





**The Microbiology of Drinking Water (2002) - Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)**

**Methods for the Examination of Waters and Associated Materials**

This booklet contains six methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157).

- A The enumeration of coliform bacteria and *Escherichia coli* by a two membrane filtration technique.
- B The enumeration of coliform bacteria and *Escherichia coli* by a single membrane filtration technique.
- C The enumeration of coliform bacteria and *Escherichia coli* by a multiple tube most probable number technique.
- D The enumeration of coliform bacteria and *Escherichia coli* by a defined substrate most probable number technique.
- E The detection of coliform bacteria and *Escherichia coli* by a presence-absence technique.
- F The detection of *Escherichia coli* O157:H7 by selective enrichment and immuno-magnetic separation.

Within this series there are separate booklets dealing with different topics concerning the microbiology of drinking water. Other booklets include

- Part 1 - Water quality and public health
- Part 2 - Practices and procedures for sampling
- Part 3 - Practices and procedures for laboratories
- Part 5 - A method for the isolation and enumeration of Enterococci by membrane filtration
- Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
- Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques
- Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
- Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques
- Part 10 - Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such by validation of the method.

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## 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 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 Environment Agency. At present, there are nine 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 and physical methods
- 4 Metals and metalloids
- 5 General non-metallic substances
- 6 Organic impurities
- 7 Biological methods
- 8 Biodegradability and inhibition methods
- 9 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 main 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. An index of methods is available from the Secretary.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood  
*Secretary*

January 2002

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### 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 1999 (SI 1999/437). 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 publications are; "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 "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

## **A The enumeration of coliform bacteria and *Escherichia coli* by a two membrane filtration technique**

### **A1 Introduction**

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. They provide the most sensitive means for detecting faecal contamination, for assessing the effectiveness of water treatment and disinfection, and for monitoring water quality in distribution. The significance of *E. coli* and coliform bacteria in water treatment and supply are described elsewhere<sup>(1)</sup> in this series.

### **A2 Scope**

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate turbidity. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method.

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

### **A3 Definitions**

In the context of this method, organisms which are oxidase-negative, produce acid from lactose, and form all shades and sizes of yellow colonies on membrane filters (after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C) are regarded as coliform bacteria.

Coliform bacteria are considered to be members of genera or species within the Family Enterobacteriaceae, capable of growth at 37 °C, that possess  $\beta$ -galactosidase. This definition includes anaerogenic (ie non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia*, *Yersinia*, *Buttiauxella* and *Leclercia*.

Organisms which are oxidase-negative, produce acid from lactose, and indole from tryptophan, and form all shades and sizes of yellow colonies on membrane filters (after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C or 44 °C) are regarded as *E. coli*.

For the purposes of water examination, *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce  $\beta$ -glucuronidase. Strains which possess these characteristics at 37 °C but do not express them at 44 °C may also be *E. coli*. When identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

#### **A4 Principle**

Paired aliquots of sample are filtered, and the membrane filters (with isolated bacteria) placed on absorbent pads saturated with broth, or placed on agar, containing lactose and phenol red as an indicator of acidity. Isolation of presumptive colonies is followed by confirmation tests for the production of acid from lactose, negative oxidase reaction and, where necessary, indole formation.

#### **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 indicator organisms. The method also allows species of non-coliform organisms to grow, high numbers of which may inhibit growth of coliform bacteria. The maximum number of colonies that should be counted from a single membrane filter is approximately 100.

For treated drinking water it may be convenient to incubate a single membrane filter at 37 °C. In this case, an immediate operational response should be made to any presumptive positive result on the assumption that any colonies isolated might be *E. coli*. Operational decisions should, therefore, not be delayed until confirmation tests for coliform bacteria and *E. coli* have been completed. As presumptive *E. coli* cannot be distinguished from other presumptive coliform bacteria on a single membrane, and may be present in much lower numbers than the other coliform bacteria, all presumptive colonies should be subject to confirmation testing to ensure detection of any *E. coli* that may be present.

#### **A6 Health and safety**

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

#### **A7 Apparatus**

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(2)</sup> in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, either static temperature or temperature cycling) are required. Other items include:

- A7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.5H<sub>2</sub>O per 100 ml of sample, or equivalent).

- A7.2 Incubators capable of maintaining temperatures of  $30.0 \pm 1.0$  °C,  $37.0 \pm 1.0$  °C and  $44.0 \pm 0.5$  °C, or cyclical incubators fitted with timers, capable of attaining these temperatures.
- A7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.
- A7.4 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based, 0.45 µm nominal pore size. If broth medium is used then appropriate absorbent pads are required.
- A7.5 Smooth-tipped forceps.
- A7.6 Water baths (or incubators) set at  $37.0 \pm 1.0$  °C or  $44.0 \pm 0.5$  °C and test tube racks.

## A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified.

### A8.1 *Membrane lauryl sulphate broth*<sup>(4)</sup>

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v aqueous solution)	50 ml
Sodium lauryl sulphate - specially pure	1 g
Distilled, deionised or similar grade water	1 litre

Add the ingredients to the water and mix gently to avoid the formation of froth. The final pH of the sterile medium should be  $7.4 \pm 0.2$  and, to achieve this, it may be necessary to adjust the pH to about 7.6 before sterilisation. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH. Distribute the medium in loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. The bottles may need to be removed from the autoclave as soon as possible after autoclaving in order to avoid possible breakdown of the lactose and reduction in the pH. When cooled, the screw cap should be more tightly sealed.

The media may be used in an agar form, as membrane lauryl sulphate agar, by the addition of agar (usually 10 - 13 g of a suitable quality) to the above formulation before autoclaving begins. Petri dishes containing the agar medium can be stored for up to one week at temperatures between 2 - 8 °C, protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium.

The broth medium should be used as soon as possible but can be stored for up to one month at temperatures between 2 - 8 °C. During refrigerated storage, sodium lauryl

sulphate may precipitate out of solution. Before use, allow the broth to come to room temperature and mix well before dispensing into Petri dishes.

#### A8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except the phenol red indicator solution, in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Add the indicator solution and distribute in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

#### A8.3 *Tryptone water for the indole test*

The use of certain peptones that give satisfactory results in tests carried out at 37 °C may not be satisfactory for the indole test at 44 °C<sup>(5)</sup>. Care should, therefore, be taken in the appropriate selection of reagents.

Tryptone	20 g
Sodium chloride	5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Distribute in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

#### A8.4 *Kovacs' reagent for the indole test*<sup>(6)</sup>

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol (3-methylbutan-1-ol) (analytical grade reagent free from organic bases)	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol and slowly add the hydrochloric acid. Protect from light and store at temperatures between 2 - 8 °C. The reagent should be pale yellow or straw-coloured when freshly prepared. Some types of amyl alcohol are unsatisfactory and give a dark colour with the aldehyde.

#### A8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution and maximum recovery diluent.

## **A9 Analytical procedure**

### *A9.1 Sample preparation*

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, 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 treated waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with Ringer's solution or maximum recovery diluent before filtration. Paired volumes of each sample are filtered and incubated separately at 37 °C and at 44 °C.

### *A9.2 Sample processing*

If membrane lauryl sulphate broth is used, for each sample, place an absorbent pad into each of two empty sterile Petri dishes. Aseptically, add sufficient membrane lauryl sulphate broth to saturate the pad, allow the medium to soak into the pad and pour off and discard any excess medium. If this is not done, confluent growth may result.

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. 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 bacteria 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 so that as little air as possible is drawn through the membrane filter.

Remove the funnel and transfer the membrane filter carefully to one of the pads saturated with membrane lauryl sulphate broth, or to a Petri dish containing well-dried membrane lauryl sulphate agar (for example, Petri dishes left at room temperature for 2 hours or at 37 °C for 30 minutes, prior to use). 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. Repeat the process with the second volume of sample, transferring the membrane filter to the other saturated pad or second Petri dish containing well-dried membrane lauryl sulphate agar.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, 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 re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling distilled, deionised or similar grade water for at least one minute. 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. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

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.

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 placed in an incubator at 30 °C for 4 hours. One dish is then transferred to an incubator at 37 °C for 14 hours (for coliform bacteria) and the other dish to an incubator at 44 °C for 14 hours (for *E. coli*). Alternatively, cyclical temperature incubators can be used. Accurate temperature control and even temperature distribution are essential. False positive results may be obtained if lower incubation temperatures are used and some organisms may fail to multiply at higher incubation temperatures. If an early indication of a result is required for any reason, the membrane filters may be examined after a total incubation time of 12 hours but must be returned to the incubator for the full incubation period of 18 hours.

### A9.3 *Reading of results*

After the total incubation period of 18 hours, examine the membrane filters under good light, if necessary with a hand lens. Colours are liable to change on cooling and standing, hence, within 15 minutes of being removed from the incubator, count all yellow colonies (however faint) irrespective of size. The number of colonies counted on the membrane filter incubated at 37 °C is regarded as the number of presumptive coliform bacteria and the number of colonies counted on the membrane filter incubated at 44 °C is regarded as the number of presumptive *E. coli*. It is important to note whether pink colonies (from non-target organisms) are present in numbers that may interfere with the growth of coliform bacteria. If the growth of pink colonies is considered to be such that they may be obscuring lactose-fermenting colonies, a further sample should be taken and re-submitted for examination. Any portion of sample retained in the refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, a count of the number of colonies on the membrane filter should not be reported of the re-examined sample, as this may not reflect the number of colonies in the original sample when first examined. In addition, appropriate dilution of the sample, and MPN or presence-absence methods should also be considered.

#### A9.4 Confirmation tests

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of yellow colonies (however faint). If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present or, at least ten colonies should be sub-cultured if more than ten are present. Colonies should always be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be sub-cultured. Where a number of colonies of different appearance are clearly distinguishable, a note of the number of each morphological type should also be made. If only a single membrane, incubated at 37 °C, has been analysed, then all presumptive colonies should be sub-cultured for confirmation testing, as the presence of low numbers of *E. coli* could be missed if only a representative number of colonies are tested. The data and information obtained from the sub-cultured isolates are then used to calculate the confirmed counts of coliform bacteria and *E. coli*.

When colonies are sub-cultured for confirmation, they should be tested for confirmation as coliform bacteria and as *E. coli*, whether initially isolated at 37 °C or at 44 °C. This is important because presumptive coliform colonies isolated at 37 °C may confirm as *E. coli*. Conversely, presumptive *E. coli* colonies isolated at 44 °C may not confirm as *E. coli* but may confirm as coliform bacteria. This is particularly important when colonies are isolated on only one of the two membranes incubated at 37 °C and at 44 °C. *E. coli* are coliform bacteria, and if the confirmed count for *E. coli* from the 44 °C membrane filter is greater than the confirmed count for coliform bacteria from the 37 °C membrane filter, then the higher count must be recorded as the confirmed count for coliform bacteria. In the case where, for example, zero coliform bacteria have been isolated at 37 °C but 2 *E. coli* have been isolated at 44 °C then results should be reported as 2 coliform bacteria and 2 *E. coli*.

