ENVIRONMENT AGENCY

The Microbiology of Sewage Sludge (2003) - Part 3 -Methods for the isolation and enumeration of *Escherichia coli*, including verocytotoxigenic *Escherichia coli*

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Methods for the Examination of Waters and Associated Materials

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This booklet contains four methods for the isolation and enumeration of *Escherichia coli* (including verocytotoxigenic *E. coli*).

A The isolation and enumeration of *Escherichia coli* by a chromogenic membrane filtration technique.
B The isolation and enumeration of *Escherichia coli* by a multiple tube most probable number technique.
C The enumeration of *Escherichia coli* by a defined substrate most probable number technique.
D The enumeration of verocytotoxigenic *E. coli*, including *Escherichia coli* O157 by membrane filtration with a chromogenic detection medium.

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

Part 1 - An overview of the treatment and use in agriculture of sewage sludge in relation to its impact on the environment and public health
Part 2 - Practices and procedures for sampling and sample preparation
Part 4 - Methods for the detection, isolation and enumeration of S*almonellae*

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 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, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

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

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

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

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the 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 and details on how to obtain copies are available from the Agency's internet web-page (www.environment-agency.gov.uk/nls) or 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

March 2003

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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 isolation and enumeration of *Escherichia coli* by a chromogenic membrane filtration technique

A1 Introduction

Sewage sludge may contain pathogenic micro-organisms such as *Salmonella* species and *Escherichia coli* O157 originating from the intestinal tracts of humans and animals. Hence, there is a risk, albeit small, that the use of such sludge in agriculture may cause outbreaks of disease due to the transmission of these organisms through the food chain. For these reasons, there is a need to monitor the efficacy of sludge treatment processes and storage practices to control the levels of pathogens in sludge and its application to agricultural land.

Escherichia coli (*E. coli*) are Gram-negative bacteria, present in large numbers in sewage, originating from faecal material. Tests for *E. coli* are an important routine microbiological examination carried out on sewage sludge. Enumeration of *E. coli* in sludge (before and after treatment) provides a sensitive means of assessing the pathogen removal in sludge in preparation for its use on agricultural land and to demonstrate compliance with regulatory standards. The significance of *E. coli* is described in more detail elsewhere[1] in this series.

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A2 Scope

The method is suitable for the examination of untreated and conventionally treated sludges, including samples of crude, primary settled, lagoon-stored, thickened, caked and mesophilic anaerobic digested sludges where limited microbial reduction is expected. Depending on the sludge matrix, different preparative techniques may be required prior to using this method. These techniques are described elsewhere[2] in this series.

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

A3 Definitions

In the context of this method, organisms which produce acid from lactose, and produce β-glucuronidase forming green colonies on membrane filters after incubation for 4 hours at 30 °C followed by 14 hours at 44 °C are regarded as *E. coli* bacteria.

For the purposes of the examination of water and associated materials, *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 β-glucuronidase.

A4 Principle

A sample of sludge is homogenised, serially diluted with maximum recovery diluent and filtered through a membrane filter. The membrane filter is placed on an agar medium and *E. coli* are enumerated on the filter after incubation for 4 hours at 30 °C followed by 14 hours at 44 °C. The agar medium contains lactose, phenol red (as an indicator of acidity) and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) either as the cyclohexylammonium salt or the sodium salt, which when hydrolysed, indicates the presence of β-glucuronidase. Colonies that are β-glucuronidase-positive and ferment lactose are regarded as *E. coli*. No further confirmation should be required. If necessary, confirmation tests demonstrating the production of acid from

lactose, the formation of indole from tryptophan at 44 °C and an oxidase-negative reaction may be carried out.

A5 Limitations

Enumeration of colonies by this method will exclude a proportion of strains of *E. coli* that are unable to grow at 44 °C, or that fail to ferment lactose. A small number of strains of *E. coli* do not express β-glucuronidase activity on primary isolation or are β-glucuronidase-negative.

This method is not suitable for sludge samples that have been lime-treated or where enhanced microbial reduction is expected. These samples should be examined using an appropriate multiple tube most probable number (MPN) technique. Sludges with high solids content (greater than 20 % m/v) tend to block the membrane filter at minimal dilutions, or may mask or inhibit the growth of the target organisms. This will limit the level at which *E. coli* will be detected and enumerated. The maximum number of colonies that should be counted on a single membrane filter is approximately 100.

A6 Health and safety

Sewage and sewage sludge samples can contain hazardous and flammable substances. They may also contain pathogenic organisms and are liable to undergo biological action. Consequently, these samples should be handled with care. Gases that can be produced by microbiological activity are potentially flammable and once generated within the sample container will cause the container to become pressurised. Infectious material and/or pathogenic aerosols will, therefore, be of concern and may be potentially hazardous if containers explode. Glass bottles should not be used, wherever possible[4].

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[5] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3]. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, either static temperature or temperature cycling) are required. Other items include:

A7.1 Sterile sample containers of appropriate volume, made of suitable material.

A7.2 Incubators capable of maintaining temperatures of 30.0 ± 1.0 °C and 44.0 ± 0.5 °C, or cycling 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.

A7.5 Smooth-tipped forceps.

A7.6 Vortex mixer.

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A7.7 Stomacher.

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.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

A8.1 *Membrane lactose glucuronide agar*[6, 7]

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
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (cyclohexylammonium or sodium salt)	0.2 g
Distilled, deionised or similar grade water	1 litre

Suspend the ingredients, except 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of a 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 suitable volumes into Petri dishes, and allow the medium 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 period may result in a deterioration of the performance of the medium. The pH of the medium after sterilisation should be 7.4 ± 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.

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A8.2 *Maximum recovery diluent*

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

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in appropriate volumes into 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 7.0 ± 0.2. The sterilised diluent should be stored at room temperature in the dark and used within one month.

A8.3 *Other media*

Standard and commercial formulations of other media and reagents that may be used, for example if confirmation is required, include oxidase reagent, lactose peptone water, tryptone water, Kovacs' reagent, nutrient agar (NA) and MacConkey agar (MA).

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. Prepare an initial 10-fold dilution of the original sample by weighing 10 g of sewage or sludge sample aseptically in an appropriate container. Add approximately 45 ml of maximum recovery diluent (MRD) and transfer to a sterile stomacher bag. Homogenise the suspension for 2 minutes at slow speed. Transfer the homogenised sample back to the container. Add sufficient MRD to the stomacher bag to bring the total volume of MRD to 90 ml. Rinse the stomacher bag and add the washings to the container. Vortex the suspension to ensure thorough mixing.

It is important that several serial dilutions are prepared and filtered so that the number of colonies on at least one of the membrane filters lies within the counting range of 20 - 80. Prepare appropriate dilutions of the homogenised initial sample dilution with MRD. It may be appropriate to include intermediate dilutions (for example, 5 ml of well-mixed dilution to 5 ml of MRD) as well as sequential or serial 10-fold (decimal) dilutions (i.e.1 ml of dilution added to 9 ml of MRD).

Treated sludges, or samples containing particulate material likely to puncture the stomacher bag during processing, may be homogenized by shaking in an orbital shaker at 200 ± 10 rpm for 1 hour.

