appropriate.

A11 Expression of results

The number of *Bacillus atrophaeus* colonies is expressed in colony forming units per volume of sample. For most samples the volume is typically 100 ml.

A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *B. atrophaeus* NCTC 10073). Petri dishes should be incubated for 44 ± 4 hours at 30 °C. In addition the same strain should be used for internal quality control with each batch of samples analysed. Petri dishes should be incubated for 44 ± 4 hours at 30 °C. Further details are given elsewhere[4] in

this series. Care should be taken when handling high concentrations of spores 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.

A13 References

1. The use of *Serratia indica* and *Bacillus subtilis* var. *niger* spores, *Journal of Applied Bacteriology*, E B Pike, A W J Bufton and D J Gould, 1969, **32**, 206-216

2. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

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

4. 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.

B The enumeration of *Enterobacter cloacae* bacteriophage by a semi-solid overlay method.

B1 Introduction

Microbial tracers have been used to study the movement of water, retention times and dispersion characteristics in rivers and marine environments, and can provide information on the investigation of sources of pollution. A number of tracers have been developed for environmental use and include bacteriophages specific for *Enterobacter cloacae*. Bacteriophages can be grown to high titres and, in addition, there is a simple and inexpensive method for their assay.

It is important to ensure background levels in the testing environment are absent or very low, as they can occur naturally. Therefore, all sources of water to be tested should be monitored for the presence of the bacteriophages before a tracing exercise commences. Details of the uses of this tracer are given elsewhere in this series[(1)].

B2 Scope

This method is suitable for the detection of *Ent. cloacae* bacteriophage in all types of water including river waters, estuarine and sea waters, untreated and treated wastewater. The volume of sample, or diluted sample, depends upon the concentration of tracer organism in the sample but generally 0.1 - 1 ml of sample is analysed for bacteriophages. Where the concentration of tracers is known to be high, dilution will be necessary in order to obtain a countable number of bacteriophage plaques.

B3 Definition

Enterobacter cloacae bacteriophages are defined as those viruses which will infect and replicate in the host *Ent. cloacae* bacterium. Subsequently, they produce plaques in semi-solid overlay containing the host bacterium.

B4 Principle

The sample and the host bacterium are mixed together in a semi-solid overlay which is poured onto a plate of blood agar base. Upon infection of the host the bacteriophage replicates within the cell and replicated bacteriophage are expelled via cell lysis. These released bacteriophage particles infect surrounding host cells and the cycle is repeated. The area of cell lysis results in plaque formation within the semi-solid agar overlay. Bacteriophages produce characteristic plaques in the overlay that are easily counted after overnight incubation. The number of plaques produced relates directly to the number of bacteriophage particles in the original sample.

B5 Limitations

The method is suitable for most types of aqueous samples. It is not suitable for samples where *Ent. cloacae* bacteriophage are likely to be present in moderate numbers as a background count.

Where high numbers of bacteriophage may be expected following tracer release serial ten-fold dilutions should be made to obtain a countable number of plaques. The

maximum number of plaques that should be counted from a single agar plate is approximately 100. Counts can be obtained from plates containing more than 100 plaques providing that isolated plaques are present and that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as estimated counts.

B6 Health and safety

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

Bacteriophages are removed by water treatment and inactivated by disinfection with chlorine. As they are host-specific, they are unlikely to infect plants, animals or humans and are not considered a risk to public health. The host bacterium should not be a recognised pathogenic organism, for example *Salmonella* species, and every effort should be made to ensure that suspensions for use in the environment are free of residual host cells.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Principally appropriate incubators (fan assisted, static temperature) are required. Other items include:

B7.1 Sterile sample containers of appropriate volume, made of suitable material.

B7.2 Incubator capable of maintaining a temperature of 37.0 ± 1.0 °C.

B7.3 Water bath or incubator capable of maintaining a temperature of approximately 45 - 50 °C.

