Methods for the Examination of Waters and Associated Materials

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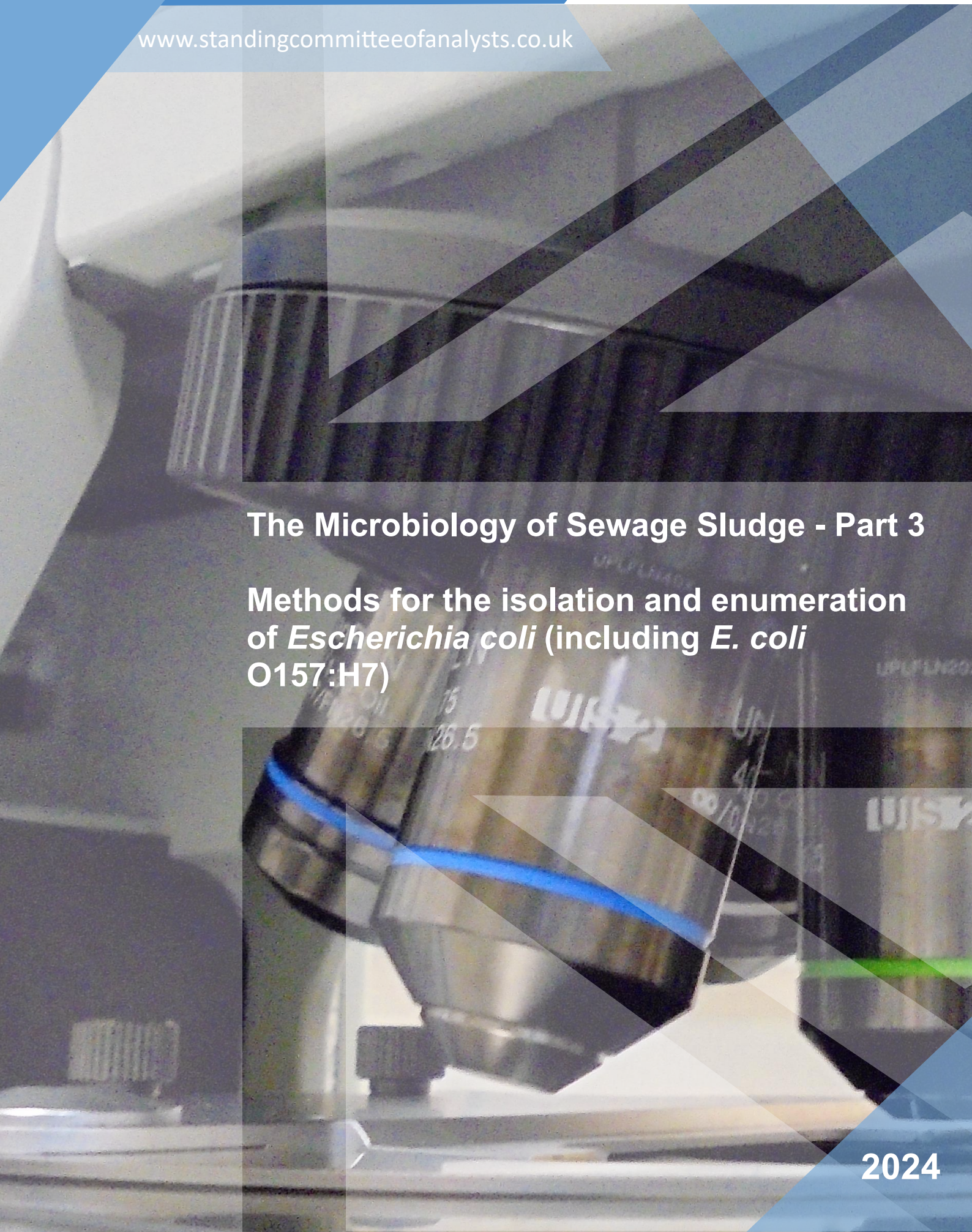

The Microbiology of Sewage Sludge - Part 3

Methods for the isolation and enumeration of *Escherichia coli* (including *E. coli* O157:H7)

2024

Methods for the isolation and enumeration of *Escherichia coli* (including *E. coli* O157:H7)

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.