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A9.2 *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. Pipette the required volume of diluted sample into the funnel. When the volume of diluted sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile MRD 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.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing well-dried medium (for example, Petri dishes containing membrane lactose glucuronide agar 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. Where several dilutions of the same sample are to be examined, the funnel may be re-used without being placed in a boiling water bath provided that the highest dilution of sample is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling 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, or is suspected of being, contaminated, or a membrane filter becomes damaged. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

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 a temperature of 30 °C for 4.0 ± 0.5 hours, then transferred to an incubator at 44 °C for at least 14 hours, but no more than 20 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 certain strains of *E. coli* may fail to grow at higher incubation temperatures.

A9.3 *Reading of results*

After the total incubation period of 18 - 24 hours, examine the membrane filters under good light, if necessary with a hand lens. Count all green colonies (however faint) irrespective of size within 15 minutes of being removed from the incubator, as the colouration of the colonies may change on cooling and standing. All green colonies are regarded as *E. coli*. It is important to note the relative number of yellow colonies (i.e. non-*E. coli*, coliform bacteria) and pink colonies (i.e. non-target organisms) present on the membrane filter, as these may interfere with the growth and detection of *E. coli*. In addition, any blue colonies (i.e. possibly lactose-negative *E. coli*) should be regarded as *E. coli*.

A9.4 *Confirmation tests*

The specificity of the membrane lactose glucuronide agar for *E. coli* is such that, following performance verification within the laboratory, confirmation of green colonies as *E. coli* should not be required. The combination of the selectivity of temperature and specificity of β-glucuronidase are sufficient for most practical purposes.

If confirmation is deemed necessary, procedures described elsewhere in this series[8] should be used. Depending on the intended purpose of the analysis in terms of the required accuracy, sub-culture a suitable number of green, and if necessary, yellow colonies (however faint). Occasionally, blue colonies may be observed and recorded. Blue colonies may be lactose-negative *E. coli* and should, therefore, be classed as *E. coli*.

If the aim of confirmation is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten colonies are present or, at least ten colonies should be sub-cultured if more than 10 colonies 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 morphological appearance are clearly distinguishable, a note of the number of each type should be made. The data and information from the sub-cultured isolates are then used to calculate the confirmed number of colonies of *E. coli*.

An understanding of the confirmation rate of yellow colonies is important for initial performance verification purposes, as some colonies may confirm as *E. coli* (for example, some strains do not express β-glucuronidase, and other strains appear negative when first isolated). The proportion of these may vary between the nature, type or source of the sludge. Rarely, green colonies may not confirm as *E. coli.*

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Colonies for confirmation tests should be sub-cultured as soon as practicable, preferably within 60 minutes, after removal of the Petri dishes from the incubator. After counting, Petri dishes may be stored in the incubator prior to sub-culturing.

A10 Calculations

A10.1 Count of *E. coli*

The number, N, of green and if present, blue colonies at a specific dilution is used to calculate the number of *E. coli* per g of sample (in terms of wet weight or dry solids, as required). The calculation takes into account the dilution used and the volume filtered. Where the result is expressed on a count per g of dried sludge, the percent dry solids content needs to be determined[9]. The following equations may be used:

a) For count, C_w, per g of original (wet) sludge

$$C_w = \frac{N \times b \times d}{a}$$

Where C_w is the number of *E. coli* in 1 g of the original (wet) sludge;
N is the number of green and blue colonies counted on the membrane filter;
a is volume of sample filtered through the membrane filter (typically, 1 ml);
b is initial dilution factor for the sludge in MRD (in this case, 10); and
d is the dilution factor for the serial dilutions in MRD.

b) For count, C_d, per g dry solids, i.e. count per g of dried sludge:

$$C_d = \frac{C_w \times 100}{e}$$

Where C_d is the number of *E. coli* in 1 g of the dried sludge;
C_w is the number of *E. coli* in 1 g of the original (wet) sludge; and
e is the percent dry solids content of the original (wet) sludge.

For example, if 10 g of original sludge is initially diluted 10-fold, and a serial dilution of 5 ml to 10 ml is made, i.e. a 2-fold dilution, and 1 ml of the final dilution taken for filtration, and 27 green colonies counted on the membrane filter, then

$$C_w = \frac{27 \times 10 \times 2}{1} = 540$$

If the percent dry solids content of the original (wet) sludge is 7.5 %, then

$$C_d = \frac{C_w \times 100}{7.5} = 7200$$

A10.2 *Confirmed E. coli*

Where confirmation is deemed necessary, the number of confirmed *E. coli* is calculated as the count of colonies (whether green, yellow or blue) regarded as presumptive *E. coli* multiplied by the proportion of the isolates that confirm before applying the calculations above. In this case,

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confirmed colonies are those that are lactose-positive in lactose peptone water at 44 °C, positive for the production of indole in tryptone water at 44 °C, and oxidase-negative.

A11 Expression of results

Counts of *E. coli* present in sludge are expressed as colony forming units per wet or dry weight of sample. Typically, results are reported as colony forming units per g of dried sludge.

A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli*) and non-target bacteria (for example, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). Petri dishes should be incubated for 24 hours at 37 °C, or 44 °C as appropriate. Further details are given elsewhere[3].

For larger batches of samples, or for monitoring of routine performance, it may be appropriate to examine one or more samples, in duplicate. A comparison of the counts obtained can then be undertaken.

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 Sewage Sludge (2003) - Part 2 - Practices and procedures for sampling and sample preparation, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

4. This text is based on Resolution 74 by CEN TC 292 - Wastes - Working Group 5, the agreed text of which was adopted by CEN TC 308 - Characterisation of sludges - for the section on "General Hazards" associated with sludge material and waste.

5. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

6. A medium detecting β-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Letters in Applied Microbiology*, D P Sartory and L Howard, 1992, **15**, pp273-276.

7. A Survey of *E.coli* in UK Sludges. UK Water Industry Research Limited, Report Reference No. 99/SL/06/3. 1999, pp11-15.

8. Standing Committee of Analysts, 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*, in this series, Environment Agency.

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9. Standing Committee of Analysts, The Conditionability, Filterability, Settleability and Solids Content of Sludges (1984) A Compendium of Methods and Tests, *Methods for the Examination of Waters and Associated Materials*, in this series, ISBN 0117517879.

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B The isolation and enumeration of *Escherichia coli* by a multiple tube most probable number technique

B1 Introduction

Sewage sludge may contain pathogenic micro-organisms such as *Salmonella* species and *Escherichia coli* O157 originating from the intestinal tracts of humans and animals. Hence, there is a risk, albeit small, that the use of such sludge in agriculture may cause outbreaks of disease due to the transmission of these organisms through the food chain. For these reasons, there is a need to monitor the efficacy of sludge treatment processes and storage practices to control the levels of pathogens in sludge and its application to agricultural land.

Escherichia coli (*E. coli*) are Gram-negative bacteria, present in large numbers in sewage, originating from faecal material. Tests for *E. coli* are an important routine microbiological examination carried out on sewage sludge. Enumeration of *E. coli* in sludge (before and after treatment) provides a sensitive means for assessing pathogen removal from sludge in preparation for its use on agricultural land and to demonstrate compliance with regulatory standards. The significance of *E. coli* is described in more detail elsewhere[1] in this series.