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[3]. 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 *Growth medium for bacteriophage hosts*

Brain heart infusion	20.0 g
Casamino acids	20.0 g
Yeast extract	1.0 g
Potassium dihydrogen phosphate	5.0 g
Magnesium sulphate	1.0 g
Glycerol	20 ml
Water	1 litre

Suspend the ingredients in the water, dissolve by stirring and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in suitable volumes (10 ml) into suitable screw-capped containers and sterilize by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The prepared medium can be kept in the dark at room temperature and used within one month.

B8.2 *Agar base for agar overlay assay (blood agar base)*

Beef extract powder	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring the mixture. The pH of the medium should be 7.4 ± 0.2. Dispense the resulting solution in suitable volumes in screw-capped containers and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Cool the molten medium to approximately 50 °C and dispense the medium into sterile Petri dishes. Once the agar has set the Petri dishes should be stored at a temperature in the range 5 ± 3 °C and used within one month. Petri dishes should be brought to room temperature before use but need not be dried.

Alternatively, after autoclaving, the medium can be stored in screw capped containers in the dark at room temperature and should be used within one month. For use, melt the agar by boiling and cool as above before dispensing to Petri dishes.

B.8.3 *Semi-solid agar overlay*

Nutrient broth number 2	11.2 g
Sodium chloride	7.0 g
Agar number 1	8.0 g
Distilled, deionised or similar grade water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring the mixture. The pH of the medium should be 7.0 ± 0.2. Dispense the resulting solution in volumes of 4 ml in screw-capped containers, for example universal containers, and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be 7.0 ± 0.2. The medium should be stored in the dark at room temperature with the caps securely tightened and used within one month. Any bottles showing signs of desiccation should be discarded. For use, melt the overlay agar by boiling and maintain at a temperature of approximately 45 - 50 °C.

Note: The concentration of agar given will produce a suitable gel strength and plaque size with 0.1 - 1.0 ml of sample.

B8.4 *Other media*

Standard and commercial formulations of other reagents used in this method include quarter strength Ringer's solution and maximum recovery diluent.

B9 Analytical procedure

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.

B9.1 *Sample processing*

Inoculate the host strain (for example, *Enterobacter cloacae* ATCC 23355/NCTC 13380) into approximately 10 ml of growth medium in a sterile universal container and incubate overnight at 37 ± 1 °C. Add 0.1 ml of host culture to the molten overlay agar medium cooled to 45 - 50 °C. Add 0.1 - 1.0 ml of the sample, mix carefully avoiding formation of air bubbles and pour the resulting suspension onto the agar base, ensuring the agar base is covered with overlay by gently tilting the Petri dish. Leave the dishes on the bench for 15 minutes for the overlay to set. Once the agar overlay has set, invert the Petri dish and incubate at 37 ± 1 °C for 12 - 24 hours. To minimise temperature variation the Petri dishes should not be stacked more than three high. Plaques are often visible within 4 - 6 hours of starting incubation. Plates can therefore be checked after this time to determine whether dilutions of samples are required to give a countable number of plaques. Once samples have been tested, they should be stored at 5 ± 3 °C for dilution and re-testing if necessary.

If a large number of samples are to be examined, several overnight broth cultures of the host bacterium should be prepared. Alternatively, host broth cultures can be inoculated at the start of the working day for use later the same day providing there is sufficient growth. This allows one culture to be discarded after the examination of a given number of samples, for example 30. This procedure minimises the risk of the host becoming contaminated with bacteriophage and all the test samples showing confluent lysis. Similarly, it is also good practice to include a negative control after the analysis of a given number of samples, for example 20, to confirm that the host has not become contaminated. A positive control consisting of host and a suitably diluted suspension of *Ent. cloacae* bacteriophage should be incubated with each batch of samples. Broth cultures should not be more than 24 hours old.

B9.2 *Reading of results*

After incubation, record the number of plaques (areas of clearing, see Figure B1) for each sample. Typical plaques are small (1 mm in diameter) and can be seen within 6 hours of starting incubation.