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Blue Book No.
Microbiology of Water and Associated Materials Series
286. Methods for the isolation and enumeration of *Escherichia coli* (including *E. coli* O157:H7)
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Within this series there are separate booklets, each dealing with different topics concerning the microbiology of drinking water, including:

Microbiology of Drinking Water

- Part 1 – Water quality, epidemiology and public health
- Part 2 – Practices and procedures for sampling
- Part 4 – Methods for the Isolation and enumeration of coliform bacteria and Escherichia coli (including E. coli O157:H7)
- Part 5 – Methods for the isolation and enumeration of enterococci
- 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
- Part 8 – Methods for the Isolation and enumeration of Aeromonas and Pseudomonas aeruginosa
- 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
- Part 11 – The Determination of Taste and Odour in Drinking Water
- Part 12 – Methods for the isolation and enumeration of micro-organisms associated with taste, odour and related aesthetic problems
- Part 13 – The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration
- Part 14 – Methods for the isolation identification and enumeration of Cryptosporidium oocysts and Giardia cysts

The Microbiology of Water and Associated Materials

- Practices and Procedures for Laboratories
- The determination of Legionella bacteria in waters and other environmental samples. Part 1 – Rationale of surveying and sampling
- The determination of Legionella bacteria in waters and other environmental samples. Part 2 – The determination of Legionella bacteria in waters and other environmental samples. Culture Methods for their detection and enumeration
- The determination of Legionella bacteria in waters and other environmental samples. Part 3 – Method for their detection and quantification by polymerase chain reaction (qPCR) and protocol for method validation
- The Identification of Microorganisms using MALDI-TOF Mass Spectrometry
- Bacteriological Swabs

The Microbiology of Recreational and Environmental Waters

- Part 1 – Water quality, epidemiology and public health
- Part 2 – Practices and procedures for sampling
- Part 3 – Methods for the isolation and enumeration of Escherichia coli (including E. coli O157:H7)
- Part 4 – Methods for the isolation and enumeration of enterococci
- Part 5 – Methods for the isolation and enumeration of sulphite-reducing clostridia and Clostridium perfringens
- Part 6 – Methods for the isolation and enumeration of Staphylococcus aureus
- Part 7 – Methods for the isolation and enumeration of Aeromonas and Pseudomonas aeruginosa
- Part 8 – Methods for the isolation and enumeration of Salmonella and Shigella
- Part 9 – Methods for the isolation of Yersinia, Vibrio and Campylobacter by selective enrichment
- Part 10 – A method for the isolation and enumeration of sorbitol-fermenting bifidobacteria by membrane filtration
- Part 11 – Methods for the isolation and enumeration of somatic and F-specific bacteriophages and bacteriophages infecting Bacteroides fragilis
- Part 12 – Methods for the concentration of enteric viruses and the detection and enumeration of enteroviruses by suspended cell assay
- Part 13 – Methods for the isolation and enumeration of microbial tracers

The Microbiology of Sewage Sludge

- Part 1 – An overview of the treatment and use in agriculture or sewage sludge in relation to its impact on the environment and public health
- Part 2 – Practices and procedures for sampling and sample preparation
- Part 3 – Methods for the isolation and enumeration of Escherichia coli, including verocytotoxigenic Escherichia coli
- Part 4 – Methods for the detection, isolation and enumeration of Salmonellae

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, wastewater and effluents as well as sewage sludges 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 (SCA) - Established 1972 by the Department of the Environment.

At present, there are several working groups, each responsible for one section or aspect of water quality analysis:

1. General principles of sampling and accuracy of results
2. Microbiological methods
3. Inorganic and physical methods, metals and metalloids
4. Organic methods
5. Biological, biodegradability and inhibition methods
6. Radiochemistry 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 appear on our website – the library for which serves as a record of the bona fide methods developed and produced by the Standing Committee of Analysts.

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Users should ensure they are aware of the most recent version they seek.

Warning to users

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

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

Numerous publications are available giving practical details on first aid and laboratory safety.

These should be consulted and be readily accessible to all analysts. Amongst such resources are:

HSE: Information about health and safety at work

RSC: Laboratory best practices

The Approved List of Biological Agents. (2023) Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE)

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. Rapid information on numbers of *E. coli* removed through the sludge treatment process and the numbers remaining in the final product, are of great importance since it is not advisable to store the product for more than a few days before applying to land, as the bacteriostatic effect of some treatments (such as lime) can be reduced during storage. The significance of *E. coli* is described in more detail elsewhere(1) in this series.

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, mesophilic anaerobic digested sludges, composted sludge and may be appropriate for enhanced treated sludge (note limitations section A5) 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, microorganisms 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, as do some strains of *Shigella* and *Salmonella*.