B2 Scope

The method is suitable for the examination of untreated, conventionally treated and enhanced treated sludges. Samples of conventionally treated sludge may include lagoon stored, thickened and mesophilic anaerobic digested sludges. The method is particularly suitable for the examination of enhanced treated sludges where treatment is designed to significantly reduce bacterial levels. Enhanced treated sludges may include sludge derived from treatment processes such as thermal drying, pasteurisation, thermophilic digestion and lime stabilisation. Depending on the sludge matrix, different preparative techniques may be required prior to using this method. These techniques are described elsewhere[2] in this series. For example, lime-treated sludge samples require neutralisation before proceeding with liquid enrichment.

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

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B3 Definitions

In the context of this method, organisms that, after selective enrichment at 36 °C, grow at 44 °C in brilliant green bile broth and are indole-positive in tryptone water at 44 °C, are regarded as *E. coli*.

For the purposes of the examination of water and associated materials, *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 β-glucuronidase.

B4 Principle

E. coli are grown in a liquid selective enrichment medium containing sodium lauryl sulphate and lactose with bromocresol purple as an indicator of acidity. Following incubation, confirmation tests are carried out to demonstrate growth at 44 °C in the presence of brilliant green and 2 % bile, and the formation of indole from tryptophan.

A sample of sludge (typically 10 g) is homogenised with maximum recovery diluent and various volumes (or dilutions) of the homogenate added to a series of tubes, or bottles, containing liquid enrichment broth. The neutralisation of lime sludge samples is carried out after the homogenisation stage, but before the various volumes of suspension are added to the tubes of enrichment broth. After incubation, some of the tubes, or bottles, should exhibit no characteristic growth in the medium and other tubes or bottles should show characteristic growth in the medium. This will depend on the volumes of homogenate added to the series of tubes. From the number of tubes exhibiting characteristic growth within the medium, confirmation that positive reactions are due to the presence of *E. coli*, is obtained by sub-culture to tubes containing confirmation media. From the number of tubes confirming, the most probable number of *E. coli* in the sludge (expressed on a wet or dry weight basis) can be estimated using appropriate probability tables, see Table B1.

B5 Limitations

Enumeration of colonies by this method will exclude a number of strains of *E. coli* that are unable to grow at 44 °C, or that fail to ferment lactose. In addition, strains that are indole-negative will not be confirmed as *E. coli*. False-positive results may be observed from thermotolerant indole-positive *Klebsiella* species.

The method is not suitable, in terms of the quantity of media and tubes required, for sludges where a relatively high number of organisms may be expected. A minimum period of 48 hours is required to obtain a confirmed result. In some cases, this may extend to 4 - 5 days.

B6 Health and safety

Sewage and sewage sludge samples can contain hazardous and flammable substances. They may also contain pathogenic organisms and are liable to undergo biological action. Consequently, these samples should be handled with care. Gases that can be produced by microbiological activity are potentially flammable and once generated within the sample container will cause the container to become pressurised. Infectious material and/or pathogenic aerosols will, therefore, be of concern and may be potentially hazardous if containers explode. Glass bottles should not be used, wherever possible[4].

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[5] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [3]. Principally, incubators with fan assisted air circulation and circulating water baths are required. Other items include:

B7.1 Suitable sample containers of appropriate volume, made of suitable material[2].

B7.2 Incubators (or water baths) capable of maintaining temperatures of 36.0 ± 1.0 °C and 44.0 ± 0.5 °C.

B7.3 Suitable sterile glass or disposable plastic bottles and/or test tubes with trays or racks.

B7.4 Vortex mixer.

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B7.5 Stomacher.

B7.6 Laboratory pH meter.

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.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

B8.1 *Lauryl tryptose broth*[6, 7] *with bromocresol purple*[8]

Double-strength medium:

Tryptose	40.0 g
Lactose	10.0 g
Sodium chloride	10.0 g
Dipotassium hydrogen phosphate	5.5 g
Potassium dihydrogen phosphate	5.5 g
Sodium lauryl sulphate (specially pure)	200 mg
Bromocresol purple (1 % m/v ethanolic solution)	1 ml
Distilled, deionised or similar grade water to	1 litre

Mix the ingredients in the water and distribute into suitable tubes, or bottles, in suitable volumes, typically, 10 ml. Cap the containers and sterilise at 115 °C for 10 minutes.

Single-strength medium can be prepared by diluting unsterilised double-strength medium with an equal volume of deionised, distilled or similar grade water. Distribute into suitable tubes, or bottles, in suitable volumes, typically, 10 ml. Sterilise at 115 °C for 10 minutes.

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

B8.2 *Brilliant green bile broth*

Peptone	10 g
Lactose	10 g
Ox bile (dehydrated)	20 g
Brilliant green (0.1 % m/v aqueous solution)	13 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the peptone in 500 ml of water. Dissolve the dehydrated ox bile in 200 ml of water (this solution should have a pH value of between 7.0 and 7.5) and add to the peptone solution. Add water to make a volume of 975 ml. Dissolve the lactose in this solution and adjust the pH to 7.4 ± 0.2. Add the brilliant green solution, mix well, and make to 1 litre with water.

Distribute the medium into test tubes, in suitable volumes, typically 5 - 10 ml. Autoclave at 115 °C for 10 minutes.

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The sterile medium 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 out at 36 °C may not be satisfactory for the indole test at 44 °C[9]. 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 ± 0.2. Distribute in 5 ml volumes into suitable containers. Cap the containers and autoclave at 115 °C for 10 minutes.

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

B8.4 *Kovacs' reagent for the indole test*[10]

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

Dissolve the aldehyde in the amyl alcohol and slowly add the 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.

B8.5 *Maximum recovery diluent*

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

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in appropriate volumes into 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 7.0 ± 0.2. The sterilised diluent should be stored at room temperature in the dark and used within one month.

B8.6 *Other media*

Standard and commercial formulations of other media and reagents used in this method include 2N hydrochloric acid, and if further confirmation of *E. coli* is required, oxidase reagent, nutrient agar (NA) and MacConkey agar (MA). A suitable for test for β-glucuronidase activity may be also be required.

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B9 Analytical procedure

B9.1 Sample *preparation*

The volumes, and dilutions, of samples should be chosen so that some of the tubes, or bottles, show growth within the medium and others do not. Prepare an initial 10-fold dilution of the original sample by weighing 10 g of sewage sludge sample aseptically in an appropriate container. Add approximately 45 ml of maximum recovery diluent (MRD) and transfer to a sterile stomacher bag. Homogenise the suspension for 2 minutes at slow speed. Transfer the homogenised sample back to the container. Add sufficient MRD to the stomacher bag to bring the total volume of MRD to 90 ml. Rinse the stomacher bag and add the washings to the container. Vortex the suspension to ensure thorough mixing. This suspension is labelled dilution "A".

With certain types of sludge, for example, thermally dried granules, it may be necessary to pre-treat the sample[(2)] by grinding or crushing, to break down large compacted particles, thus increasing the surface area of the sludge. The addition of a re-hydration step prior to stomaching may also be effective. In these cases, the sludge should be weighed directly into the stomacher bag, 90 ml of MRD added, and the suspension allowed to stand at room temperature for 30 – 60 minutes, prior to stomaching. After vortex mixing as above, this suspension is labelled dilution "A".

Enhanced treated sludges, or samples containing particulate material likely to puncture the stomacher bag during processing, may be homogenized by shaking in an orbital shaker at 200 ± 10 rpm for 1 hour.