Colonies for confirmation tests should be sub-cultured as soon as practicable, preferably within 60 minutes, as colony colours can fade after removal of the Petri dishes from the incubator. After counting, Petri dishes may be stored in the appropriate incubator prior to sub-culturing to allow retention of colour and identification of colony.

##### A9.4.1 Confirmation for coliform bacteria

The confirmation procedure outlined is based upon the demonstration of lactose fermentation as being indicative of the possession of  $\beta$ -galactosidase enzyme. Alternative procedures based upon the direct detection of this enzyme, for example using the substrate, ortho-nitrophenyl- $\beta$ -D-galactopyranoside, may be more appropriate.

From the membrane filter incubated at 37 °C, sub-culture to lactose peptone water (LPW) each colony to be tested and incubate at 37 °C. After 6 hours, the LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from

the membrane filter incubated at 37 °C. Examine the LPW after 24 hours for acid production and, if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA plates at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate. Pure cultures are essential for the oxidase test and it may be necessary to make further sub-cultures. These should be made from characteristic coliform colonies obtained from the MA plate.

Typically, coliform bacteria produce pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. *In situ* oxidase tests carried out directly on colonies on the membrane filter may not be suitable. Oxidase may diffuse from oxidase-positive colonies to adjacent oxidase-negative colonies and oxidase production may be inhibited by acid produced from lactose. Also, further sub-culture may be unsuccessful due to the toxic effects of the oxidase reagent. Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests<sup>(7)</sup>. Commercial test kits may be used following appropriate performance verification at the laboratory.

Some species of *Bacillus* and *Staphylococcus* may grow on membrane lauryl sulphate broth producing yellow colonies. These can be readily recognised by colony characteristics on MA, and by Gram staining.

#### A9.4.2 Confirmation of *E. coli*

From the membrane filter incubated at 44 °C sub-culture yellow colonies to two tubes of LPW and one tube of tryptone water (TW). Incubate one of the LPW tubes and the TW tube at 44 °C for 24 hours. After 24 hours, examine for the production of acid in the LPW tube and indole in the TW tube. The other LPW tube is incubated at 37 °C for 6 hours. After the 6 hour incubation, the 37 °C LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from the membrane filter incubated at 44 °C. Examine the 37 °C LPW after 24 hours for acid production, and if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate.

In addition, from the membrane filter incubated at 37 °C sub-culture yellow colonies to one tube of LPW and one tube of TW and incubate at 44 °C for 24 hours. After 24 hours, examine for the production of acid in the LPW tube and indole in the TW tube.

Typically, *E. coli* colonies are oxidase-negative, produce acid in LPW at 37 °C and at 44 °C, and produce indole in TW at 44 °C. Tests for  $\beta$ -glucuronidase may assist in the early confirmation of *E. coli*<sup>(8,9)</sup>. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

#### A9.4.2.1 *Indole test*

After incubation of the TW tubes at 44 °C, add 0.2 - 0.3 ml of Kovacs' reagent. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer.

#### A9.4.3 *Oxidase test*

Some organisms that are found in water may conform to the definition of coliform bacteria in most respects, but are able to produce acid from lactose only at temperatures below 37 °C. *Aeromonas* species, which occur naturally in water, possess optimum growth at temperatures between 30 - 35 °C but may produce acid from lactose at 37 °C. These organisms are of uncertain public health significance and are distinguishable from coliform bacteria by a positive oxidase reaction. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA. Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA onto the treated filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction.

Commercial test kits for oxidase testing are available and should be used in accordance with manufacturer's instructions and following appropriate performance verification at the laboratory.

On each occasion where oxidase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example *E. coli*).

## **A10 Calculations**

### A10.1 *Presumptive coliform bacteria and E. coli*

The number of presumptive coliform bacteria and *E. coli* is generally expressed as the number of colonies per 100 ml of sample. Calculate the presumptive count as follows:

$$\text{Presumptive count/100 ml} = \frac{\text{Number of colonies counted on membrane filter} \times 100}{\text{Volume of sample filtered (ml)}}$$

The count from the 37 °C incubation is regarded as presumptive coliform bacteria and that from the 44 °C incubation as presumptive *E. coli*.

### A10.2 *Confirmed coliform bacteria and E. coli*

The number of confirmed coliform bacteria is calculated by multiplying the number of presumptive coliform bacteria by the proportion of the isolates that are both lactose-positive (in LPW) and oxidase-negative.

The number of confirmed *E. coli* is calculated by multiplying the number of presumptive *E. coli* by the proportion of the isolates that are lactose-positive (in LPW), produce indole from TW at 44 °C and are oxidase-negative.

On rare occasions, a significant number of isolates from the 37°C incubation may confirm as *E. coli* and the count calculated may be higher than that calculated for the 44 °C incubation. For these examples, the higher count from the 37°C incubation should be reported.

#### **A11 Expression of results**

Counts for presumptive and confirmed coliform bacteria and *E. coli* are expressed in colony forming units per volume of sample. For drinking water, 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 *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example *Pseudomonas aeruginosa*). Petri dishes should be incubated for 24 hours at 37 °C or 44 °C as appropriate. Further details are given elsewhere<sup>(2)</sup> in this series.

#### **A13 References**

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality 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*, in this series, Environment Agency.
3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
4. Membrane filtration media for the enumeration of coliform bacteria and *Escherichia coli* in water: comparison of Tergitol 7 and lauryl sulphate with Teepol 610, by a Joint Committee of the Public Health Laboratory Service and Standing Committee of Analysts. *Journal of Hygiene*, 1980, **85**, 181-191.
5. The standardisation and selection of bile salt and peptone for culture media used in the bacteriological examination of water. *Proceedings of the Society for Water Treatment and Examination*, Burman, N.P., 1955, **4**, 10-26.
6. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. *Zeitschrift für Immunitätsforschung und experimentelle Therapie*, Kovács, N., 1928, **55**, 311-315.

7. *Cowan and Steels' Manual for the Identification of Medical Bacteria*, 3rd edition. (Editors, Barrow G.I. & Feltham R.K.A.). London, Cambridge University Press, 1993.
8. Fluorogenic assay for immediate confirmation of *Escherichia coli*. *Applied and Environmental Microbiology*, Feng, P.C.S. & Hartman, P.A., 1982, **43**, 1320-1329.
9. Glycosidase profiles of members of the family Enterobacteriaceae. *Journal of Clinical Microbiology*, Kampfer, P., Rauhoff, O. & Dott, W., 1991, **29**, 2877-2879.

## **B The enumeration of coliform bacteria and *Escherichia coli* by a single membrane filtration technique**

### **B1 Introduction**

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. They provide the most sensitive means for detecting faecal contamination, for assessing the effectiveness of water treatment and disinfection, and for monitoring water quality in distribution. The significance of *E. coli* and coliform bacteria in water treatment and supply are described elsewhere<sup>(1)</sup> in this series.

### **B2 Scope**

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate turbidity. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method.

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

### **B3 Definitions**

In the context of this method, organisms which are oxidase-negative, produce acid from lactose, and form all shades and sizes of yellow colonies on membrane filters (after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C) are regarded as coliform bacteria. In addition, organisms, which conform to the definition of *E. coli* below, are also coliform bacteria.

Coliform bacteria are considered to be members of genera or species within the Family Enterobacteriaceae, capable of growth at 37 °C, that possess  $\beta$ -galactosidase. This definition includes anaerogenic (ie non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia*, *Yersinia*, *Buttiauxella* and *Leclercia*.

Isolates which produce acid from lactose and produce  $\beta$ -glucuronidase forming green colonies after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C are regarded as *E. coli*. Most strains of *E. coli* express  $\beta$ -glucuronidase, as do some strains of *Shigella* and *Salmonella*.

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Strains which possess these characteristics at 37 °C but do not express them at 44 °C may also be *E. coli*. When identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

## **B4 Principle**

Organisms are isolated on a membrane filter placed on an agar medium containing lactose, phenol red as an indicator of acidity, and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (BCIG) either as the cyclohexylammonium or sodium salt for the indication of the production of  $\beta$ -glucuronidase. Isolation of colonies is followed by confirmation tests for acid production from lactose, negative oxidase reaction and, where necessary, indole formation.

## **B5 Limitations**

This 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 indicator organisms. The method also allows species of non-coliform organisms to grow, high numbers of which may inhibit growth of coliform bacteria. The maximum number of colonies that should be counted from a single membrane is 100. The growth of high numbers of coliform and non-coliform bacteria from untreated waters may inhibit the production of  $\beta$ -glucuronidase by *E. coli*.

## **B6 Health and safety**

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

## **B7 Apparatus**

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(2)</sup> in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, either static temperature or temperature cycling) are required. Other items include:

- B7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  per 100 ml of sample, or equivalent).
- B7.2 Incubators capable of maintaining temperatures of  $30.0 \pm 1.0$  °C and  $37.0 \pm 1.0$  °C, or cycling incubators, fitted with timers, capable of attaining these temperatures.
- B7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.
- B7.4 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based 0.45  $\mu\text{m}$  nominal pore size.
- B7.5 Smooth-tipped forceps.

B7.6 Water baths (or incubators) set at  $37.0 \pm 1.0$  °C or  $44.0 \pm 0.5$  °C and test tube racks.

## B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified.

### B8.1 *Membrane lactose glucuronide agar*<sup>(4, 5)</sup>

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v solution)	50 ml
Sodium lauryl sulphate	1.0 g
Sodium pyruvate	0.5 g
Agar	10.0 g
BCIG	0.2 g
Distilled, deionised or similar grade water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of solution consisting of 2.5 ml of 95% v/v aqueous ethanol and 0.5 ml of 1 molar sodium hydroxide solution. Add this solution to the medium. The sodium salt of BCIG can be added directly to the medium. Mix the solution well and autoclave at 121 °C for 15 minutes. Allow the solution to cool, distribute in Petri dishes and allow to solidify. Petri dishes containing the agar medium may be stored at temperatures between 2 - 8 °C for up to one week, protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium. The pH after sterilisation should be  $7.4 \pm 0.2$ . The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH.

### B8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except the phenol red indicator solution, in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Add the indicator solution and distribute in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

### B8.3 *Tryptone water for the indole test*

The use of certain peptones that give satisfactory results in tests carried at 37 °C may not be satisfactory for the indole test at 44 °C<sup>(6)</sup>. Care should, therefore, be taken in the appropriate selection of reagents.