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 ± 0.25 hours at 30 ± 1.0 °C followed by 14 ± 1 hours at 44 ± 1.0 °C – not exceeding 20 hours in total. 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 ± 1.0 °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 (β-glucuronidase negative). A small number of strains of *Salmonella* are able to produce blue colonies on MLGA.

This method may not be suitable for sludge samples that have been lime treated or where enhanced microbial reduction is expected. A multiple tube most probable number (MPN) technique may be more appropriate. Where solid content remains sufficiently diluted or is partially removed the effects should be minimal. This approach should be verified under the users own laboratory conditions.

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 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 from 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 may include:

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

A7.2 Incubators capable of maintaining temperatures of 30 ± 1.0 °C and 44 ± 1.0 °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.

A7.7 Stomacher, with appropriate bags.

A7.8 Timer

A7.9 Orbital shaker

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 in Petri dishes, and allow the medium to solidify. Petri dishes containing the agar medium may be stored at a temperature in the range 5 ± 3 °C and protected against dehydration. Storage time of up to 3 weeks has been demonstrated as suitable for the medium for this test, (note Appendix 1). The validation, however, was not carried out using sewage sludge and storage longer than 1 week should be verified by the laboratory. Storage beyond this period may result in a deterioration of 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.

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 5 ± 3 °C in the dark and used within three months.

Where small volumes (for example 9 ml) of diluent, are required to dilute samples, these volumes should ideally be dispensed aseptically into sterile containers after the diluent has been sterilised. They should ideally be used immediately, but may be stored, for example at 5 ± 3 °C in the dark, they should then be used as soon as possible due to the risk of contaminant growth and deterioration. Additional guidance

on the production, dispensing, control and use of smaller volumes of diluents for the purposes of serial dilution can be found in section 6.8 of MWAM Practices and Procedures for Laboratories (2017).

A8.3 2N hydrochloric acid, 2N Sodium hydroxide where pH adjustment is necessary

A8.4 *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

Laboratories analysing sludge samples for the presence of both *E. coli* and *Salmonella* may wish to consider the combination of preparation stages for efficient processing, by multiplying up the amount of initial tenfold dilution prepared to cover both analyses. (10)

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 falls, if possible, between 20 and 80.

Prepare an initial tenfold dilution of the original sample by following steps:

- Weigh 10 ± 0.1 g of sewage or sludge sample aseptically in an appropriate container (or directly into a stomacher bag)
- Add 45 ml of maximum recovery diluent (MRD) and transfer to a sterile stomacher bag (if not already) and homogenise at 200 ± 10 rpm for 2 mins ± 10 seconds. Adjustments made to the timing / speed are acceptable, provided they are verified by the user.
- Transfer the homogenised sample back to the container.
- Add a further 45ml MRD to the stomacher bag (to bring the total volume of MRD to 90 ± 1.0 ml).
- Rinse the stomacher bag and add the washings to the container. Vortex the suspension to ensure thorough mixing.

Alternative methods of preparing the initial tenfold dilution can be used e.g. weigh 10 ± 1.0g of sludge sample aseptically into an appropriate container and transfer to a sterile stomacher bag containing a separator sieve, add 90 ± 1.0ml MRD and homogenise at 200 ± 10 rpm for 2 mins ± 10 seconds. Adjustments made to the timing / speed are acceptable, provided they are verified by the user. The sample remains within the stomacher bag and serial dilutions can be made directly from the stomacher bag.

The use of stomacher bags containing sieve sleeves may be advantageous in reducing solid content prior to filtration, reducing masking or inhibition of organisms on the membrane.

If using stomacher bags containing sieves, ensure the inner sieve is moved sufficiently to allow aseptic removal of aliquots for serial dilution.

Laboratories may choose to transfer the homogenised sample to a new suitable sterile container prior to serial dilution to reduce the risk of contamination.

Samples that have been subjected to pH changes, e.g. lime treated samples, are likely to require pH adjustment after homogenisation to pH 7.0 ± 0.2 by the addition of hydrochloric acid, laboratories may choose to transfer the sample from the stomacher bag to a more suitable sterile container. The process of pH adjustment should ensure that additional bacterial load is not added to the sample or transferred to other samples, therefore aseptic technique is essential at this stage.

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 falls within the counting range of 20 – 80 colonies. 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 tenfold (decimal) dilutions (i.e.1 ml of dilution added to 9 ml of MRD).