B9.3 *Confirmation tests*

Plaques may be confirmed by picking into fresh overlay agar medium with host strain and replating when, after incubation, confluent lysis should be observed (see Figure B2).

Figure B1 Plaques of *Enterobacter cloacae* in an agar overlay assay

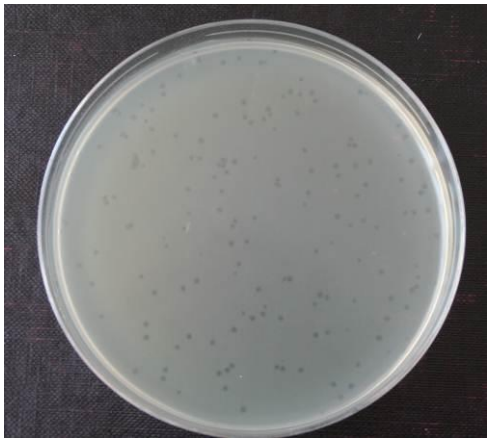
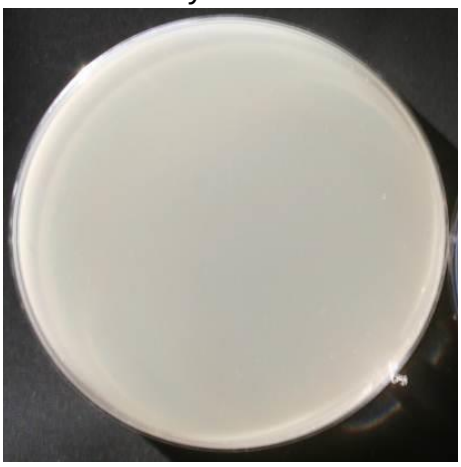
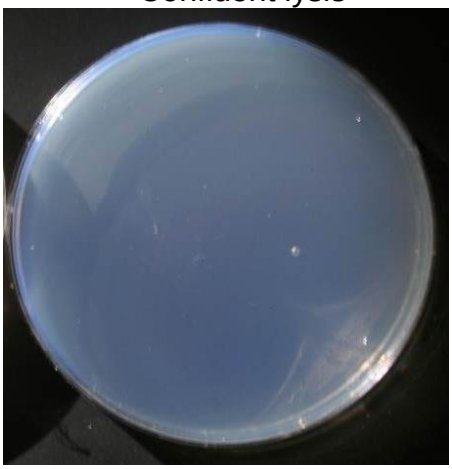


Figure B2 Plates showing no lysis and confluent lysis

No lysis



Confluent lysis



B10 Calculations

The number of bacteriophage is generally expressed as the number of plaque forming units per 1 ml of sample. Calculate the count as follows:

$$\text{Count/1 ml} = \frac{\text{Number of plaques counted on an agar plate x DF}}{\text{Volume of sample analysed (ml)}}$$

where DF is the dilution factor, if appropriate.

B11 Expression of results

Counts are expressed as 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 an appropriate reference strain of host bacterium (for example, *Enterobacter cloacae* ATCC 23355/NCTC 13380) and with the target bacteriophage (for example, ATCC 23355 B-1). Petri

dishes should be incubated for 12 to 24 hours at 37 °C. In addition the same strain should be used for internal quality control with each batch of samples analysed. Petri dishes should be incubated for 12 to 24 hours at 37 °C. Further details are given elsewhere[3] in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.

3. 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.

C The enumeration of *Escherichia coli* bacteriophage MS2 by a semi-solid overlay method.

C1 Introduction

Microbial tracers have been used to study the movement of water, retention times and dispersion characteristics in rivers and marine environments, and can provide information on the investigation of sources of pollution. A number of tracers have been developed for environmental use and include bacteriophages specific for *Escherichia coli*. Bacteriophages can be grown to high titres and, in addition, there is a simple and inexpensive method for their assay.