Tryptone	20 g
Sodium chloride	5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Distribute in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

### B8.4 *Kovacs' reagent for the indole test*<sup>(7)</sup>

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol (3-methylbutan-1-ol) (analytical grade reagent free from organic bases)	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the aldehyde in the amyl alcohol and slowly add the acid. Protect from light and store at 2 - 8 °C. The reagent should be pale-yellow or straw-coloured after preparation. Some types of amyl alcohol are unsatisfactory and give a dark colour with the aldehyde.

### B8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution and maximum recovery diluent.

## **B9 Analytical procedure**

### B9.1 *Sample preparation*

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, 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 treated waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with Ringer's solution or maximum recovery diluent before filtration.

### B9.2 *Sample processing*

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. 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 bacteria 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 so that as little air as possible is drawn through the membrane filter.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing well-dried membrane lactose glucuronide agar (for example, Petri dishes left at room temperature for 2 hours or at 37 °C for 30 minutes, prior to use). 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.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, 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 re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling distilled, deionised or similar grade water for at least one minute. 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. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

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.

The Petri dishes are inverted and placed in an incubator at 30 °C for 4 hours then transferred to an incubator at 37 °C for 14 hours. Alternatively, a cycling temperature incubator can be used. Accurate temperature control and even temperature distribution are essential. False positive results may be obtained if lower incubation temperatures are used and some organisms may fail to multiply at higher incubation temperatures. If an early indication of a result is required for any reason, the membrane filter may be examined after a total incubation time of 12 hours but must be returned to the incubator for the full incubation period of 18 hours.

### B9.3 *Reading of results*

After the total incubation period of 18 hours, examine the membrane filters under good light, if necessary with a hand lens. Count all yellow and green colonies (however faint) irrespective of size within 15 minutes of being removed from the incubator, as the yellow coloration may change on cooling and standing. All yellow colonies are presumptive non-*E. coli* coliform bacteria and green colonies are *E. coli*. The total combined count of yellow and green colonies is regarded as the number of coliform bacteria. It is important to note whether pink colonies (from non-target organisms) are present in numbers that may interfere with the growth of coliform bacteria and *E. coli*. If the growth of pink colonies is considered to be such that they may be obscuring lactose-fermenting colonies, a further sample should be taken and re-submitted for examination. Any portion of sample retained in the refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, a count of the number of colonies on the membrane filter should not be reported of the re-examined sample, as this may not reflect the number of colonies in the original sample when first examined. In addition, appropriate dilution of the sample and MPN or presence-absence methods should also be considered.

### B9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of yellow and green colonies (however faint). The specificity of the green colonies on membrane lactose glucuronide agar being *E. coli* is very high, and, following suitable confirmation of performance within the laboratory, confirmation of green colonies may not be needed. Occasionally, blue colonies may be noted and recorded. These colonies may be lactose-negative *E. coli*, but are more commonly strains of *Aeromonas*. Blue colonies should, therefore, initially be classed as presumptive coliform bacteria and be subjected to confirmatory testing. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present or, at least ten colonies should be sub-cultured if more are present. Colonies should always be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be sub-cultured. Where a number of colonies of different appearance are clearly distinguishable, a note of the number of each morphological type should also be made. The data and information from the sub-cultured isolates are then used to calculate the confirmed counts of coliform bacteria and *E. coli*.

When colonies are sub-cultured for confirmation, they should be tested for confirmation as coliform bacteria and as *E. coli*. This is important because yellow colonies may confirm as *E. coli* (as some strains do not express  $\beta$ -glucuronidase, and other strains appear negative when first isolated). Occasionally, green (presumptive *E. coli*) colonies may not confirm as *E. coli* but may, nevertheless, confirm as coliform bacteria.

Colonies for confirmation tests should be sub-cultured as soon as practicable, preferably within 60 minutes, as colony colours can fade after removal of the Petri

dishes from the incubator. After counting, Petri dishes may be stored in the incubator prior to sub-culturing to allow retention of colour and identification of colony.

#### B9.4.1 *Confirmation for coliform bacteria*

The confirmation procedure outlined is based upon demonstration of lactose fermentation as being indicative of possession of the  $\beta$ -galactosidase enzyme. Alternative procedures based upon the direct detection of this enzyme, for example using the substrate, ortho-nitrophenyl- $\beta$ -D-galactopyranoside, may be more appropriate.

Sub-culture to lactose peptone water (LPW) each colony to be tested and incubate at 37 °C. After 6 hours, the LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from the membrane filter. Examine the LPW after 24 hours for acid production and, if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA plates at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate. Pure cultures are essential for the oxidase test and it may be necessary to make further sub-cultures. These should be made from characteristic coliform colonies obtained from the MA plate.

Typically, coliform bacteria produce pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. *In situ* oxidase tests carried out directly on colonies on the membrane filter may not be suitable. Oxidase may diffuse from oxidase-positive colonies to adjacent oxidase-negative colonies and oxidase production may be inhibited by acid produced from lactose. Also, further sub-culture may be unsuccessful due to the toxic effects of the oxidase reagent. Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests<sup>(8)</sup>. Commercial test kits may be used following appropriate performance verification at the laboratory.

Some species of *Bacillus* and *Staphylococcus* may grow on membrane lactose glucuronide agar producing yellow colonies. These can be readily recognised by colony characteristics on MA, and by Gram staining.

#### B9.4.2 *Confirmation of E. coli*

The specificity of membrane lactose glucuronide agar for *E. coli* is such that, following performance verification within the laboratory, confirmation of green colonies as *E. coli* may not be required.

Sub-culture yellow and, if required, green colonies from the membrane filter to two tubes of LPW and one tube of tryptone water (TW). Incubate one of the LPW tubes and the TW tube at 44 °C for 24 hours. After 24 hours, examine for the production of acid in the LPW tube and indole in the TW tube. The other LPW tube is incubated at 37 °C for 6 hours. After the 6 hour incubation, the 37 °C LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then

returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from the membrane filter. Examine the 37 °C LPW after 24 hours for acid production, and if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate.

Typically, *E. coli* colonies are oxidase-negative, produce acid in LPW at 37 °C and at 44 °C, and indole in TW at 44 °C. Tests for  $\beta$ -glucuronidase may assist in the early confirmation of *E. coli* <sup>(9, 10)</sup>. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

#### B9.4.2.1 *Indole test*

After incubation of the TW tubes at 44 °C add 0.2 - 0.3 ml of Kovacs' reagent. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer.

#### B9.4.3 *Oxidase test*

Some organisms that are found in water may conform to the definition of coliform bacteria in most respects, but are able to produce acid from lactose only at temperatures below 37 °C. *Aeromonas* species, which occur naturally in water, possess optimum growth at temperatures between 30 - 35 °C but may produce acid from lactose at 37 °C. These organisms are of uncertain public health significance and are distinguishable from coliform bacteria by a positive oxidase reaction. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA. Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA onto the treated filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction.

Commercial test kits for oxidase testing are available and should be used in accordance with manufacturers' instructions and following appropriate performance verification at the laboratory.

On each occasion where oxidase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example, *E. coli*).

## **B10 Calculations**

### B10.1 *Presumptive coliform bacteria and E. coli*

The number of presumptive coliform bacteria and *E. coli* is generally expressed as the number of colonies per 100 ml of sample. Calculate the presumptive count as follows:

$$\text{Presumptive count/100 ml} = \frac{\text{Number of colonies counted on membrane filter} \times 100}{\text{Volume of sample filtered (ml)}}$$

The count of the total number of yellow and green colonies on a membrane filter is regarded as the presumptive coliform bacteria count and the number of green colonies is regarded as the presumptive *E. coli* count.

#### B10.2 *Confirmed coliform bacteria and E. coli*

The number of confirmed coliform bacteria is calculated by multiplying the number of presumptive coliform bacteria by the proportion of the isolates that are both lactose-positive (in LPW) and oxidase-negative

The number of confirmed *E. coli* is calculated by multiplying the number of presumptive *E. coli* by the proportion of the isolates that are lactose-positive (in LPW), produce indole from tryptone water at 44 °C and are oxidase-negative, combined with any proportion of yellow colony isolates that subsequently confirm as *E. coli*.

### **B11 Expression of results**

Counts for presumptive and confirmed coliform bacteria and *E. coli* are expressed in colony forming unit per volume of sample. For drinking water the volume is typically 100 ml.

### **B12 Quality assurance**

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example, *Pseudomonas aeruginosa*). Petri dishes should be incubated for 24 hours at 37 °C, or 44 °C as appropriate. Further details are given elsewhere<sup>(2)</sup> in this series.

### **B13 References**

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality 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*, in this series, Environment Agency.
3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

4. A medium detecting  $\beta$ -glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Letters in Applied Microbiology*, Sartory, D.P. & Howard, L., 1992, **15**, 273-276.
5. Standing Committee of Analysts, Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
6. The standardisation and selection of bile salt and peptone for culture media used in the bacteriological examination of water. *Proceedings of the Society for Water Treatment and Examination*, Burman, N.P., 1955, **4**, 10-26.
7. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. *Zeitschrift für Immunitätsforschung und experimentelle Therapie*, Kovács, N., 1928, **55**, 311-315.
8. *Cowan and Steels' Manual for the Identification of Medical Bacteria*, 3rd edition. (Editors, Barrow G.I. & Feltham R.K.A.). London, Cambridge University Press, 1993.
9. Fluorogenic assay for immediate confirmation of *Escherichia coli*. *Applied and Environmental Microbiology*, Feng, P.C.S. & Hartman, P.A., 1982, **43**, 1320-1329.
10. Glycosidase profiles of members of the family Enterobacteriaceae. *Journal of Clinical Microbiology*, Kampfer, P., Rauhoff, O. & Dott, W., 1991, **29**, 2877-2879.

## **C The enumeration of coliform bacteria and *Escherichia coli* by a multiple tube most probable number technique**

### **C1 Introduction**

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. They provide the most sensitive means for detecting faecal contamination, for assessing the effectiveness of water treatment and disinfection, and for monitoring water quality in distribution. The significance of *E. coli* and coliform bacteria in water treatment and supply are described elsewhere<sup>(1)</sup> in this series.

### **C2 Scope**

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate to high turbidity.

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

### **C3 Definitions**

In the context of the method, organisms which are oxidase-negative and produce acid from lactose within 48 hours at 37 °C in a chemically defined medium are regarded as coliform bacteria.

Coliform bacteria are considered to be members of genera or species within the Family Enterobacteriaceae, capable of growth at 37 °C, that possess  $\beta$ -galactosidase. This definition includes anaerogenic (ie non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia*, *Yersinia*, *Buttiauxella*, *Leclercia*.

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce  $\beta$ -glucuronidase. Strains which possess these characteristics at 37 °C but do not express them at 44 °C may also be *E. coli*. When identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

### **C4 Principle**

Bacteria are grown in a liquid medium containing lactose and bromocresol purple as an indicator of acidity. This is followed by confirmation tests for acid production from lactose, negative oxidase reaction and, where necessary, indole formation.