For lime-treated samples, the pH of dilution "A" should be adjusted to pH 7.0 ± 0.2 by the addition of hydrochloric acid before proceeding. A laboratory pH meter may be used provided precautions are taken to minimise contamination, either from the meter or as a result of cross-contamination between samples. It may be useful to record the initial pH before addition of the hydrochloric acid. Before measuring the pH, dilution "A" should be thoroughly mixed between additions of hydrochloric acid, and sufficient time should be allowed between additions to ensure equilibration.

B9.2 *Volumes of sample for inoculation*

A series of different volumes of sample is inoculated into tubes, or bottles, of medium.

A volume (typically, 10 ml) of well-mixed dilution "A" is added to 90 ml of MRD. This suspension is well mixed for 5-9 seconds with a vortex mixer, and labelled dilution "B".

To a series of 5 tubes each containing 10 ml of double-strength lauryl tryptose broth with bromocresol purple, aseptically add 10 ml of dilution "A" to each tube. This constitutes the first series of MPN tubes and each tube contains the equivalent of 1 g of original (wet) sludge.

Inoculate a second series of tubes, comprising 5 tubes each containing 10 ml of single-strength lauryl tryptose broth with bromocresol purple, by adding 1 ml of dilution "A" to each tube. This constitutes the second series of MPN tubes and each tube contains the equivalent of 0.1 g of original (wet) sludge.

Inoculate a third series of tubes, comprising 5 tubes each containing 10 ml of single-strength lauryl tryptose broth with bromocresol purple, by adding 1 ml of dilution "B" to each tube. This constitutes the third series of MPN tubes and each tube contains the equivalent of 0.01 g of original (wet) sludge.

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Inoculate a fourth series of tubes, comprising 5 tubes each containing 10 ml of single-strength lauryl tryptose broth with bromocresol purple, by adding 0.1 ml of dilution "B" to each tube. This constitutes the fourth series of MPN tubes and each tube contains the equivalent of 0.001 g of original (wet) sludge.

Further series of tubes may be prepared, as described above, by making suitable additional dilutions of the sludge, if required.

Ideally, sufficient tubes should be prepared so that, after incubation, some of the tubes or bottles exhibit growth within the medium and some of the tubes exhibit no characteristic growth within the medium.

B9.3 *Sample processing*

After the tubes, or bottles, of medium have been inoculated with the appropriate volumes and dilutions of sample, each tube, or bottle, is capped or sealed and placed in an incubator at 36 °C. After 18 - 24 hours, the tubes are removed from the incubator and examined for acid production (as demonstrated by the presence of a yellow colouration). The number of tubes showing growth within the medium is recorded. After a further period of incubation of 24 ± 2 hours, the tubes are re-examined and results recorded. All the tubes that exhibit characteristic growth within the medium are regarded as presumptively positive for *E. coli* and are retained for confirmatory testing. Some tubes may exhibit growth within the medium (as demonstrated by the presence of turbidity) without a colour change. These tubes are regarded as negative, as are those tubes exhibiting no characteristic growth in the medium.

B9.4 *Confirmation tests for E. coli*

For each tube or bottle showing a positive reaction within the medium, sub-culture to brilliant green bile broth and incubate at 44 °C for 18 - 24 hours. In addition, for each tube or bottle showing a positive reaction within the medium, inoculate a tube of tryptone water (TW) and incubate at 44 °C for 21 ± 3 hours. After incubation, remove the TW from the incubator and carry out an indole test on the TW. The presence of *E. coli* is demonstrated by turbidity (growth) in brilliant green bile broth and the production of indole in TW.

Tests for β-glucuronidase may help in the early confirmation of *E. coli*[11, 12]. Suitable commercial test-kits may be used following appropriate performance verification within the laboratory.

B9.4.1 *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.

B9.5 *Reading of results*

The number of presumptive positive tubes of selective enrichment medium within each series of tubes is recorded (as demonstrated by characteristic growth within the medium and the production of a yellow colouration). Subsequently, the number of tubes confirmed as containing *E. coli* is recorded.

Three consecutive series of tubes are chosen whereby some of the tubes have been confirmed as containing *E. coli* and some have been shown to be negative for the presence of *E. coli*. From the results, the MPN of *E. coli* in the sample is determined from probability tables, see Table B1.

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B10 Calculations

The number of tubes containing lauryl tryptose broth with bromocresol purple showing a positive reaction that are confirmed as containing *E. coli* is counted and recorded. By reference to the appropriate probability tables, see Table B1, the MPN of *E. coli* present in 10 g of original (wet) sludge can be determined.

For example, if a 15-tube series comprising 5 x 1 g, 5 x 0.1 g and 5 x 0.01 g of original (wet) sludge yields *E. coli* in each consecutive series of tubes, and 3, 2 and 0 positive tubes are recorded respectively, and each of these tubes confirm, then from Table B1, the MPN of confirmed *E. coli*, C_w, is 13 per 10 g of original (wet) sludge. Further examples are given in Table B2.

To obtain the result, C_d, expressed on a dry weight basis[13], i.e. per g of dried sludge,

$$C_d = \frac{C_w \times 100}{10 \times e}$$

Where C_d is the MPN of *E. coli* per g of dried sludge;
C_w is the MPN of *E. coli* (from probability tables) in 10 g of the original (wet) sludge; and
e is the percent dry solids content of the original (wet) sludge.

For lime-treated sludges, and other sludges incorporating some form of pre-treatment, the quantity of lime (or other chemical used in the pre-treatment process) may make a significant contribution to the dry weight.

B11 Expression of results

The MPN of *E. coli* present in sludges may be expressed on a wet or dry weight basis. Typically, results are reported as MPN per g dried sludge.

B12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (*E. coli*) and non-target bacteria (for example, *Enterobacter aerogenes* or *Klebsiella pneumoniae*). Tubes or bottles should be incubated for 24 hours at 36 °C or 44 °C as appropriate. Further details are given elsewhere[3]. The pH meter used for neutralisation of lime-treated sludges should be calibrated using freshly prepared buffer solutions prior to use.

For larger batches of samples, or for monitoring of routine performance, it may be appropriate to examine one or more samples, in duplicate. A comparison of the counts obtained can then be undertaken.

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Appendix B1 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 10 g of original (wet) sludge. 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[14]. A procedure for estimating these confidence intervals for other dilution series has also been published[15]. 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[16]. The MPR in Table B1 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 the tubes of medium.

Table B1 shows data for a 15-tube series of 5 x 1 g, 5 x 0.1 g and 5 x 0.01 g of original (wet) sludges, but gives only those values of the more likely combinations of positive and negative reactions. For example, positive reactions in the 0.01 g tubes would not be expected if all of the tubes containing 1 g and 0.1 g 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[14, 17, 18]. For example, the organisms may not have been uniformly distributed throughout the sample, or toxic or inhibitory substances may have been present.

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Calculation of MPN

The number of positive reactions for each series of tubes is recorded and, from the relevant table, the MPN of organisms present in 10 g 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 B2.

(i) Use only three consecutive series 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 B2).

(iii) If less than three series of dilutions give positive results, begin with the series containing the largest volume of sample (see example (d) in Table B2).

(iv) If only one series of tubes gives a positive reaction, use this dilution and the one higher and one lower (see example (e) in Table B2).