It is important to ensure background levels in the testing environment are absent or very low. Therefore, all sources of water to be tested should be monitored for the presence of the bacteriophage before a tracing exercise commences. Details of the uses of this tracer are given elsewhere in this series[(1)].

C2 Scope

This method is suitable for the detection of *E. coli* MS2 bacteriophage in all types of water including river waters, estuarine and sea waters, untreated and treated wastewater. The volume of sample, or diluted sample, depends upon the concentration of tracer organism in the sample but generally 0.1 - 1 ml of sample is analysed for bacteriophages. Where the concentration of tracers is known to be high, dilution will be necessary in order to obtain a countable number of bacteriophage plaques.

C3 Definition

Escherichia coli MS2 bacteriophages are defined as those viruses which will infect and replicate in the host *E. coli* strain, usually K12. They attach and infect through the F-specific pilus of *E. coli*. Subsequently, they produce plaques in semi-solid overlay containing the host bacterium.

C4 Principle

The sample and the host bacterium are mixed together in a semi-solid overlay which is poured onto a plate of blood agar base. Upon infection of the host the bacteriophage replicates within the cell and replicated bacteriophage are expelled via cell lysis. These released bacteriophage particles infect surrounding host cells and the cycle is repeated. The area of cell lysis results in plaque formation within the semi-solid agar overlay. Bacteriophages produce characteristic plaques in the overlay that are easily counted after overnight incubation. The number of plaques produced relates directly to the number of bacteriophage particles in the original sample.

C5 Limitations

The method is suitable for most types of aqueous samples. It is not suitable for samples where *E. coli* bacteriophage are likely to be present in moderate numbers as a background count.

Where high numbers of bacteriophages may be expected following tracer release serial ten-fold dilutions should be made to obtain a countable number of colonies. The maximum number of plaques that should be counted from a single agar plate is approximately 100. Counts can be obtained from plates containing more than 100 plaques providing that isolated plaques are present and that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as estimated counts.

C6 Health and safety

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

Bacteriophages are removed by water treatment and inactivated by disinfection with chlorine. As they are host-specific, they are unlikely to infect plants, animals or humans and are not considered a risk to public health. The host bacterium should not be a recognised pathogenic organism, for example *Salmonella* species and every effort should be made to ensure that suspensions for use in the environment are free of residual host cells.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Principally appropriate incubators (fan assisted, static temperature) are required. Other items include:

C7.1 Sterile sample containers of appropriate volume, made of suitable material.

C7.2 Incubator capable of maintaining a temperature of 37.0 ± 1.0 °C.

C7.3 Water bath or incubator capable of maintaining a temperature of approximately 45 - 50 °C.

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[3]. 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.

C8.1 *Growth medium for bacteriophage hosts*

Brain heart infusion	20.0 g
Casamino acids	20.0 g
Yeast extract	1.0 g
Potassium dihydrogen phosphate	5.0 g
Magnesium sulphate	1.0 g

Glycerol	20 ml
Water	1 litre

Suspend the ingredients in the water, dissolve by stirring and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in suitable volumes (10 ml) into suitable screw-capped containers and sterilize by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The prepared medium can be kept in the dark at room temperature and used within one month.

C8.2 *Agar base for agar overlay assay (blood agar base)*

Beef extract powder	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring the mixture. The pH of the medium should be 7.4 ± 0.2. Dispense the resulting solution in suitable volumes in screw-capped containers and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Cool the molten medium to approximately 50 °C and dispense the medium into sterile Petri dishes. Once the agar has set the Petri dishes should be stored at a temperature in the range 5 ± 3 °C and used within one month. Petri dishes should be brought to room temperature before use but need not be dried.

Alternatively, after autoclaving, the medium can be stored in screw capped containers in the dark at room temperature and should be used within one month. For use, melt the agar by boiling and cool as above before dispensing to Petri dishes.