In this method, measured volumes of sample, or dilution of sample, are added to a series of tubes or bottles containing liquid differential medium. If, within the series,

some of the tubes or bottles exhibit no characteristic growth in the medium following incubation, and other tubes or bottles exhibit some characteristic growth, then the most probable number of organisms in 100 ml of sample can be estimated from appropriate probability tables, see appendix C1. Confirmation that positive reactions (ie those tubes or bottles showing characteristic growth) are due to a particular organism can be obtained by sub-culture to tubes of confirmation media. This may involve incubation at higher temperatures, depending on the particular organism.

## **C5 Limitations**

This method is suitable for all types of water and related samples and is particularly suitable for the examination of sludges and waters containing sediment.

## **C6 Health and safety**

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

## **C7 Apparatus**

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

C7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  per 100 ml of sample, or equivalent).

C7.2 Incubators (or water baths) capable of maintaining temperatures of  $37.0 \pm 1.0$  °C and  $44.0 \pm 0.5$  °C.

C7.3 Suitable bottle or test tube racks.

## **C8 Media and reagents**

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified.

Minerals modified glutamate medium<sup>(4, 5)</sup> is used for the isolation of coliform bacteria from waters. The use of MacConkey broth may not be suitable because of variations in the inhibitory properties of different batches of bile salts.

### C8.1 *Minerals modified glutamate medium*

Lactose	20.0 g
L (+) Glutamic acid sodium salt	12.7 g
L (+) Arginine monohydrochloride	40 mg
L (-) Aspartic acid	48 mg
L (-) Cystine	40 mg
Sodium formate	500 mg
Dipotassium hydrogen phosphate	1.8 g
Ammonium chloride	5.0 g
Magnesium sulphate heptahydrate	200 mg
Calcium chloride dihydrate	20 mg
Iron(III) citrate	20 mg
Thiamine (Aneurin hydrochloride)	2 mg
Nicotinic acid	2 mg
Pantothenic acid	2 mg
Bromocresol purple (1 % m/v ethanolic solution)	2 ml
Distilled, deionised or similar grade water to	1 litre

This formulation enables double-strength medium to be prepared. This is conveniently prepared in quantities of 10 (or more) litres. If the medium is not to be distributed in tubes immediately, the lactose and thiamine should be omitted and added before dispensing.

Several of the ingredients are more conveniently added as separate solutions and these may be prepared as follows:

#### SOLUTION 1

L (+) Arginine monohydrochloride	400 mg
L (-) Aspartic acid	480 mg
Distilled, deionised or similar grade water	50 ml

Heat the ingredients in the water to dissolve.

#### SOLUTION 2

L (-) Cystine	400 mg
5M Sodium hydroxide	10 ml
Distilled, deionised or similar grade water	90 ml

Heat the ingredients in the water to dissolve.

#### SOLUTION 3

Nicotinic acid	20 mg
Pantothenic acid	20 mg
Distilled, deionised or similar grade water	5 ml

Dissolve the ingredients in the water without heating the solution.

#### SOLUTION 4

Iron(III) citrate	200 mg
Distilled, deionised or similar grade water	10 ml

Heat the ingredients in the water to dissolve.

#### SOLUTION 5

Calcium chloride dihydrate	5 g
Distilled, deionised or similar grade water	100 ml
Concentrated hydrochloric acid	0.1 ml

Dissolve the ingredients in the water without heating the solution and sterilise at 121°C for 20 minutes. Store as a stock solution.

#### SOLUTION 6

Thiamine	100 mg
Distilled, deionised or similar grade water	99 ml

Prepare a sterile 0.1 % m/v solution of thiamine in distilled, deionised or similar grade water. (This can be carried out by adding the contents of an ampoule of thiamine (100 mg) to 99 ml of sterile distilled water.)

The above solutions should be stored at temperatures between 2 - 8 °C and any remaining, unused solution should be discarded after 6 weeks.

To prepare 10 litres of double-strength medium, dissolve the appropriate quantities of L (+) glutamic acid sodium salt, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulphate heptahydrate in 9 litres of hot distilled water. Add the whole of solutions 1, 2, 3 and 4, and 4 ml of solution 5. Adjust the pH to  $6.9 \pm 0.2$  or higher if necessary, so that the final pH (when completely prepared and after sterilisation is  $6.7 \pm 0.2$ ). After adjustment of the pH, add 20 ml of a 1 % m/v ethanolic solution of bromocresol purple. Dilute to a final volume of 10 litres.

If the medium is not required for immediate use, dispense the mixed solution (medium without lactose and thiamine) into suitable containers in 500 ml volumes. Autoclave the solutions at 115 °C for 10 minutes and store for not more than one month in the dark at room temperature.

For use, add the necessary amounts of lactose and solution 6, (10 g and 1 ml respectively). Allow the added lactose to dissolve and distribute into suitable tubes or bottles in 10 ml and 50 ml volumes. Cap the containers and sterilise at 115 °C for 10 minutes.

Prepare single-strength medium by diluting the double-strength medium with an equal volume of distilled water and distribute in 5 ml volumes in tubes. Sterilise at 115 °C for 10 minutes.

The sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

### C8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except the phenol red indicator solution, in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Add the indicator solution and distribute in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

### C8.3 *Tryptone water for the indole test*

The use of certain peptones that give satisfactory results in tests carried out at 37 °C may not be satisfactory for the indole test at 44 °C<sup>(6)</sup>. Care should, therefore, be taken in the appropriate selection of reagents.

Tryptone	20 g
Sodium chloride	5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Distribute in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

### C8.4 *Kovacs' reagent for the indole test*<sup>(7)</sup>

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol (3-methylbutan-1-ol) (analytical grade reagent free from organic bases)	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol and slowly add the hydrochloric acid. Protect from light and store at temperatures between 2 - 8 °C. The reagent should be pale yellow or straw-coloured when freshly prepared. Some types of amyl alcohol are unsatisfactory and give a dark colour with the aldehyde.

### C8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution and maximum recovery diluent.

## **C9 Analytical procedure**

### *C9.1 Volumes of sample for inoculation*

A series of different volumes of sample is inoculated into tubes or bottles of minerals modified glutamate medium.

For waters expected to be of good quality, use 1 x 50 ml and 5 x 10 ml volumes of sample. Add the 50 ml and 10 ml volumes of sample to equal volumes of double-strength medium.

For waters expected to be of doubtful or unknown quality, use 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Add the 50 ml and 10 ml volumes of sample to equal volumes of double-strength medium and the 1 ml volumes of sample to 5 ml of single-strength medium.

For waters expected to be of a more polluted nature, use 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample. Add the 10 ml volumes of sample to equal volumes of double-strength medium and the 1 ml and 0.1 ml volumes of sample to 5 ml of single-strength medium.

For waters expected to be heavily polluted, dilutions (in a suitable diluent, for example, quarter-strength Ringer's solution or maximum recovery diluent) of a hundred-fold, or a thousand-fold or higher, may need to be prepared. Suitable volumes of these diluted samples should then be used and added to 5 ml of single-strength medium.

For all types of samples and in all cases, sufficient volumes of sample should be added to the medium so that, after incubation, some of the tubes or bottles exhibit growth within the medium and some of the tubes or bottles exhibit no growth within the medium. Sterile pipettes are used to transfer the sample to the tube or bottle containing the minerals modified glutamate medium.

### *C9.2 Sample processing*

After the tubes or bottles of minerals modified glutamate medium have been inoculated with the appropriate volume of sample, or diluted sample, each tube or bottle is capped or sealed and placed in an incubator at 37 °C. After 18 - 24 hours, the tubes or bottles are examined for acid production (as demonstrated by the presence of a yellow coloration). After a further 24 hours, the tubes or bottles are re-examined and results recorded. All tubes or bottles that exhibit positive (characteristic) growth within the medium are retained for confirmatory testing. Some tubes or bottles may exhibit growth without a colour change. In these cases, these tubes are regarded as negative.

### *C9.3 Reading of results*

The number of tubes or bottles for each series of volume of sample is recorded where a positive reaction is given, as demonstrated by growth within the medium and the

production of a yellow coloration. After this, confirmation tests may be carried out as required.

When dilutions of sample have been used, a consecutive series of volumes is chosen whereby some of the tubes or bottles show a positive reaction and some show a negative reaction. From the results, the MPN of bacteria in the sample is determined from probability tables, see appendix C1.

#### C9.4 *Confirmation Tests*

Whenever positive tubes or bottles are used in confirmatory tests, they should be tested for confirmation both as coliform bacteria and as *E. coli*.

##### C9.4.1 *Confirmation for coliform bacteria*

The confirmation procedure outlined is based upon demonstration of lactose fermentation as being indicative of the possession of the  $\beta$ -galactosidase enzyme. Alternative procedures based upon the direct detection of this enzyme, for example using the substrate, ortho-nitrophenyl- $\beta$ -D-galactopyranoside, may be more appropriate.

For each tube or bottle showing growth within the medium, sub-culture to MA and NA. Incubate the plates of MA and NA at 37 °C for 18 - 24 hours. If a pure culture is obtained on NA then perform the oxidase test as described in section C9.4.3. If the isolate is oxidase-negative, then perform the test for lactose fermentation or possession of  $\beta$ -galactosidase. If there is any doubt about the purity of the culture then sub-culture at least one typical coliform colony from MA to NA, incubate at 37 °C for 18 - 24 hours and carry out the oxidase test. Typically coliform bacteria produce pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts.

For each isolate to be tested, sub-culture to lactose peptone water (LPW) and incubate at 37 °C. Examine for acid production after 24 hours. If the results are negative, re-examine after a further 24 hours. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests<sup>(8)</sup>. Commercial test kits may be used following appropriate performance verification at the laboratory.

Some species of *Bacillus* may grow in minerals modified glutamate medium producing yellow coloration. These can be readily recognised by colony characteristics on MA, and by Gram staining.

##### C9.4.2 *Confirmation of E. coli*

As well as conducting tests for confirmation of coliform bacteria, for each tube or bottle showing growth within the medium, sub-culture to MA and incubate at 44 °C for 18 - 24 hours. Inoculate typical coliform colonies into tubes of tryptone water (TW) and incubate at 44 °C for 24 hours. The presence of *E. coli* is demonstrated by

the production of indole in TW. Tests for  $\beta$ -glucuronidase may assist in the early confirmation of *E. coli*<sup>(9, 10)</sup>. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

#### C9.4.2.1 *Indole test*

After incubation of the TW tubes at 44 °C, add 0.2 - 0.3 ml of Kovacs' reagent. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer.