In the event that sample processing cannot continue promptly, serial dilutions must be refrigerated at 5 ± 3 °C for not longer than 2 hours to prevent changes to the bacterial content of the sample.

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 (or other appropriate sterile 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 70 kPa 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 medium (for example, Petri dishes containing membrane lactose glucuronide agar left at room temperature for 2 hours or at 37 ± 1.0 °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 to disinfect it. If the funnel is to be re-used, disinfect the funnel by immersing it in boiling distilled, deionised or similar grade water for at least one minute. Other alternatives to boiling water baths, such as steaming or UV may be used if verified as appropriate. 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.

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 suitable sterile 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 ± 1.0 °C for 4.0 ± 0.25 hours, then transferred to an incubator at 44 ± 1.0 °C for at least 14 hours, but no more than 20 hours in total. 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. Examine the plates as soon as possible following removal from the incubator, but within 15 mins of removal, as the colouration of the colonies may change on cooling and standing. Count all green colonies (however faint) irrespective of size. All green colonies are regarded as *E. coli*. In addition, any blue colonies (i.e. possibly lactose negative *E. coli*) should be 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*.

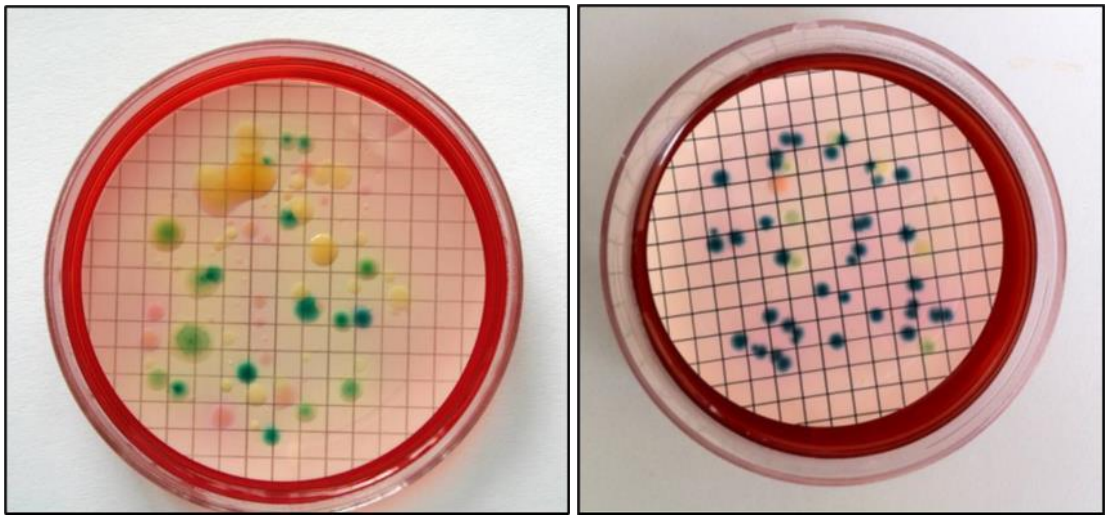

Figure A1: Green colonies of *E. coli* on membrane lactose glucuronide agar. The green colour can vary from pale green to dark green (almost blue) and different mixtures can be seen in environmental samples.

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.

Colonies for confirmation tests should be subcultured 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 subculturing.

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 subcultured if fewer than ten colonies are present or, at least ten colonies should be subcultured 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 subcultured. 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 subcultured isolates are then used to calculate the confirmed count 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 dependent on the nature, type or source of the sludge. Rarely, green colonies may not confirm as *E. coli.*

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 as a count per g of dried sludge, the percentage 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

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

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 e.g.:

- An additional tenfold dilution step - d = 10
- Two additional tenfold dilutions steps - d = 100) etc.
- An additional twofold dilution step – d = 2
- Two additional twofold dilution steps – d = 4 etc.

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 tenfold, and a serial dilution of 5 ml to 10 ml is made, i.e. a twofold 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 percentage 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 or blue) regarded as presumptive *E. coli* multiplied by the proportion of isolates that confirm before applying the calculations above. In this case, confirmed colonies are those which ferment lactose in lactose peptone water at 44 ± 1.0 °C, produce indole in tryptone water at 44 ± 1.0 °C, and are oxidase negative. Alternative confirmation methodologies can be used if appropriately validated, e.g., MALDI-TOF[11], VITEK, API etc

A11 Expression of results

Counts of *E. coli* present in sludge are expressed in 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 ± 1.0 °C, or 44 ± 1.0 °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

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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. Rapid information on numbers of *E. coli* removed through the sludge treatment process and the numbers remaining in the final product, are of great importance since it is not advisable to store the product for more than a few days before applying to land, as the bacteriostatic effect of some treatments (such as lime) can be reduced during storage. 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)].