C8.3 *Semi-solid agar overlay*

Nutrient broth number 2	11.2 g
Sodium chloride	7.0 g
Agar number 1	8.0 g
Water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring the mixture. The pH of the medium should be 7.0 ± 0.2. Dispense the resulting solution in volumes of 4 ml in screw-capped containers, for example universal containers, and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be 7.0 ± 0.2. The medium should be stored in the dark at room temperature with the caps securely tightened and used within one month. Any bottles showing signs of desiccation should be discarded. For use, melt the overlay agar by boiling and maintain at a temperature of approximately 45 - 50 °C.

Note: The concentration of agar given will produce a suitable gel strength and plaque size with 0.1 - 1.0 ml of sample.

C8.4 *Other media*

Standard and commercial formulations of other reagents used in this method include quarter strength Ringer's solution and maximum recovery diluent.

C9 Analytical procedure

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.

C9.1 *Sample processing*

Inoculate the host strain into approximately 10 ml of growth medium in a sterile universal container and incubate overnight at 37 ± 1 °C. Add 0.1 ml of host culture to the molten overlay agar medium cooled to 45 - 50 °C. Add 0.1 - 1.0 ml of the sample, mix carefully avoiding formation of air bubbles and pour the resulting suspension onto the agar base, ensuring the agar base is covered with overlay by gently tilting the Petri dish. Leave the dishes on the bench for 15 minutes for the overlay to set. Once the agar overlay has set, invert the Petri dish and incubate at 37 ± 1 °C for 12 - 24 hours. To minimise temperature variation the Petri dishes should not be stacked more than three high. Plaques are often visible within 4 - 6 hours of starting incubation. Plates can therefore be checked after this time to determine whether dilutions of samples are required to give a countable number of plaques. Once samples have been tested, they should be stored at 5 ± 3 °C for dilution and re-testing if necessary.

If a large number of samples are to be examined, several overnight broth cultures of the host bacterium should be prepared. Alternatively, host broth cultures can be inoculated at the start of the working day for use later the same day providing there is sufficient growth. This allows one culture to be discarded after the examination of a given number of samples, for example 30. This procedure minimises the risk of the host becoming contaminated with bacteriophage and all the test samples showing confluent lysis. Similarly, it is also good practice to include a negative control after the analysis of a given number of samples, for example 20, to confirm that the host has not become contaminated. A positive control consisting of host and a suitably diluted suspension of MS2 bacteriophage should be incubated with each batch of samples. Broth cultures should not be more than 24 hours old.

C9.2 *Reading of results*

After incubation, record the number of plaques (areas of clearing, see Figure C1) for each sample. Typical plaques are small (1-2 mm in diameter) often with an opaque halo around the perimeter. They can be seen within 4 hours of starting incubation.

C9.3 *Confirmation tests*

Plaques may be confirmed by picking into fresh overlay agar medium with host strain and replating when, after incubation, confluent lysis should be observed (see Figure C2).

C10 Calculations

The number of bacteriophage is generally expressed as the number of plaque forming units per 1 ml of sample. Calculate the count as follows:

$$\text{Count/1 ml} = \frac{\text{Number of plaques counted on an agar plate x DF}}{\text{Volume of sample analysed (ml)}}$$

where DF is the dilution factor, if appropriate.