#### C9.4.3 *Oxidase test*

Some organisms that are found in water may conform to the definition of coliform bacteria in most respects, but are able to produce acid from lactose only at temperatures below 37°C. *Aeromonas* species, which occur naturally in water, possess optimum growth at temperatures between 30 - 35 °C but may produce acid from lactose at 37 °C. These organisms are of uncertain public health significance and are distinguishable from coliform bacteria by a positive oxidase reaction. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA.

Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA onto the prepared filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction.

Commercial test kits for oxidase testing are available and should be used in accordance with manufacturer's instructions and following appropriate performance verification at the laboratory.

On each occasion that oxidase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example, *E. coli*).

## **C10 Calculations**

### C10.1 *Presumptive coliform bacteria*

The number of minerals modified glutamate medium tubes or bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in appendix C1, the MPN of presumptive coliform bacteria and *E. coli* present in 100 ml of sample is determined. For example, if in a 15-tube test comprising 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample, the number of tubes showing positive reactions in each consecutive series is 3, 2 and 0 respectively, then, from Table C4, the MPN is 13 organisms per 100 ml.

### C10.2 *Confirmed coliform bacteria and E. coli*

Confirmed coliform bacteria are calculated by reference to the appropriate table in appendix C1 for the number of tubes or bottles that yield isolates that produce typical coliform colonies on MA, produce acid from LPW at 37 °C and are oxidase-negative.

Confirmed *E. coli* are calculated by reference to the appropriate table in appendix C1 for the number of tubes or bottles that yield isolates that produce typical coliform colonies on MA, produce acid from LPW at 44 °C, produce indole from TW at 44 °C and are oxidase-negative.

### C11 **Expression of results**

Presumptive coliform bacteria and confirmed coliform bacteria and *E. coli* counts are expressed as MPN per volume of sample. For drinking water, the volume is typically 100 ml.

### C12 **Quality assurance**

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example, *Pseudomonas aeruginosa*). Tubes or bottles should be incubated for 24 hours at 37 °C or 44 °C as appropriate. Further details are given elsewhere<sup>(2)</sup> in this series.

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## Appendix C1                      Tables of most probable numbers

From the various combinations of positive and negative reactions for the different volumes examined, the following tables indicate the MPN of bacteria in 100 ml of sample. It is important to realise that the MPN is only an estimate, based on statistical probabilities and that the actual number may lie within a range of values.

Approximate 95 % confidence intervals, which demonstrate the range of possible numbers (the MPR) which could yield the number of positive reactions, have been published<sup>(11)</sup>. A procedure for estimating these confidence intervals for other dilution series has also been published<sup>(12)</sup>. These confidence intervals are seldom of practical use when reporting results because they apply to the accuracy of the method and not the likely variability of organisms at the sampling source<sup>(13)</sup>. The MPR in tables C1 - C3 illustrates those situations where the method becomes relatively imprecise, particularly when nearly all the tubes show growth within the medium. In these situations, further dilutions should have been prepared and added to tubes of medium.

Table C1 gives the MPN (and where applicable the MPR) for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample. Similarly table C2 gives the MPN (and where applicable the MPR) for an 11-tube series comprising 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Table C3 shows data for a 15-tube series of 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of samples but gives only those values of the more likely combinations of positive and negative reactions. For example, positive reactions in the 0.1 ml tubes would not be expected if all of the 10 ml and 1 ml tubes were negative. Hence, MPN and MPR values for a combination of results like for instance 0, 0, 2 etc are not tabulated. If these unlikely combinations are observed in practice with greater than expected frequencies, then this might indicate that the statistical assumptions underlying the MPN estimation are not correct<sup>(11, 14, 15)</sup>. For example, the organisms may not have been uniformly distributed throughout the sample, or toxic substances may have been present.

### Calculation of MPN

The number of positive reactions for each set of tubes is recorded and, from the relevant table, the MPN of organisms present in 100 ml of the sample is determined.

Where a series of dilutions of the sample is used, then the following rules should be applied, as illustrated by the numbers in bold, underlined, italic type in table C4.

- (i) Use only three consecutive sets of dilutions for calculating the MPN.
- (ii) Wherever possible, select three consecutive dilutions where the results are neither all positive nor all negative. The most efficient statistical estimate will result when about half the tubes are positive (see examples (a), (b) and (c) in table C4).
- (iii) If less than three sets of dilutions give positive results, begin with the set containing the largest volume of sample (see example (d) in table C4).
- (iv) If only one set of tubes gives a positive reaction, use this dilution and the one higher and one lower (see example (e) in table C4).

**Table C1** MPN and MPR per 100 ml of sample for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample

Number of tubes giving a positive reaction		MPN per 100 ml	MPR* per 100 ml
1 x 50 ml	5 x 10 ml		
0	0	None found	
0	1	1	
0	2	2	
0	3	3	
0	4	4	4-5
0	5	6	
1	0	1	
1	1	2	
1	2	5	4-5
1	3	9	8-10
1	4	15	13-18
1	5	>18**	

\* These numbers are at least 95 % as probable as the MPN.

\*\* There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 18.

**Table C2** MPN and MPR per 100 ml of sample for an 11-tube series of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
1 x 50 ml	5 x 10 ml	5 x 1 ml		
0	0	0	None found	
0	0	1	1	
0	1	0	1	
0	1	1	2	
0	2	0	2	
0	2	1	3	
0	3	0	3	
1	0	0	1	
1	0	1	2	
1	1	0	2	
1	1	1	4	
1	1	2	6	
1	2	0	4	4-5
1	2	1	7	6-7
1	2	2	9	9-10
1	3	0	8	7-9
1	3	1	10	10-11
1	3	2	13	12-13
1	3	3	17	15-18
1	4	0	12	11-14
1	4	1	16	15-19
1	4	2	21	19-24
1	4	3	27	24-30
1	4	4	33	30-38
1	5	0	23	20-27
1	5	1	33	29-40
1	5	2	53	44-65
1	5	3	91	75-110
1	5	4	160	134-190
1	5	5	>180**	

\* These numbers are at least 95 % as probable as the MPN.

\*\* There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 180.

**Table C3 MPN and MPR per 100 ml of sample for a 15-tube series containing 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample**

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
5 x 10 ml	5 x 1 ml	5 x 0.1 ml		
0	0	0	None found	
0	0	1	2	
0	1	0	2	
1	0	0	2	
1	0	1	4	
1	1	0	4	
2	2	0	4	
2	0	1	5	
2	1	0	5	
2	1	1	7	
2	2	0	7	7-9
2	3	0	11	
3	0	0	7	
3	0	1	9	
3	1	0	9	
3	1	1	13	
3	2	0	13	
3	2	1	16	14-16
3	3	0	16	14-16
4	0	0	11	11-13
4	0	1	14	14-16
4	1	0	16	14-16
4	1	1	20	18-20
4	2	0	20	18-22
4	2	1	25	23-27
4	3	0	25	23-27
4	3	1	31	29-34
4	4	0	32	29-34
4	4	1	38	34-41
5	0	0	22	20-23
5	0	1	29	25-34
5	0	2	41	36-50
5	1	0	31	27-36
5	1	1	43	36-50
5	1	2	60	50-70
5	1	3	85	70-95
5	2	0	50	40-55
5	2	1	70	60-80
5	2	2	95	80-110
5	2	3	120	105-135
5	3	0	75	65-90
5	3	1	110	90-125
5	3	2	140	120-160
5	3	3	175	155-200
5	3	4	210	185-240
5	4	0	130	110-150
5	4	1	170	150-200
5	4	2	220	190-250
5	4	3	280	240-320
5	4	4	345	300-390
5	5	0	240	200-280
5	5	1	350	290-420
5	5	2	540	450-600
5	5	3	910	750-1100
5	5	4	1600	1350-1900
5	5	>1800**		

\* These numbers are at least 95 % as probable as the MPN.

\*\* There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 1800.

**Table C4** Examples of the derivation of the MPN from the numbers of positive reactions in a series of dilutions\*

Example in text	Volume of sample (ml)					MPN per 100 ml
	10	1	0.1	0.01	0.001	
(a)	<u>5</u>	<u>3</u>	<u>2</u>	0		140
(b)	5	<u>5</u>	<u>3</u>	<u>2</u>	0	1400
(c)	5	<u>5</u>	<u>2</u>	<u>0</u>	0	500
(d)	<u>3</u>	<u>1</u>	<u>0</u>	0		9
(e)	<u>0</u>	<u>1</u>	<u>0</u>	0		2

\* Numbers in bold, underlined, italic type indicate which results should be used in determining the MPN.

## **D The enumeration of coliform bacteria and *Escherichia coli* by a defined substrate most probable number technique**

### **D1 Introduction**

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. They provide the most sensitive means for detecting faecal contamination, for assessing the effectiveness of water treatment and disinfection, and for monitoring water quality in distribution. The significance of *E. coli* and coliform bacteria in water treatment and supply are described elsewhere<sup>(1)</sup> in this series.

### **D2 Scope**

This method comprises a most probable number (MPN) technique and is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate to high turbidity.

Users wishing to employ this method, or similar methods from other manufacturers, should verify the performance under their own laboratory conditions<sup>(2)</sup>. Details of evaluation trials are reported elsewhere<sup>(3)</sup>.

### **D3 Definitions**

Defined substrate media are chemically defined formulations containing substrates for the specific detection of diagnostic enzymes associated with a particular group of organisms.

In the context of this method, organisms which are oxidase-negative and produce  $\beta$ -galactosidase, as demonstrated by the production of a yellow colour through the enzymatic cleavage of ortho-nitrophenyl- $\beta$ -D-galactopyranoside in a defined substrate medium, are regarded as coliform bacteria. In addition, organisms which produce  $\beta$ -glucuronidase, as demonstrated by the production of a yellow colour and blue-white fluorescence (under long wavelength ultra-violet illumination) through the enzymatic cleavage of 4-methylumbelliferyl- $\beta$ -D-glucuronide in a defined substrate medium, are regarded as *E. coli*.

Coliform bacteria are considered to be members of genera or species within the Family Enterobacteriaceae, capable of growth at 37 °C, that possess  $\beta$ -galactosidase. This definition includes anaerogenic (ie non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia*, *Yersinia*, *Buttiauxella*, *Leclercia*.

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce  $\beta$ -glucuronidase. Strains which possess these characteristics at 37 °C but do not express them at 44 °C may also be *E. coli*. When

identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

#### **D4 Principle**

Organisms are grown in a defined liquid medium containing substrates for the specific detection of the enzymes  $\beta$ -galactosidase and  $\beta$ -glucuronidase. The dehydrated medium is dissolved in 100 ml of sample, or dilution of sample, which is then added to a 51-well reaction pouch. This is then sealed and incubated at 37 °C for up to 22 hours. If, within the pouch, some of the wells exhibit no growth in the medium after incubation, while other wells exhibit some growth, then the most probable number of organisms in 100 ml of sample can be estimated from appropriate probability tables, see table D1.