Table B1 MPN and MPR per 10 g of original (wet) sludge for a 15-tube series containing 5 x 1 g, 5 x 0.1 g and 5 x 0.01 g of sample

Number of tubes giving a positive reaction			MPN per 10 g	MPR* per 10 g
5 x 1 g	5 x 0.1 g	5 x 0.01 g		
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 infinite. The true count is likely to exceed 1800.

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Table B2 Examples of the derivation of the MPN from the numbers of positive reactions in a series of dilutions*

Example in text	Wet weight of sample (g)					MPN per 10 g
	1	0.1	0.01	0.001	0.0001	
(a)	***5***	***3***	***2***	0		140
(b)	5	***5***	***3***	***2***	0	1400
(c)	5	***5***	***2***	***0***	0	500
(d)	***3***	***1***	***0***	0		9
(e)	***0***	***1***	***0***	0		2

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

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C The enumeration of *Escherichia coli* by a defined substrate most probable number technique

C1 Introduction

Sewage sludge may contain pathogenic micro-organisms such as *Salmonella* species and *E. coli* O157 originating from the intestinal tracts of humans and animals. Hence, there is a risk, albeit small, that the use of such sludge in agriculture may cause outbreaks of disease due to the transmission of these organisms through the food chain. For these reasons, there is a need to monitor the efficacy of sludge treatment processes and storage practices to control the levels of pathogens in sludge and its application to agricultural land.

Escherichia coli (*E. coli*) are Gram-negative bacteria, present in large numbers in sewage, originating from faecal material. Tests for *E. coli* are an important routine microbiological examination carried out on sewage sludge. Enumeration of *E. coli* in sludge provides a sensitive means for assessing pathogen removal from sludge in preparation for its use on agricultural land and to demonstrate compliance with regulatory standards. The significance of *E. coli* is described in more detail elsewhere[1] in this series.

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C2 Scope

This method, which is an example of the defined substrate techniques that are available, is suitable for the examination of untreated, conventionally treated and enhanced treated sludges. Samples of conventionally treated sludge may include lagoon stored, thickened and mesophilic anaerobic digested sludges. Enhanced treated sludges may include sludge derived from treatment processes such as pasteurised, thermophilic digestion and lime stabilisation. Depending on the sludge matrix, different preparative techniques may be required prior to using this method. These techniques are described elsewhere[2] in this series.

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

C3 Definitions

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

In the context of this method, organisms are regarded as *E. coli* if they produce the enzyme β-glucuronidase at 37.0 ± 1.0 °C. The enzyme β-glucuronidase is demonstrated by the production of a blue-white fluorescence (under long wavelength ultra-violet illumination) as a result of the enzymatic cleavage of 4-methyl-umbelliferyl-β-D-glucuronide (MUG).

For the purposes of the examination of water and associated materials, *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 β-glucuronidase.

C4 Principle

A sample of sludge is initially homogenised[2] and then serially diluted with distilled, deionised or similar grade water. After incubation in a defined liquid medium containing specific substrates for the

detection of the enzymes β-galactosidase and β-glucuronidase, the organisms are then enumerated. The dehydrated medium is dissolved in 99 ml of sterile deionised water to which 1 ml of the diluted sludge sample is added. The suspension is then added to a multiple well reaction pouch containing discrete wells of a defined volume. This pouch acts as a simplified multiple tube MPN system, where the number of wells in the reaction pouch exhibiting growth in the medium will be dependent on the number and distribution of organisms in the diluted sample. The reaction pouch is sealed and incubated at a temperature 37.0 ± 1.0 °C for a minimum period of 18 hours, and up to a maximum period of 22 hours. If, within the pouch, some but not all of the wells exhibit growth within the medium, then the most probable number of organisms in 1 ml of the diluted sample can be estimated from appropriate probability tables, an example of which is given in Table C1.

C5 Limitations

Enumeration by this method will exclude a small proportion of strains of *E. coli* that are unable to grow at 37 °C. A small proportion of strains of *E. coli* do not express β-glucuronidase activity on primary isolation or are β-glucuronidase-negative.

C6 Health and safety

Sewage and sewage sludge samples can contain hazardous and flammable substances. They may also contain pathogenic organisms and are liable to undergo biological action. Consequently, these samples should be handled with care. Gases that can be produced by microbiological activity are potentially flammable and once generated within the sample container will cause the container to become pressurised. Infectious material and/or pathogenic aerosols will, therefore, be of concern and may be potentially hazardous if containers explode. Glass bottles should not be used, wherever possible[4].

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[5] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

When ultra-violet lamps are utilized, eye protection appropriate to the ultra-violet source should be used.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [3]. An example of the methodology for this type of defined substrate most probable number technique is presented and is based upon a commercially available system. Some of the equipment listed is specific to this system. Alternative systems may be available for which other equipment may be required. Items include:

C7.1 Incubator capable of maintaining a temperature of 37.0 ± 1.0 °C.

C7.2 Sterile 100 ml plastic bottles containing proprietary anti-foaming agent, as supplied by the manufacturer of the test system, or suitable equivalent.

C7.3 MPN reaction pouches as supplied by the manufacturer (for example a 51-well system providing a countable range of up to 200 organisms, or a 97-well system providing a countable range of up to 2419 organisms) and associated heat-sealing equipment.

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C7.4 Ultra-violet long wavelength (365 - 366 nm) lamp, and viewer.

C7.5 Colour and fluorescence comparator as supplied by the manufacturer.

C8 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.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

C8.1 *Colilert® 18 medium*[6]

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

C8.2 *Maximum recovery diluent*

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

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in appropriate volumes into 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 7.0 ± 0.2. The sterilised diluent should be stored at room temperature in the dark and used within one month.

C8.3 *Other media*

Standard and commercial formulations of other media and reagents used in this method include 2N hydrochloric acid, nutrient agar (NA) and oxidase reagent.

C9 Analytical procedure

C9.1 *Sample preparation*

The volume and dilutions of samples should be chosen so that the media in some of the wells exhibit growth and others do not. Prepare an initial 10-fold dilution of the original sample by weighing 10 g of sewage or sludge aseptically in an appropriate container. Add 90 ml of maximum recovery diluent (MRD) and homogenise the diluted sample using an appropriate technique based on the characteristics of the matrix[2].

After homogenising the diluted (MRD) sample, lime-treated samples need to be adjusted to a pH value of 7.0 ± 0.2 by the addition of hydrochloric acid.

Conventionally treated sludges may be homogenized by stomaching. Transfer the diluted sample into a sterile stomacher bag and homogenise by stomaching for 2 minutes at slow speed. Transfer the homogenised sample back to the original container. Vortex the suspension to ensure thorough mixing.

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Enhanced treated sludges, or samples containing particulate material likely to puncture the stomacher bag during processing, may be homogenised by shaking in an orbital shaker at 200 ± 10 rpm for 1 hour.

Several serial dilutions should be prepared from the homogenised sample to ensure some wells exhibit growth within the medium and some wells do not. Serial dilutions, typically about 100-fold, of the homogenised sample are prepared in sterile distilled, deionised or similar grade water.