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, as do some strains of *Shigella* and *Salmonella*.

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 ± 0.1°C in the presence of brilliant green and 2% bile and the formation of indole from tryptophan.

A sample of sludge (typically 10 ± 0.1 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 in appendix B1.

B5 Limitations

The estimation of the concentration of *E. coli* 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 or combustible 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 may 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 ± 1.0 °C and 44 ± 1.0°C.

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

B7.4 Vortex mixer.

B7.5 Stomacher with appropriate bags.

B7.6 Laboratory pH meter.

B7.7 2N hydrochloric acid, 2N Sodium hydroxide where pH adjustment is necessary

B7.8 Timer

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
Potasium 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 5 ± 3 °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.

The sterile medium can be stored for up to one month at temperatures between 5 ± 3 °C.

B8.3 *Tryptone water for the indole test*

The use of certain peptones that give satisfactory results in tests carried out at 37 ± 1.0 °C may not be satisfactory for the indole test at 44 ± 1.0 °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 5 ± 3 °C.

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

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 temperatures between 5 ± 3 °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 5 ± 3 °C in the dark and used within three months.

Where small volumes (for example 9 ml) of diluent, are required to dilute samples, these volumes should ideally be dispensed aseptically into sterile containers after the diluent has been sterilised. They should ideally be used immediately, but may be stored, for example at 5 ± 3 °C in the dark, they should then be used as soon as possible due to the risk of contaminant growth and deterioration. Additional guidance on the production, dispensing, control and use of smaller volumes of diluents for the purposes of serial dilution can be found in section 6.8 of MWAM Practices and Procedures for Laboratories (2017).

B8.6 *Other media*

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

A suitable for test for β-glucuronidase activity may be also be required.

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 tenfold dilution of the original sample by weighing 10 ± 0.1 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 at 200 ± 10 rpm for 2 mins ± 10 seconds. Adjustments made to the timing / speed are acceptable, provided they are verified by the user. Transfer the homogenised sample back to the container. Add sufficient MRD to the stomacher bag to bring the total volume of MRD to 90 ± 1.0 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, 10 ± 0.1 g of sludge should be weighed directly into the stomacher bag, 90 ± 1.0 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".

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.

In the event that sample processing cannot continue promptly, serial dilutions must be refrigerated at 5 ± 3 °C for not longer than 2 hours to prevent changes to the bacterial content of the sample.

B9.2 *Volumes of sample for inoculation*

A series of different volumes of sample are inoculated into tubes, or bottles, of medium. The following dilution methodologies are provided as examples.

A volume (typically, 10 ml) of well-mixed dilution “A” is added to 90 ± 1.0 ml of MRD. This suspension is well mixed for 5 - 10 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.

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 ± 1.0 °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 coloration). 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 ± 1.0 °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 ± 1.0 °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 ± 1.0°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 coloration). 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.

B10 Calculations

The number of tubes containing lauryl tryptose broth with bromocresol purple showing a positive reaction that were 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 pretreatment, the quantity of lime (or other chemical used in the pretreatment 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, *Klebsiella aerogenes* or *Klebsiella pneumoniae*). Tubes or bottles should be incubated for 24 hours at 36 ± 1.0 °C or 44 ± 1.0 °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 buffers 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 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.

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.

MPN tables can be found within ISO 7218

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 Example of 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.

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.

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. Rapid information on numbers of *E. coli* removed through the sludge treatment process and the numbers remaining in the final product, are of great importance since it is not advisable to store the product for more than a few days before applying to land, as the bacteriostatic effect of some treatments (such as lime) can be reduced during storage. The significance of *E. coli* is described in more detail elsewhere[(1)] in this series.

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 β-galactosidase and β-glucuronidase enzymes at 37 °C. β-galactosidase is demonstrated by the production of a yellow colour as a result of enzymatic cleavage

of ortho-nitrophenyl-β-D-galactopyranoside (ONPG). The enzyme β-glucuronidase is demonstrated by the production of a blue-white fluorescence (under long wavelength ultraviolet illumination) as a result of 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 added to Maximum Recovery Diluent (MRD) to produce a 1:10 dilution, homogenised[(2)] and then serially diluted with MRD.
1 ml of the resulting diluted sludge sample is pipetted into a vessel and filled to the line with sterile deionised water, followed by the addition of the dehydrated media. Alternatively, 10 ± 0.1 g of sewage or sludge may be added to a vessel containing 90 ± 1.0 ml of MRD and the dehydrated media added.