Figure C1 Plaques of MS2 in an agar overlay assay

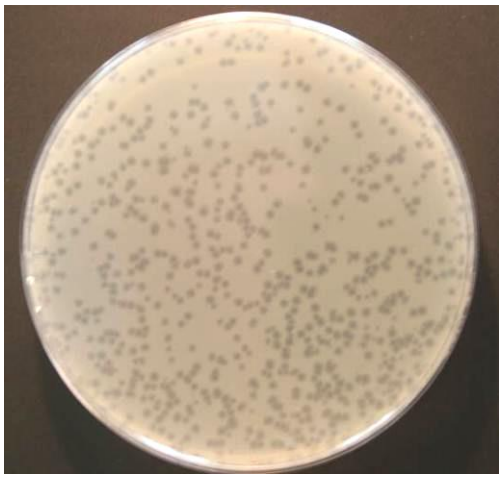
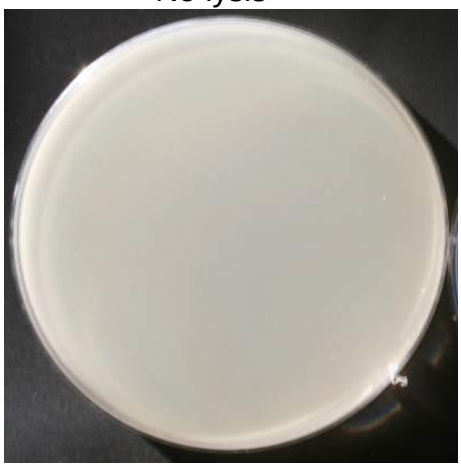
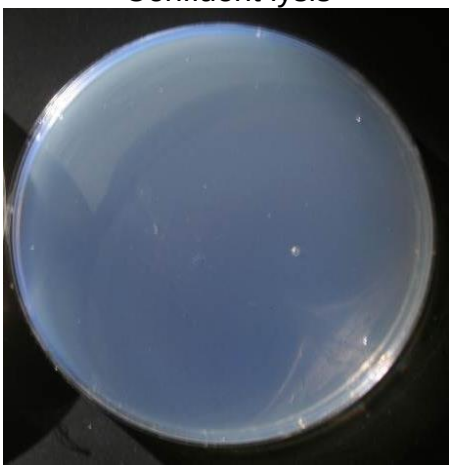


Figure C2 Plates showing no lysis and confluent lysis

No lysis



Confluent lysis



C11 Expression of results

Counts are expressed as plaque forming units (pfu) per volume of sample. For most samples, the volume is typically 1 ml.

C12 Quality assurance

New batches of media and reagents should be tested with an appropriate reference strain of host bacterium (for example *E. coli* NCTC 12486) and with the target bacteriophage (for example MS2 NCTC 12847). Petri dishes should be incubated for

12 to 24 hours at 37 °C. In addition the same strain should be used for internal quality control with each batch of samples analysed. Petri dishes should be incubated for 12 to 24 hours at 37 °C. Further details are given elsewhere[3] in this series.

C13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.

3. 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.

D The enumeration of *Serratia marcescens* bacteriophage by a semi-solid overlay method.

D1 Introduction

Microbial tracers have been used to study the movement of water, retention times and dispersion characteristics in rivers and marine environments, and can provide information on the investigation of sources of pollution. A number of tracers have been developed for environmental use and include bacteriophages specific for *Serratia marcescens*. Bacteriophages can be grown to high titres and, in addition, there is a simple and inexpensive method for their assay.

It is important to ensure background levels in the testing environment are absent or very low. Therefore, all sources of water to be tested should be monitored for the presence of the bacteriophage before a tracing exercise commences. Details of the uses of this tracer are given elsewhere in this series[(1)].

D2 Scope

This method is suitable for the detection of *Serratia marcescens* bacteriophage in all types of water including river waters, estuarine and sea waters, untreated and treated wastewater. The volume of sample, or diluted sample, depends upon the concentration of tracer organism in the sample but generally 0.1 - 1 ml of sample is analysed for bacteriophages. Where the concentration of tracers is known to be high, dilution will be necessary in order to obtain a countable number of bacteriophage plaques.

D3 Definition

Serratia marcescens bacteriophages are defined as those viruses which will infect and replicate in the host *Serr. marcescens* bacterium. Subsequently, they produce plaques in semi-solid overlay containing the host bacterium.