C9.2 *Sample processing*

An appropriate dilution of the sample is taken and 1 ml of diluted sample is added to a sterile bottle containing anti-foaming agent and 99 ml of sterile distilled, deionised or similar grade water. Buffered solutions should not be used as adverse reactions may occur. Following the manufacturer's instructions, the contents of one sachet of medium are then aseptically added to the bottle. After capping the bottle, the contents are gently agitated to ensure dissolution of the medium. The bottle is then allowed to stand, typically, for a few minutes, to enable dispersion of any air bubbles. The contents of the bottle are then added to the MPN reaction pouch, which is then sealed. Exposure of the inoculated reaction pouch to direct sunlight should be avoided as this may result in hydrolysis of the specific substrates, thus, causing false-positive reactions to occur. The time between the inoculation of the reaction pouch and the beginning of the incubation stage should be as short as possible and no greater than 2 hours. Sealed MPN reaction pouches are then incubated at a temperature of 37.0 ± 1.0 °C for not less than 18 hours and not more than 22 hours.

C9.3 *Reading of results*

After incubation, the reaction pouch is examined and the number of wells that exhibit a blue-white fluorescence under ultra-violet light (365 nm) is recorded. The degree of fluorescence exhibited by samples is compared with manufacturer's scales to ensure correct interpretation of results. In certain cases, it may be relevant to note the number of wells exhibiting a yellow colour due to the enzymatic cleavage of ortho-nitrophenyl-β-D-galactopyranoside (ONPG) indicating the presence β-galactosidase

C9.4 *Confirmation tests*

This method is reported to be highly specific for *E. coli* as demonstrated by the production of a blue-white fluorescence, as a result of the enzymatic cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUG) indicating the presence β-glucuronidase. Hence, confirmation tests are not usually required. Should there be any doubt as to the type of response detected, then medium from those wells should be sub-cultured and confirmatory tests undertaken. If confirmation is required, procedures are described elsewhere in this series[7]. Depending on the intended purpose of the analysis in terms of the required accuracy, sub-culture the medium from a suitable number of wells exhibiting blue-white fluorescence and if necessary, yellow colouration. The medium should be sub-cultured as soon as practicable, within 60 minutes after removal of the reaction pouch from the incubator. After counting, the reaction pouch may be stored in a refrigerator if necessary.

C10 Calculations

The number of wells showing a positive reaction, (i.e. showing blue-white fluorescence, with or without yellow colouration within the medium) is counted and recorded. The MPN of *E. coli* is determined by reference to appropriate probability tables, see Table C1. For example, with the 51-

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well MPN reaction pouch system, if there are 15 wells showing blue-white fluorescence, then from probability tables, the MPN of *E.coli* is determined as 18 per 100 ml of solution examined.

The MPN of *E. coli* in the sludge, expressed on a wet or dry weight basis, can then be calculated. The calculation takes into account the volume and dilutions used and, for the number of *E. coli* per g of dry solids, the percent dry solids content[8]. The following equations may be used:

a) For the MPN, C_w, per g of original (wet) sludge

$$C_w = \frac{N \times b \times d}{a}$$

Where C_w is the number of *E. coli* in 1 g of the original (wet) sludge;
N is the MPN of *E. coli* obtained from probability tables (per 100 ml examined);
a is volume of diluted sample (typically, 1 ml);
b is initial dilution factor for the sludge in MRD (in this case, 10); and
d is the dilution factor for the serial dilutions in deionised or distilled water.

b) For the MPN, C_d, per g of dry solids, i.e. per g of dried sludge

$$C_d = \frac{C_w \times 100}{e}$$

Where C_d is the MPN of *E. coli* in 1 g of the dried sludge;
C_w is the MPN of *E. coli* in 1 g of the original (wet) sludge; and
e is the percent dry solids content of the original (wet) sludge.

For example, if 10 g of original (wet) sludge is initially diluted 10-fold, and a serial dilution of 5 ml to 10 ml, i.e. a 2-fold dilution is made, and 1 ml of the final dilution taken for addition to the medium, and 15 wells counted showing blue-white fluorescence, then

$$C_w = \frac{18 \times 10 \times 2}{1} = 360$$

If the percent dry solids content of the original (wet) sludge is 7.5 %, then

$$C_d = \frac{C_w \times 100}{7.5} = 4800$$

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C11 Expression of results

The MPN of *E. coli* present in sludge is expressed as the number of *E. coli* on a wet weight or dry weight basis. Typically, results are reported as the MPN of *E. coli* per g of dried sludge.

C12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli*) and non-target bacteria (for example, *Klebsiella pneumoniae*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*). Further details are given elsewhere[3].

For larger batches of samples, or for monitoring of routine performance, it may be appropriate to examine one or more samples, in duplicate. A comparison of the numbers obtained can then be undertaken.

C13 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 Sewage Sludge (2003) - Part 2 - Practices and procedures for sampling and sample preparation, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

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

4. This text is based on Resolution 74 by CEN TC 292 - Wastes - Working Group 5, the agreed text of which was adopted by CEN TC 308 - Characterisation of sludges - for the section on "General Hazards" associated with sludge material and waste.

5. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

6. IDEXX Laboratories, Milton Court, Churchfield Road, Chalfont St Peter, Buckinghamshire, SL9 9EW

7. Standing Committee of Analysts, 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*, in this series, Environment Agency.

8. Standing Committee of Analysts, The Conditionability, Filterability, Settleabliity and Solids Content of Sludges (1984) A Compendium of Methods and Tests, *Methods for the Examination of Waters and Associated Materials*, in this series, ISBN 0117517879.

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Table C1 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	

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D The enumeration of verocytotoxigenic *Escherichia coli*, including *Escherichia coli* O157 by membrane filtration with a chromogenic detection medium

Details of this method are included for information purposes only, as an example of the defined substrate techniques that are available. Information on the routine use of this method would be welcomed to access its capabilities.

D1 Introduction

Verocytotoxigenic *Escherichia coli*, including *E. coli* O157, are pathogenic strains of *E. coli* causing illness in humans and animals. When present in sewage sludge used for agriculture purposes, they present a risk, albeit a small one, of causing illnesses via transmission through the food chain. The significance of *E. coli* is described elsewhere[1] in this series.

Verocytotoxigenic *E. coli* (VT *E. coli*), in particular the serotype *E. coli* O157:H7 are recognised causes of haemorrhagic colitis, an illness characterised by symptoms of bloody diarrhoea with severe abdominal pain. Additionally, certain strains of *E. coli* O157:H7 may cause haemolytic uraemic syndrome, which is characterised by anaemia with renal failure, which can be fatal. VT *E. coli* produce toxins called verotoxins (namely, VT1 and VT2) or toxins similar to those produced by *Shigella dysenteriae*, and Shigella-like toxins (SLT-I and STL-II) which are cytotoxic to vero cells in cell culture. VT *E. coli* do not produce the enzyme β-glucuronidase, an enzyme often used as an identification parameter for non-pathogenic strains of *E. coli*.

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D2 Scope

This method is suitable for the examination of untreated, conventionally treated and enhanced treated sludges. Samples of conventionally treated sludge may include lagoon stored, thickened and mesophilic anaerobic digested sludges. Enhanced treated sludges may include sludges derived from treatment processes such as pasteurisation, thermophilic digestion, lime-stabilisation and composting. Depending on the sludge matrix, different preparative techniques may be required prior to using this method. These techniques are described elsewhere[2] in this series.