#### **D5 Limitations**

This method is suitable for most types of aqueous samples. Those with high turbidities, however, may mask or impede colour development. The presence of very high numbers of *Aeromonas* may result in false positive reactions.

#### **D6 Health and safety**

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

When ultra-violet lamps are used gloves and either goggles or a face shield suitable for use with appropriate ultra-violet-emitting sources should be worn.

#### **D7 Apparatus**

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(2)</sup> in this series. Principally, fan assisted incubators are required. An example of the methodology for this type of method is presented and is based upon a commercially available system. Some of the equipment listed is specific to this system and alternative systems may be available for which other equipment may be required. Other items include:

- D7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  per 100 ml of sample, or equivalent).
- D7.2 Incubator capable of maintaining a temperature of  $37.0 \pm 1.0$  °C.
- D7.3 Sterile 100 ml plastic bottles containing anti-foaming agent as supplied by the manufacturer of the test system or suitable equivalent.

D7.4 MPN reaction pouches as supplied by the manufacturer (for example, a 51-well system) and associated heat-sealing equipment.

D7.5 Ultra-violet long wavelength (365 - 366 nm) lamp, and viewer.

D7.6 Colour and fluorescence comparator as supplied by the manufacturer.

## **D8 Media and reagents**

Commercial formulations of these media and reagents are available. The performance of all media and reagents should be verified prior to their use. Variations in the preparation and storage of media should also be verified.

D8.1 *Colilert® 18 medium*<sup>(5)</sup>.

The medium is a commercially available formulation provided in sachets and is suitable for single samples. The medium is a chemically defined formulation with minimal nutrients and substrates for the specific detection of the enzymes  $\beta$ -galactosidase and  $\beta$ -glucuronidase.

## **D9 Analytical procedure**

D9.1 *Sample preparation*

The volume, or dilution, of samples should be chosen so that not all the wells show a positive response. For treated waters, 100 ml of sample will generally be appropriate, whilst for contaminated waters, appropriate dilutions should be prepared, and 100 ml of diluted sample used. When preparing dilutions use sterile distilled, deionised or similar grade water. Buffered solutions should not be used as they may adversely affect the performance.

D9.2 *Sample processing*

The sample, or appropriate dilution, (usually 100 ml) is decanted into a sterile bottle containing anti-foaming agent. Following the manufacturer's instructions, the contents of one sachet of medium is then aseptically added. After capping the bottle, the contents are gently agitated to ensure dissolution of the medium and then the bottle is left to stand, typically, for a few minutes to allow completion of dissolution and dispersal of any air bubbles. The contents of the bottle are then added to the MPN reaction pouch, which is then sealed in the apparatus provided by the manufacturer to produce a 51-well reaction pouch. Prolonged exposure of the inoculated reaction pouch to direct sunlight should be avoided as this may result in hydrolysis of the specific substrates causing false-positive reactions. The time between the inoculation of the reaction pouch and the beginning of the incubation stage should be as short as possible and no longer than 2 hours.

Sealed MPN reaction pouches are then incubated, 'well-side' down, at 37 °C for not less than 18 hours and not more than 22 hours.

### D9.3 *Reading of results*

After incubation, the pouch is examined and the number of wells that have a sufficient yellow colour, compared against the manufacturer's comparator, is recorded. The pouch is then re-examined under an ultra-violet long wavelength lamp and the number of wells, that produce a blue-white fluorescence of sufficient intensity compared against the manufacturer's comparator, is recorded. If the pouch is examined before the completion of 22 hours incubation and this examination reveals borderline responses, then it should be returned to the incubator for the remaining incubation period. After 22 hours incubation, the pouch is removed and re-examined as before.

### D9.4 *Confirmation tests*

This method is reported to be highly specific for coliform bacteria (yellow coloration in the wells) and *E. coli* (blue-white fluorescence in the wells). Hence, confirmation tests are not usually required. Should there be any doubt as to the type of organism and response detected, then wells showing a positive response should be sub-cultured and confirmatory tests undertaken.

## **D10 Calculations**

### D10.1 *Confirmed coliform bacteria and E. coli*

The MPN of coliform bacteria is determined by reference to appropriate probability tables, see for example table D1. This is derived from the number of wells showing a positive, yellow coloration. For example, if there are 31 wells showing a yellow coloration in the reaction pouch, then from table D1, the MPN of coliform bacteria is 48 per 100 ml of sample, or diluted sample, examined. Any dilution needs to be taken into account.

The MPN of *E. coli* is determined by reference to the same probability table. This is derived from the number of wells showing a positive blue-white fluorescence. For example, if there are 12 wells showing a blue-white fluorescing in the reaction pouch then, from table D1, the MPN of *E. coli* is 14 per 100 ml of sample, or diluted sample examined. Any dilution needs to be taken into account.

## **D11 Expression of results**

Confirmed coliform bacteria and *E. coli* counts are expressed as MPN counts per volume of sample. For drinking water, the volume is typically 100 ml.

## **D12 Quality assurance**

New batches of media should be tested with appropriate reference strains of target bacteria (for example, *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*). Pouches should be incubated for 18 - 22 hours at 37 °C. Further details are given elsewhere<sup>(2)</sup> in this series.

## D13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality 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*, in this series, Environment Agency.
3. Standing Committee of Analysts, Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
4. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
5. IDEXX Laboratories, Milton Court, Churchfield Road, Chalfont St Peter, Buckinghamshire, SL9 9EW.

**Table D1 MPN (and 95% confidence intervals) per 100 ml for a 51-well defined substrate medium reaction pouch**

Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits	Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits
0	0	0-4	26	36	25-54
1	1	0-6	27	38	26-57
2	2	1-7	28	41	28-60
3	3	1-9	29	43	30-63
4	4	2-11	30	45	32-66
5	5	2-12	31	48	33-69
6	6	3-14	32	50	35-73
7	8	4-16	33	53	38-76
8	9	5-17	34	56	40-80
9	10	5-19	35	59	42-84
10	11	6-21	36	62	45-89
11	12	7-22	37	66	47-94
12	14	8-24	38	70	50-99
13	15	9-26	39	74	53-105
14	16	10-28	40	78	56-111
15	18	11-29	41	83	60-118
16	19	12-31	42	89	64-126
17	21	13-33	43	95	68-135
18	22	14-35	44	101	73-146
19	24	15-37	45	109	79-159
20	25	17-39	46	118	85-175
21	27	18-42	47	130	93-195
22	29	19-44	48	145	102-224
23	31	20-46	49	165	115-272
24	32	22-49	50	201	136-388
25	34	23-51	51	>201	

## **E The detection of coliform bacteria and *Escherichia coli* by a presence-absence technique**

### **E1 Introduction**

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. They provide the most sensitive means for detecting faecal contamination, for assessing the effectiveness of water treatment and disinfection, and for monitoring water quality in distribution. The significance of *E. coli* and coliform bacteria in water treatment and supply are described elsewhere<sup>(1)</sup> in this series.

### **E2 Scope**

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution.

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

### **E3 Definitions**

In the context of the method, organisms which are oxidase-negative and produce acid from lactose within 48 hours at 37 °C in a chemically defined medium are regarded as coliform bacteria.

Coliform bacteria are considered to be members of genera or species within the Family Enterobacteriaceae, capable of growth at 37 °C, that possess  $\beta$ -galactosidase. This definition includes anaerogenic (ie non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia*, *Yersinia*, *Buttiauxella*, *Leclercia*.

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce  $\beta$ -glucuronidase. Strains which possess these characteristics at 37 °C but do not express them at 44 °C may also be *E. coli*. When identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

### **E4 Principle**

The presence-absence test is basically a simple modification of the multiple tube technique. The test incorporates a single volume of medium (usually 100 ml) instead of a series of tubes or bottles of different volumes. Several media have been evaluated<sup>(3)</sup> and procedures are based on the principle that coliform bacteria and *E. coli* should be absent in 100 ml of drinking water. A positive result, therefore, indicates that presumptive coliform bacteria or *E. coli* may be present.

## **E5 Limitations**

Presence-absence tests are not quantitative. They only provide an indication of the presence or absence of presumptive coliform bacteria or *E. coli*. Where a positive result or characteristic growth is recorded, an immediate operational response should be made on the assumption that any positive growth may contain *E. coli*. Operational decisions should, therefore, not be delayed until confirmation tests for coliform bacteria and *E. coli* have been completed.

## **E6 Health and safety**

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

Whilst the first stage of this test (ie the filling and incubation of bottles) can be conducted at non-laboratory sites, the method involves the growth of cultures of potentially pathogenic bacteria. Therefore, if used at such sites the tests should be conducted in suitably equipped facilities.

## **E7 Apparatus**

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

- E7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  per 100 ml of sample, or equivalent).
- E7.2 Incubators (or water baths) capable of maintaining temperatures of  $37.0 \pm 1.0$  °C and  $44.0 \pm 0.5$  °C.

## **E8 Media and reagents**

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified.

### E8.1 *Minerals modified glutamate medium*<sup>(5)</sup>

Lactose	20.0 g
L (+) Glutamic acid sodium salt	12.7 g
L (+) Arginine monohydrochloride	40 mg
L (-) Aspartic acid	48 mg
L (-) Cystine	40 mg
Sodium formate	500 mg
Dipotassium hydrogen phosphate	1.8 g
Ammonium chloride	5.0 g
Magnesium sulphate heptahydrate	200 mg
Calcium chloride dihydrate	20 mg
Iron(III) citrate	20 mg
Thiamine (Aneurin hydrochloride)	2 mg
Nicotinic acid	2 mg
Pantothenic acid	2 mg
Bromocresol purple (1 % m/v ethanolic solution)	2 ml
Distilled, deionised or similar grade water to	1 litre

This formulation enables double-strength medium to be prepared. This is conveniently prepared in quantities of 10 (or more) litres. If the medium is not to be distributed in tubes immediately, the lactose and thiamine should be omitted and added before dispensing.

Several of the ingredients are more conveniently added as separate solutions and these may be prepared as follows:

#### SOLUTION 1

L (+) Arginine monohydrochloride	400 mg
L (-) Aspartic acid	480 mg
Distilled, deionised or similar grade water	50 ml

Heat the ingredients in the water to dissolve.

#### SOLUTION 2

L (-) Cystine	400 mg
5M Sodium hydroxide	10 ml
Distilled, deionised or similar grade water	90 ml

Heat the ingredients in the water to dissolve.

#### SOLUTION 3

Nicotinic acid	20 mg
Pantothenic acid	20 mg
Distilled, deionised or similar grade water	5 ml

Dissolve the ingredients in the water without heating the solution.

#### SOLUTION 4

Iron(III) citrate	200 mg
Distilled, deionised or similar grade water	10 ml

Heat the ingredients in the water to dissolve.