D4 Principle

The sample and the host bacterium are mixed together in a semi-solid overlay which is poured onto a plate of blood agar base. Upon infection of the host the bacteriophage replicates within the cell and replicated bacteriophage are expelled via cell lysis. These released bacteriophage particles infect surrounding host cells and the cycle is repeated. The area of cell lysis results in plaque formation within the semi-solid agar overlay. Bacteriophages produce characteristic plaques in the overlay that are easily counted after overnight incubation. The number of plaques produced relates directly to the number of bacteriophage particles in the original sample.

D5 Limitations

The method is suitable for most types of aqueous samples. It is not suitable for samples where *Serr. marcescens* bacteriophage are likely to be present in moderate numbers as a background count.

Where high numbers of bacteriophages may be expected following tracer release serial ten-fold dilutions should be made to obtain a countable number of colonies. The

maximum number of plaques that should be counted from a single agar plate is approximately 100. Counts can be obtained from plates containing more than 100 plaques providing that isolated plaques are present and that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as estimated counts.

D6 Health and safety

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

Bacteriophages are removed by water treatment and inactivated by disinfection with chlorine. As they are host-specific, they are unlikely to infect plants, animals or humans and are not considered a risk to public health. The host bacterium should not be a recognised pathogenic organism, for example *Salmonella* species and every effort should be made to ensure that suspensions for use in the environment are free of residual host cells.

D7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Principally appropriate incubators (fan assisted, static temperature) are required. Other items include:

D7.1 Sterile sample containers of appropriate volume, made of suitable material.

D7.2 Incubator capable of maintaining a temperature of 37.0 ± 1.0 °C.

D7.3 Water bath or incubator capable of maintaining a temperature of approximately 45 - 50 °C.

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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[3]. 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.

D8.1 *Growth medium for bacteriophage hosts*

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Casamino acids	20.0 g
Yeast extract	1.0 g
Potassium dihydrogen phosphate	5.0 g
Magnesium sulphate	1.0 g
Glycerol	20 ml
Water	1 litre

Suspend the ingredients in the water, dissolve by stirring and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in suitable volumes (10 ml) into suitable screw-capped containers and sterilize by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The prepared medium can be kept in the dark at room temperature and used within one month.

D8.2 *Agar base for agar overlay assay (blood agar base)*

Beef extract powder	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring the mixture. The pH of the medium should be 7.4 ± 0.2. Dispense the resulting solution in suitable volumes in screw-capped containers and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.3 ± 0.2. Cool the molten medium to approximately 50 °C and dispense the medium into sterile Petri dishes. Once the agar has set the Petri dishes should be stored at a temperature in the range 5 ± 3 °C and used within one month. Petri dishes should be brought to room temperature before use but need not be dried.

Alternatively, after autoclaving, the medium can be stored in screw capped containers in the dark at room temperature and should be used within one month. For use, melt the agar by boiling and cool as above before dispensing to Petri dishes.

D8.3 *Semi-solid agar overlay*

Nutrient broth number 2	11.2 g
Sodium chloride	7.0 g
Agar number 1	8.0 g
Water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring the mixture. The pH of the medium should be 7.0 ± 0.2. Dispense the resulting solution in volumes of 4 ml in screw-capped containers, for example universal containers, and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be 7.0 ± 0.2. The medium should be stored in the dark at room temperature with the caps securely tightened and used within one month. Any bottles showing signs of desiccation should be discarded. For use, melt the overlay agar by boiling and maintain at a temperature of approximately 45 - 50 °C.

Note: The concentration of agar given will produce a suitable gel strength and plaque size with 0.1 - 1.0 ml of sample.

D8.4 *Other media*

Standard and commercial formulations of other reagents used in this method include quarter strength Ringer's solution and maximum recovery diluent.

D9 Analytical procedure

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D9.2 *Reading of results*

After incubation, record the number of plaques (areas of clearing, see Figure D1) for each sample. Typical plaques are large (2-4 mm in diameter) and can be seen within three hours of starting incubation.

D9.3 *Confirmation tests*

Plaques may be confirmed by picking into fresh overlay agar medium with host strain and replating when, after incubation, confluent lysis should be observed (see Figure D2).