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

D3 Definitions

In the context of this method VT *E. coli* are strains of *E. coli* that exhibit growth on a chromogenic medium at 36 ± 1 °C, following a resuscitation stage using modified tryptone soya broth, and do not produce the enzyme β-glucuronidase. These are regarded as presumptive VT *E. coli.* They are characterized by the production of pink colonies due to their inability to metabolise the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG), in the presence of the selective supplements cefixime and tellurite.

D4 Principle

A sample of sludge is initially homogenised[2] and then serially diluted. The diluted sludge is filtered through a membrane filter and incubated at a temperature of 36 ± 1 °C on a sterile glass fibre filter saturated with resuscitation medium comprising modified tryptone soya broth. After incubating for 6 - 18 hours, the membrane filter is further incubated at 36 ± 1 °C on chromogenic medium (for example, CHROMagar O157 or equivalent) for 24 hours. The membrane filters are

then examined and positive colonies enumerated. The presence of glucuronidase-negative *E. coli* is indicated by the presence of pink colonies. Glucuronidase-positive colonies are identified by the presence of blue colonies[4].

D5 Limitations

Sludge with high solids content (greater than 20 % m/v) tend to block the membrane filter at minimal dilutions or may mask or inhibit the growth of target organisms. This will limit the level at which *E. coli* can be detected and enumerated. The growth of high numbers of non-target bacteria on the membrane may inhibit or obscure the growth of VT *E. coli*.

There are some indications that the combination of bile salts and anti-microbial supplements in some resuscitation media[5] may inhibit the growth of injured *E. coli*.

D6 Health and safety

Sewage and sewage sludge samples can contain hazardous and flammable substances. They may also contain pathogenic organisms and are liable to undergo biological action. Consequently, these samples should be handled with care. Gases that can be produced by microbiological activity are potentially flammable and once generated within the sample container will cause the container to become pressurised. Infectious material and/or pathogenic aerosols will, therefore, be of concern and may be potentially hazardous if containers explode. Glass bottles should not be uswed, wherever possible[6].

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[7] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

Strains of *E. coli* O157:H7 that produce verotoxins are classified as "Hazard Group 3"[8]. However, where samples are not expected to contain these organisms, 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 verotoxins and suitable strains are available commercially[9]. Caution should be exercised in the disposal of contaminated materials, especially those containing *E. coli* O157:H7.

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D7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Items include:

D7.1 Incubator capable of maintaining a temperature of 36.0 ± 1.0 °C.

D7.2 Stomacher and stomacher bags with or without integral mesh filter.

D7.3 Centrifuge capable of maintaining 200 - 300 g for 1 minute.

D7.4 Sterilsed membrane filters, for example 47 mm diameter, cellulose nitrate, 0.45 μm nominal pore size.

D7.5 Course glass fibre filters, for example 47 mm diameter, 2.7 µm nominal pore size, or equivalent.

D7.6 0.2 µm sterile syringe filter.

D7.7 5 ml syringe.

D7.8 Sterilised filter-housing units and traps.

D7.9 Plate microscope.

D7.10 Blue-daylight filter.

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.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

D8.1 *CHROMagar® O157*[10]

CHROMagar 0157 agar	29 g
Distilled, deionised or similar grade water to	1000 ml

Diperse the agar in the water and heat to 100 °C, stirring until fully dissolved. Cool the solution to 45 °C prior to distributing in suitable volumes into Petri dishes. Store the Petri dishes in the dark at a temperature between 2 - 8 °C for no longer than 48 hours.

D8.2 *Modified tryptone soya broth*

Tryptone soya broth base	17 g
Tryptose	3 g
Dextrose	2.5 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.5 g
Bile salts No 3	1.5 g
Dipotassium hydrogen phosphate	1.5 g
Distilled, deionised or similar grade water to	1000 ml

Dissolve the ingredients in the water and adjust the pH of the solution to 7.4 ± 0.2. Dispense the solution in suitable volumes into suitable containers, and cap. Sterilise the solution by autoclaving at 121 °C for 15 minutes. Store the containers a temperature between 2 - 8 °C, until required.

D8.3 *Novobiocin solution*

Novobiocin (sodium salt)	1 g
Deionized, deionised or similar grade water	10 ml

Dissolve the novobiocin in the water. Filter-sterilise through an 0.2 µm filter. Store the sterile solution at a temperature between 2 - 8 °C in the dark, and use within 4 weeks.

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Additions of sterile novobiocin solution (to a final concentration of 40 mg l^{-1}) should be carried out immediately prior to use.

D8.4 *Phosphate buffered saline*

Sodium chloride	8 g
Disodium hydrogen phosphate	1.44g
Potassium dihydrogen phosphate	0.24g
Potassium chloride	0.2g
Distilled, deionised or similar grade water to	1000 ml

Dissolve the ingredients in the water and adjust the pH of the solution to 7.4 ± 0.2. Dispense the solution in suitable volumes into suitable containers, and cap. Sterilise the solution by autoclaving at 121 °C for 15 minutes. Store between 2 - 8 °C, until required.

D8.5 O*ther media*

Standard and commercial formulations of other media and reagents used in this method include 2N hydrochloric acid.

D9 Analytical procedure

D9.1 *Sample preparation*

Add 225 ml of modified tryptone soya broth (supplemented just before use with novobiocin to a final concentration of 40 μg ml^{-1}) to 25 g of sewage sludge in a suitable container. Mix thoroughly. Alternatively, add 225 ml of phosphate buffered saline to 25 g of sewage sludge in a suitable container. Mix thoroughly.

Homogenise the sample using an appropriate technique based on the characteristics of the matrix[(2)].

After homogenising the diluted sample, lime-stabilised samples should be adjusted to pH 7.0 ± 0.2 by the addition of 2N hydrochloric acid[(2)].

Conventionally treated sludges may be homogenised by stomaching in a stomacher bag. Transfer the diluted sample into a sterile stomacher bag and homogenise by stomaching for 2 minutes at slow speed.

Transfer the homogenised sample to suitable centrifuge tubes and centrifuge at 200-300 g for one minute. Decant the supernatant liquid from the centrifuge tubes and filter through a course glass-fibre filter to remove sediment material.

D9.2 *Sample processing*

The volume 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 - 80, but varies according to the number of target organisms within the sludge. Typically, the filtrate should be serially diluted 10- to 1000-fold with supplemented modified tryptone soya broth. This enables the enumeration of up to 100000 (i.e. 10^5) glucuronidase-negative *E. coli* per g of original (wet) sludge. Higher levels will require additional dilutions of the filtrate.

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Prepare the relevant number of sterile bottles according to the number of selected dilutions. Add 90 ml of sterile supplemented modified tryptone soya broth to each bottle. Using a sterile pipette, transfer 10 ml of the filtrate to the first bottle containing 90 ml of broth and mix thoroughly. Using a fresh pipette, transfer 10 ml of this diluted filtrate to a second bottle containing 90 ml of broth and mix thoroughly. Continue this process until all appropriate dilutions have been prepared.

D9.3 *Membrane filtration and resuscitation*

For each prepared dilution, filter 10 ml of the diluted sample through a cellulose nitrate filter, nominal pore size of 0.45 μm, housed in a sterile filter-housing unit. Remove each filter from the housing unit using sterile tweezers and place the membrane filter onto the surface of a glass fibre filter, contained in a Petri dish, saturated with novobiocin-supplemented modified tryptone soya broth[5]. Ensure that no air bubbles are trapped between the membrane filter and the media-saturated glass fibre filter. "Rolling" the membrane filter onto the medium helps minimise the likelihood that air bubbles will be formed. Place a lid on the Petri dish.