#### SOLUTION 5

Calcium chloride dihydrate	5 g
Distilled, deionised or similar grade water	100 ml
Concentrated hydrochloric acid	0.1 ml

Dissolve the ingredients in the water without heating the solution and sterilise at 121°C for 20 minutes. Store as a stock solution.

#### SOLUTION 6

Thiamine	100 mg
Distilled, deionised or similar grade water	99 ml

Prepare a sterile 0.1 % m/v solution of thiamine in distilled, deionised or similar grade water. (This can be carried out by adding the contents of an ampoule of thiamine (100 mg) to 99 ml of sterile distilled water.)

The above solutions should be stored at temperatures between 2 - 8 °C and any remaining, unused solution should be discarded after 6 weeks.

To prepare 10 litres of double-strength medium, dissolve the appropriate quantities of L (+) glutamic acid sodium salt, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulphate heptahydrate in 9 litres of hot distilled water. Add the whole of solutions 1, 2, 3 and 4, and 4 ml of solution 5. Adjust the pH to  $6.9 \pm 0.2$  or higher if necessary, so that the final pH (when completely prepared and after sterilisation is  $6.7 \pm 0.2$ ). After adjustment of the pH, add 20 ml of a 1 % m/v ethanolic solution of bromocresol purple. Dilute to a final volume of 10 litres.

If the medium is not required for immediate use, dispense the mixed solution (medium without lactose and thiamine) into suitable containers in 500 ml volumes. Autoclave the solutions at 115 °C for 10 minutes and store for not more than one month in the dark at room temperature.

For use, add the necessary amounts of lactose and solution 6 (10 g and 1 ml respectively). Allow the added lactose to dissolve and distribute in 100 ml volumes into suitable bottles, usually over 200 ml capacity. Cap the containers and sterilise at 115 °C for 10 minutes.

The sterile media can be stored for up to one month at room temperature.

### E8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except the phenol red indicator solution, in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Add the indicator solution and distribute in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

### E8.3 *Tryptone water for the indole test*

The use of certain peptones which give satisfactory results in tests carried out at 37 °C may not be satisfactory for the indole test at 44 °C<sup>(6)</sup>. Care should, therefore, be taken in the appropriate selection of reagents.

Tryptone	20 g
Sodium chloride	5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is  $7.5 \pm 0.2$ . Distribute in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

### E8.4 *Kovacs' reagent for the indole test*<sup>(7)</sup>

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol (3-methylbutan-1-ol) (analytical grade reagent free from organic bases)	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol and slowly add the hydrochloric acid. Protect from light and store at temperatures between 2 - 8 °C. The reagent should be pale yellow or straw-coloured when freshly prepared. Some types of amyl alcohol are unsatisfactory and give a dark colour with the aldehyde.

### E8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution and maximum recovery diluent.

## **E9 Analytical procedure**

### *E9.1 Sample preparation*

A volume of sample is added directly to the test kit containing the volume of medium. Equal volumes of sample and medium are used.

### *E9.2 Sample processing*

A volume of sample (100 ml) is added to each test kit bottle containing 100 ml of medium. For convenience, the bottle can be marked at the 200 ml level. The sample can be poured directly into the test kit bottle from a sample bottle, or run from a sampling tap provided that care is taken to avoid contamination of the sample or medium. The addition of chlorine-neutralising agents is unnecessary.

The bottle is then incubated at 37 °C and examined after 18 - 24 hours. The production of acid (demonstrated by the production of a yellow coloration) should be regarded as a presumptive positive result for coliform bacteria. If a negative result is obtained (where no acid or yellow coloration is produced) after 18 - 24 hours, the bottle should be incubated for a further 24 hours before a final result is reported.

### *E9.3 Reading of results*

After incubation, examine the bottles for the production of acid (demonstrated by a yellow coloration) which indicates a positive result. Confirmation tests should then be carried out to establish the presence of coliform bacteria and *E. coli*.

### *E9.4 Confirmation tests*

Bottles showing positive results are subjected to confirmatory tests for coliform bacteria and *E. coli*.

#### *E9.4.1 Confirmation for coliform bacteria*

The confirmation procedure outlined is based upon demonstration of lactose fermentation as being indicative of the possession of the  $\beta$ -galactosidase enzyme. Alternative procedures based upon the direct detection of this enzyme, for example using the substrate, ortho-nitrophenyl- $\beta$ -D-galactopyranoside, may be more appropriate.

Sub-culture from each bottle showing a positive result to MA and NA. Incubate the plates of MA and NA at 37 °C for 18 - 24 hours. If a pure culture is obtained on NA then perform the oxidase test as described in section E9.4.3. If the isolate is oxidase-negative, then perform the test for lactose fermentation or possession of  $\beta$ -galactosidase. If there is any doubt about the purity of the culture then sub-culture at least one typical coliform colony from MA to NA, incubate at 37 °C for 18 - 24 hours and carry out the oxidase test. Typically, coliform bacteria produce pink to red,

mucoïd or non-mucoïd, colonies on MA, often with a halo of precipitation of bile salts.

For each isolate to be tested, sub-culture to lactose peptone water (LPW) and incubate at 37 °C. Examine for acid production after 24 hours. If the results are negative, re-examine after a further 24 hours. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests<sup>(8)</sup>. Commercial test kits may be used following appropriate performance verification at the laboratory.

Some species of *Bacillus* may grow in minerals modified glutamate medium producing yellow coloration. These can be readily recognised by colony characteristics on MA, and by Gram staining.

#### E9.4.2 Confirmation of *E. coli*

As well as conducting tests for confirmation of coliform bacteria, for each tube or bottle showing growth within the medium, sub-culture to MA and incubate at 44 °C for 18 - 24 hours. Inoculate typical coliform colonies into tubes of tryptone water (TW) and incubate at 44 °C for 24 hours. The presence of *E. coli* is demonstrated by the production of indole in TW. Tests for  $\beta$ -glucuronidase may assist in the early confirmation of *E. coli*<sup>(9, 10)</sup>. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

##### E9.4.2.1 Indole test

After incubation of the TW at 44 °C, add 0.2 - 0.3 ml of Kovacs' reagent. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer.

#### E9.4.3 Oxidase test

Some organisms that are found in water may conform to the definition of coliform bacteria in most respects, but are able to produce acid from lactose only at temperatures below 37°C. *Aeromonas* species, which occur naturally in water, possess optimum growth at temperatures between 30 - 35 °C but may produce acid from lactose at 37 °C. These organisms are of uncertain public health significance and are distinguishable from coliform bacteria by a positive oxidase reaction. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA.

Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA onto the prepared filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction.

Commercial test kits for oxidase testing are available and should be used in accordance with manufacturer's instructions and following appropriate performance verification at the laboratory.

On each occasion that oxidase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example, *E. coli*).

#### **E10 Calculations**

This test indicates the presence or absence of presumptive coliform bacteria and *E. coli*.

#### **E11 Expression of results**

Presumptive and confirmed coliform bacteria and *E. coli* are expressed as being either present or absent. For computerised laboratory information systems, a positive result should be reported as greater than or equal to 1/100 ml.

#### **E12 Quality assurance**

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example, *Pseudomonas aeruginosa*). Bottles should be incubated for 24 hours at 37 °C or 44 °C as appropriate. Further details are given elsewhere<sup>(2)</sup> in this series.

#### **E13 References**

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality 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*, in this series, Environment Agency.
3. Standing Committee of Analysts, An evaluation of presence-absence tests for coliform organisms and *Escherichia coli* 1996. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
4. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
5. A minerals modified glutamate medium for the enumeration of coliform organisms in water, by the Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies, *Journal of Hygiene*, 1969, **67**, 367-374.

6. The standardisation and selection of bile salt and peptone for culture media used in the bacteriological examination of water. *Proceedings of the Society for Water Treatment and Examination*, Burman, N.P., 1955, **4**, 10-26.
7. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. *Zeitschrift für Immunitätsforschung und experimentelle Therapie*, Kovacs, N., 1928, **55**, 311-315.
8. *Cowan and Steels' Manual for the Identification of Medical Bacteria*, 3rd edition. (Editors, Barrow G.I. & Feltham R.K.A.). London, Cambridge University Press, 1993.
9. Fluorogenic assay for immediate confirmation of *Escherichia coli*. *Applied and Environmental Microbiology*, Feng, P.C.S. & Hartman, P.A., 1982, **43**, 1320-1329.
10. Glycosidase profiles of members of the family Enterobacteriaceae. *Journal of Clinical Microbiology*, Kampfer, P., Rauhoff, O. & Dott, W., 1991, **29**, 2877-2879.

## **F The detection of *Escherichia coli* O157:H7 by selective enrichment and immuno-magnetic separation**

### **F1 Introduction**

The recovery of *Escherichia coli* O157:H7 (*E. coli* O157:H7) from environmental samples is often difficult because of the altered physiological state that bacteria sometimes develop in order to survive hostile environments. Infections involving *E. coli* O157:H7 have occasionally been implicated with contaminated water, but food-borne infections are more common. The significance of *E. coli* and other coliform bacteria in water treatment and supply are described elsewhere<sup>(1)</sup> in this series.

*E. coli* O157:H7 is a recognised cause of haemorrhagic colitis, an illness characterised by bloody diarrhoea and severe abdominal pain but little or no fever. It is also one of the causes of haemolytic uraemic syndrome. Outbreaks involving *E. coli* O157:H7 have been associated with the consumption of food and contaminated water, and person-to-person contact also occurs. Symptoms can persist for up to 7 days. Strains of *E. coli* O157:H7 produce a toxin which is similar to that produced by *Shigella dysenteriae* Type 1 which is cytotoxic to Vero cells in cell culture.

### **F2 Scope**

The method is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

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

### **F3 Definitions**

In the context of this method, *E. coli* O157:H7 are strains of *E. coli* which do not ferment sorbitol, produce colourless to pale orange colonies on cefixime tellurite sorbitol MacConkey agar, and which subsequently confirm by biochemical and serological tests.

### **F4 Principle**

Organisms are isolated by membrane filtration or entrapment with filter-aid, and then selective enrichment followed by immuno-magnetic separation (IMS) and inoculation onto a selective agar medium containing sorbitol as a fermentable carbohydrate and neutral red as an indicator of acidity. Isolation of colonies is followed by selection of typical non-sorbitol-fermenting colonies for identification by biochemical and serological tests.

## **F5 Limitations**

This method neither identifies atypical sorbitol-fermenting strains of *E. coli* O157 nor other serotypes of *E. coli* that produce verocytotoxins.

This 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. In these instances, the use of several membrane filters or filter aid may be more appropriate.

When low numbers of *E. coli* O157 are present, detection is improved when larger volumes of sample are examined. However, the presence of high numbers of competing organisms may inhibit the growth or detection of *E. coli* O157.