D10 Calculations

The number of bacteriophage is generally expressed as the number of plaque forming units per 1 ml of sample. Calculate the count as follows:

$$\text{Count/1 ml} = \frac{\text{Number of plaques counted on an agar plate x DF}}{\text{Volume of sample analysed (ml)}}$$

where DF is the dilution factor , if appropriate.

Figure D1 Plaques of *Serratia marcescens* bacteriophage in an agar overlay assay

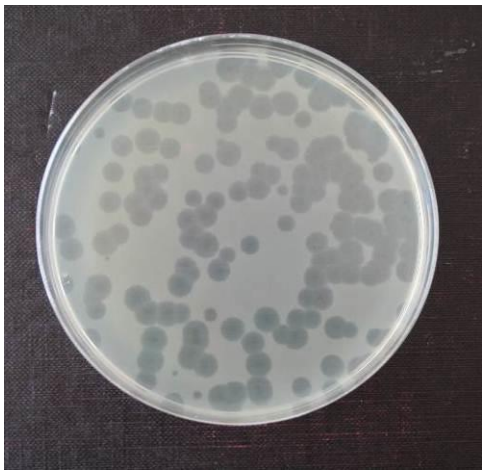
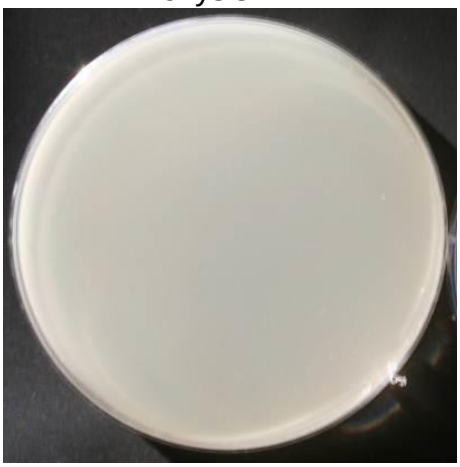
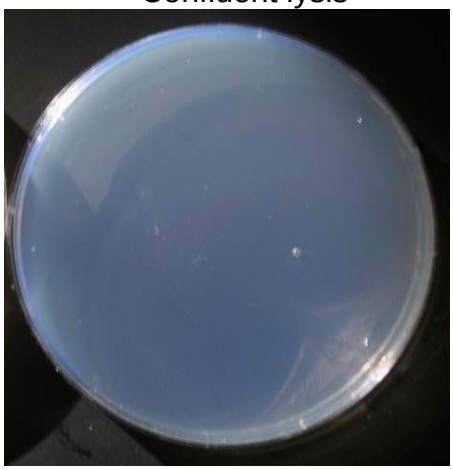


Figure D2 Plates showing no lysis and confluent lysis

No lysis



Confluent lysis



D11 Expression of results

Counts are expressed as plaque forming units (pfu) per volume of sample. For most samples, the volume is typically 1 ml.

D12 Quality assurance

New batches of media and reagents should be tested with an appropriate reference strain of host bacterium (for example *Serr. marcescens* NCIMB 10644) and with the target bacteriophage (for example *Serr. marcescens* bacteriophage NCIMB 10645).

Petri dishes should be incubated for 12 to 24 hours at 37 °C. In addition the same strain should be used for internal quality control with each batch of samples analysed. Petri dishes should be incubated for 12 to 24 hours at 37 °C. Further details are given elsewhere[3] in this series.

D13 References

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Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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Standing Committee of Analysts

Members assisting with this method

Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

Peter Boyd	Formerly Public Health England
Simon Cole	Wessex Water
Carol Francis	CREH *Analytical* Limited
David Gaskell	United Utilities
Malcolm Morgan	SCA Strategic Board
David Sartory	SWM Consulting
Helen Shapland	Wessex Water
Martin Walters	Environment Agency
John Watkins	CREH *Analytical* Limited
John Watson	South West Water
David Westwood	Formerly Environment Agency

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