Incubate the Petri dishes at 36 ± 1 ºC for 6 - 18 hours. From a practical point of view, overnight incubation is not detrimental, and enables samples to be processed within a normal working day.

After incubation, the membrane filter is carefully removed, using sterile tweezers, from the Petri dish and transferred to the agar surface of a Petri dish containing CHROMagar O157 or equivalent medium. "Rolling" the membrane filter onto the medium helps minimise the likelihood that air bubbles will be formed. Cover the membrane filter with a Petri dish lid. These Petri dishes are incubated at 36 ± 1 ºC for 24 hours. After incubation the membrane filters are examined and pink colonies counted.

D9.4 *Reading of results*

The number of pink colonies on the membrane filter is counted for example, using a plate microscope. Colonies can be discriminated more easily by illumination of the membrane filter through a blue-daylight filter.

D9.5 *Confirmation tests*

Confirmation of VT *E. coli* can be carried out using biochemical and/or antibody-based agglutination tests with commercially available test-kits following manufacturer's instructions.

Direct confirmation of an O157 serotype may be carried out by latex agglutination with commercially available test-kits following manufacturer's instructions. Alternatively, confirmation may be achieved by appropriate use of gene-probe technology. Consideration should be given to identifying selected isolates within a recognised reference laboratory undertaking this kind of confirmation and identification.

D10 Calculations

Confirmed VT E. coli

The number of confirmed VT *E. coli* at a specific dilution is used to calculate the number of VT *E. coli* present per g of original (wet) sludge or per g of dried sludge, as required. The calculation takes into account the volume and dilutions filtered and, for the number of VT *E. coli* per g of dried sludge, the percent dry solids content[11]. The following equations may be used:

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a) For count, C_w, per g of original (wet) sludge

$$C_w = \frac{N \times b \times d}{a}$$

Where C_w is the number of VT *E. coli* in 1 g of the original (wet) sludge;
N is the number of pink colonies counted on the membrane filter;
a is volume of sample filtered through the membrane filter (typically, 10 ml);
b is initial dilution factor for the sludge in supplemented medium (in this case, 10); and
d is the dilution factor for the serial dilutions in supplemented medium.

b) For count, C_d, per g dry solids, i.e. count per g of dried sludge:

$$C_d = \frac{C_w \times 100}{e}$$

Where C_d is the number of VT *E. coli* in 1 g of the dried sludge;
C_w is the number of VT *E. coli* in 1 g of the original (wet) sludge; and
e is the percent dry solids content of the original (wet) sludge.

For example, if 25 g of original sludge is initially diluted 10-fold, and 10 ml of this dilution taken for filtration, and 32 pink colonies counted on the membrane filter, then

$$C_w = \frac{32 \times 10}{10} = 32$$

If the percent dry solids content of the original (wet) sludge is 7.5 %, then

$$C_d = \frac{C_w \times 100}{7.5} = 427$$

D11 Expression of results

The number of VT *E.coli* present in sludge is expressed as the number of VT *E.coli* on a wet weight or dry weight basis. Typically, results are reported as the number of VT *E.coli* per g of dried sludge.

D12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, non-cytotoxigenic *E. coli* O157) and non-target bacteria (for example, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*).

For larger batches of samples or for monitoring of routine performance it may be appropriate to analyse one or more samples, in duplicate. A comparison of results can then be undertaken.

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.

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2. Standing Committee of Analysts, The Microbiology of Sewage Sludge (2003) - Part 2 - Practices and procedures for sampling and sample preparation, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

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

4. UKWIR (2000) Methods for the detection of pathogens in biosolids. Report No 00/SL/06/05, UK Water Industry Research, London.

5. Optimising enrichment conditions for the isolation of *Escherichia coli* O157 in soils by immunomagnetic separation. *Letters in Applied Microbiology* , N F Hepburn, M MacRae, M Johnston, J Mooney and I D Ogden, 2002, **34**, pp365-369.

6. This text is based on Resolution 74 by CEN TC 292 - Wastes - Working Group 5, the agreed text of which was adopted by CEN TC 308 - Characterisation of sludges - for the section on "General Hazards" associated with sludge material and waste.

7. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

8. Categorisation of Pathogens According to Hazard and Categories of Containment. 1995, 4th Edition and supplement 1998, Advisory Committee on Dangerous Pathogens.

9. For example, National Collection of Type Cultures 12900.

10. M-Tech Diagnostics Ltd, Unit 8, Thelwell New Road, Industrial Estate, Thelwell, Warrington, Cheshire, WA4 2LY.

11. Standing Committee of Analysts, The Conditionability, Filterability, Settleabliity and Solids Content of Sludges (1984) A Compendium of Methods and Tests, *Methods for the Examination of Waters and Associated Materials*, in this series, ISBN 0117517879.

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Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below.

Standing Committee of Analysts
Environment Agency (National Laboratory Service)
Wheatcroft Office Park
Landmere Lane, Edwalton
Nottingham, NG12 4DG
www.environment-agency.gov.uk/nls

Standing Committee of Analysts
Members assisting with these methods

R A Barrell
P Boyd
S Cole
A Davis
R Down
P Finch
A Gawler
A Godfree
A Hockin
A Jonas
C W Keevil
N L Lang
J V Lee
R Pitchers
M Rink
D Sartory
J Sellwood
S R Smith
D Taylor
K C Thompson
J Watson

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CONTACTS:

ENVIRONMENT AGENCY HEAD OFFICE

Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol BS32 4UD
Tel: 01454 624 400 Fax: 01454 624 409

www.environment-agency.gov.uk
www.environment-agency.wales.gov.uk

ENVIRONMENT AGENCY REGIONAL OFFICES

ANGLIAN
Kingfisher House
Goldhay Way
Orton Goldhay
Peterborough PE2 5ZR
Tel: 01733 371 811
Fax: 01733 231 840

MIDLANDS
Sapphire East
550 Streetsbrook Road
Solihull B91 1QT
Tel: 0121 711 2324
Fax: 0121 711 5824

NORTH EAST
Rivers House
21 Park Square South
Leeds LS1 2QG
Tel: 0113 244 0191
Fax: 0113 246 1889

NORTH WEST
PO Box 12
Richard Fairclough House
Knutsford Road
Warrington WA4 1HG
Tel: 01925 653 999
Fax: 01925 415 961

SOUTHERN
Guildbourne House
Chatsworth Road
Worthing
West Sussex BN11 1LD
Tel: 01903 832 000
Fax: 01903 821 832

SOUTH WEST
Manley House
Kestrel Way
Exeter EX2 7LQ
Tel: 01392 444 000
Fax: 01392 444 238

THAMES
Kings Meadow House
Kings Meadow Road
Reading RG1 8DQ
Tel: 0118 953 5000
Fax: 0118 950 0388

WALES
Rivers House Plas-yr-Afon
St Mellons Business Park
Fortran Road
St Mellons
Cardiff CF3 0EY
Tel: 029 2077 0088
Fax: 029 2079 8555

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ENVIRONMENT AGENCY
GENERAL ENQUIRY LINE
0845 9 333 111

ENVIRONMENT AGENCY
FLOODLINE
0845 988 1188

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0800 80 70 60

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