## **F6 Health and safety**

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

Strains of *E. coli* O157:H7 which produce verocytotoxin have been reclassified from “Hazard Group 2” to “Hazard Group 3”<sup>(4)</sup>. However, where samples are not expected to contain *E. coli* O157:H7, routine examination may be undertaken in “Hazard Group 2” containment facilities. Where substantial sub-culture work is required, this should be undertaken in “Hazard Group 3” containment facilities. In addition, those strains used as positive control strains should not produce verocytotoxin. Suitable strains are available commercially (for example, National Collection of Type Cultures 12900). Caution should be exercised in the disposal of contaminated materials, especially those containing *E. coli* O157:H7.

Disposable gloves and safety glasses should be worn throughout the IMS procedure.

## **F7 Apparatus**

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

- F7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O per 100 ml of sample, or equivalent).
- F7.2 Incubators (or water baths) capable of maintaining temperatures of 37.0 ± 1 °C and 42.0 ± 1 °C.

- F7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.
- F7.4 Sterile, membrane filters, for example, white, 47 mm diameter, cellulose-based, 0.45 µm nominal pore size.
- F7.5 Smooth-tipped forceps.
- F7.6 Vortex mixer.
- F7.7 Rotary sample mixer for IMS mixing (for example, Dynal) suitable for use with Eppendorf tubes or screw-capped tubes.
- F7.8 Magnetic particle concentrator (for example, Dynal MPC–m) suitable for use with Eppendorf tubes or screw-capped tubes.
- F7.9 Eppendorf tubes and tube opener or suitable screw-capped tubes.

## F8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified.

### F8.1 *Modified tryptone soya broth*<sup>(5)</sup>

Tryptone soya broth	30 g
Bile salts number 3	1.5 g
Dipotassium hydrogen phosphate	1.5 g
Novobiocin	20 mg
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to  $7.4 \pm 0.2$ . Dispense the resulting solution in 90 ml volumes into suitable 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  $7.4 \pm 0.2$ . The sterilised medium may be stored at room temperature in the dark, protected from dehydration, and used within one month.

### F8.2 *Buffered peptone water*<sup>(6)</sup>

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water. Dispense the resulting solution in 90 ml volumes into suitable screw-capped tubes or bottles 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  $7.2 \pm 0.2$ . Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and used within one month.

### F8.3 *Cefixime tellurite sorbitol MacConkey agar*<sup>(7)</sup>

Peptone	20.0 g
Sorbitol	10.0 g
Bile salts number 3	1.5 g
Sodium chloride	5.0 g
Neutral red	30.0 mg
Crystal violet	1 mg
Potassium tellurite	2.5 mg
Cefixime	0.05 mg
Agar	15.0 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except cefixime and potassium tellurite, in the water. To achieve this, it will be necessary to heat to boiling. Dispense in appropriate volumes into suitable screw-capped bottles 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  $7.1 \pm 0.2$ . Allow the medium to cool. This basal medium can be stored in the dark at room temperature, protected from dehydration, and used within one month. Prior to use melt the basal medium if taken from store. Allow the molten medium to cool to approximately 50 °C and add the following selective supplements which should be filter-sterilised.

- (i) Cefixime solution: Dissolve 500 mg of cefixime in 100 ml of ethanol. This may be stored at between 2 - 8 °C and used within one month. Add 1 ml of this solution to 100 ml of ethanol and add 1 ml of the resulting solution to 1 litre of the basal medium to give a final concentration of 0.05 mg/l.
- (ii) Potassium tellurite solution: Dissolve 25 mg of potassium tellurite in 10 ml of water. The filter-sterilised solution may be stored at approximately -20 °C and used within one month. Add 1 ml of this solution to 1 litre of the basal medium to give a final concentration of 2.5 mg/l.

Mix the complete medium thoroughly and pour into sterile Petri dishes and allow the agar to solidify. Petri dishes may be stored at between 2 - 8 °C, protected against dehydration, and used within one month. Dishes should be dried in a suitable oven at 45 - 50 °C for 30 minutes before use.

#### F8.4 *Modified phosphate buffered solution*

Sodium chloride	8 g
Potassium chloride	20 mg
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Polyoxyethylene-sorbitan monolaurate (for example, Tween 20)	0.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and check that the pH is  $7.4 \pm 0.2$ . Sterilise the resulting solution by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the solution should be checked to confirm a pH of  $7.4 \pm 0.2$ . Allow the solution to cool. This solution can be stored in the dark at room temperature and used within one month.

#### F8.5 *Filter-aid*<sup>(8)</sup>

Diatomaceous earth	1 g (approximately)
Distilled, deionised or similar grade water	15 ml

Weigh out appropriate amounts of filter-aid into suitable bottles and add the water. Sterilise by autoclaving at 121 °C for 15 minutes. Store in the dark at room temperature and use within 12 months.

#### F8.6 *Magnetic beads*

Para-magnetic beads coated with antibodies to *E. coli* O157 antigen<sup>(9)</sup> (for example, Dynabeads or equivalent).

#### F8.7 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA) and *E. coli* antisera and latex agglutination kits.

### **F9 Analytical procedure**

#### F9.1 *Sample preparation.*

If present in drinking water, *E. coli* O157 are likely to be found in low numbers. Hence, a sample volume of at least 1000 ml should be examined. Smaller volumes may be more appropriate for polluted source waters.

#### F9.2 *Sample processing*

The sample is filtered using either a membrane filter or with filter aid and any residue added to medium. After incubation, a portion of the medium is then used for the detection of the organism.

### F9.2.1 *Membrane filtration*

Filter an appropriate volume of sample, or diluted sample. If the sample is turbid, several membrane filters may be required.

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. 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 so that as little air as possible is drawn through the membrane filter.

Remove the funnel and transfer the membrane filter carefully to 90 ml of modified tryptone soya broth or buffered peptone water. Whereas modified tryptone soya broth is suitable for polluted waters, buffered peptone water may be more appropriate for the recovery of stressed *E. coli* O157 from drinking waters and relatively unpolluted waters<sup>(10, 11)</sup>.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, 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 re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling distilled, deionised or similar grade water for at least one minute. 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. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

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.

### F9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of a membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (15 ml) is added to the filter funnel and filtered to form an initial layer on the absorbent pad. The contents of a second aliquot of filter aid are then mixed with the sample, which is then filtered. For heavily polluted waters, additional aliquots of filter-aid may be required. When filtration is complete, remove the funnel carefully

and transfer the absorbent pad and filter-aid to modified tryptone soya broth. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 90 ml.

### F9.2.3 *Enrichment, immuno-magnetic separation and sub-culture to selective agar*

Thoroughly mix the modified tryptone soya broth or buffered peptone water from sections F9.2.1 or F9.2.2. Incubate the modified tryptone soya broth at 42 °C for 24 hours and the buffered peptone water at 37 °C for 24 hours<sup>(10, 11)</sup>. Enrichment broths should be subjected to IMS, firstly, after incubation for 6 - 7 hours, and then again at 24 hours.

Thoroughly mix the antibody-coated para-magnetic beads and transfer 20 µl of the suspension to a 1.5 ml Eppendorf tube, or suitable screw-capped tube. Add 1 ml of the thoroughly mixed incubated enrichment broth to the tube and mix again, gently, by inversion. Ensure that no air bubbles are trapped at the bottom of the tube. Place the tube onto a rotating mixer set at 30 revolutions per minute and gently mix for approximately 30 minutes. After mixing, place the tube into the magnetic particle concentrator with the associated magnetic strip in position. To concentrate the beads into a small pellet onto the side of the tube, gently invert the magnetic particle concentrator repeatedly for about 1 minute. With the magnetic strip in position, carefully open the tube and aspirate the liquid from the tube and any remaining liquid that might be inside the cap. Remove the magnetic strip from the magnetic particle concentrator and add 1 ml of modified phosphate buffered solution (F8.4) to the tube. Close the cap and gently invert to re-suspend the beads. Re-position the magnetic strip in the magnetic particle concentrator and concentrate the beads into a small pellet as before. Repeat the rinsing step with more modified phosphate buffered solution (F8.4). Re-suspend the beads in 50 µl of modified phosphate buffered solution (F8.4) and inoculate the beads onto cefixime tellurite sorbitol MacConkey agar, following manufacturer's instructions where provided, and incubate at 37 °C for 24 hours.

### F9.3 *Reading of results*

After incubation, examine the cefixime tellurite sorbitol MacConkey agar Petri dishes for typical non-sorbitol-fermenting colonies that are smooth and circular, 1 - 3 mm in diameter and colourless to pale orange in colour, usually with dark centres. Strains of *E. coli* which ferment sorbitol are pink in colour.

### F9.4 *Confirmation tests*

Inoculate typical colonies onto NA (and MA if isolate purity needs to be checked) and incubate at 37 °C for 24 hours. Isolates can then be subjected to serological identification using commercially available antisera or latex agglutination kits. Examine the slides for evidence of agglutination and carry out the tests with appropriate positive (non-verocytotoxin-producing strain of *E. coli* O157) and negative (non-O157 strain of *E. coli*) controls. Some isolates may require further identification by biochemical testing as some non-sorbitol-fermenting coliform bacteria (for example, *E. hermannii*) can cross react in the latex agglutination test. While chromogenic media can be used to demonstrate the lack of β-glucuronidase,

some strains of *E. coli* O157:H7 may produce atypical biochemical profiles and results should be interpreted with caution.

#### **F10 Calculations**

The test indicates the presence or absence of *E. coli* O157.

#### **F11 Expression of results**

*E. coli* O157 are reported as being detected or not detected in the volume of sample examined.

#### **F12 Quality assurance**

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli* O157) and non-target bacteria (for example, other *E. coli*). Further details are given elsewhere<sup>(2)</sup> in this series.

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1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
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5. Optimisation of methods for the isolation of *Escherichia coli* O157 from beefburgers, *PHLS Microbiology Digest*, Bolton, E. J., Crozier, L. & Williamson, J. K., 1995, **12**, 67-70.
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8. Concentration technique for demonstrating small amounts of bacteria in tap water. *Acta Pathologica et Microbiologica Scandinavica*, Hammarstrom, E. & Ljutov, V., 1954, **35**, 365 – 369.
9. Immuno-magnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food samples. *Epidemiology and Infection*, Wright, D. J., Chapman, P. A. & Siddons, C. A., 1994, **113**, 31-40.
10. Detection of toxin producing strains of *E. coli*, Report to the Department of the Environment. London, Drinking Water Inspectorate, DWI0674, 1996
11. Growth of starved *Escherichia coli* O157 cells in selective and non-selective media. *Microbiology and Immunology*, Sata, S., Osawa, R., Asai, Y. & Yamai, S., 1999, **43**, 217-227.

### **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.

Secretary  
Standing Committee of Analysts  
Environment Agency  
Wheatcroft Office Park  
Landmere Lane, Edwalton  
Nottingham  
NG12 4DG

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