The suspension is then poured into a multiple well reaction pouch containing discrete wells of a defined volume. 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.

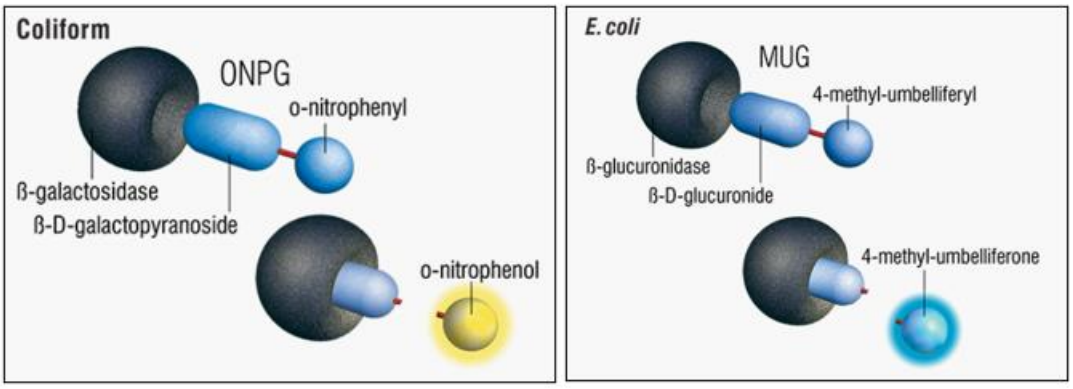

Figure C1 - Enzymatic reaction
(Courtesy of Idexx)

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 ± 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 or found on the IDEXX website (https://www.idexx.com/en/water/resources/mpn-generator/)

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 *E. coli* strains do not express β-glucuronidase activity on primary isolation or are β-glucuronidase-negative.

Some biocides and types of lime that are added to sludge can cause interactions such as autofluorescence. Sample matrices must be validated appropriately to ensure the method selected is appropriate.

Sample turbidity, suspended solids or high colour may also mask colour changes and growth. Users of this method need to understand the effect of these variables on their samples.

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 ultraviolet lamps are utilized, eye protection or safety boxes appropriate to the ultraviolet 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 the commercially available system Colilert™ by IDEXX Laboratories Inc. Some of the equipment listed is specific to this system.

Alternative systems may be available for which other equipment may be required.

Equipment required:

C7.1 Incubator capable of maintaining a temperature of 37 ± 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 201 organisms, or a 97-well system providing a countable range of up to 2419.6 organisms) and associated heat-sealing equipment.

C7.4 Ultraviolet long wavelength (365 - 366 nm) lamp, and viewer / eye protection

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

C7.6 Timer

C7.7 Orbital Shaker

C8 Media and reagents

Different commercial formulations of these media and reagents may be available.

Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method.

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

C8.1 *Colilert™ 18 medium*[6]

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 β-galactosidase and β-glucuronidase. For MPN counts the medium can be used in conjunction with Quanti-Tray® 200 and 2000 reaction pouches.

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 5 ± 3 °C in the dark and used within three months.

Where small volumes (for example 9 ml) of diluent, are required to dilute samples, these volumes should ideally be dispensed aseptically into sterile containers after the diluent has been sterilised. They should ideally be used immediately, but may be stored, for example at 5 ± 3 °C in the dark, they should then be used as soon as possible due to the risk of contaminant growth and deterioration. Additional guidance on the production, dispensing, control and use of smaller volumes of diluents for the purposes of serial dilution can be found in section 6.8 of MWAM Practices and Procedures for Laboratories (2017).

C8.3 *Other media*

Standard and commercial formulations of other media that may be useful include, MacConkey agar (MA), nutrient agar (NA) and oxidase reagent, laboratories may choose other similar media depending on the intended use.

C8.4 2N hydrochloric acid, 2N Sodium hydroxide where pH adjustment is necessary

C9 Analytical procedure

C9.1 *Sample preparation*

The volume, or dilution, of samples should be chosen so that not all the wells show a positive response. 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.

Prepare an initial tenfold dilution of the original sample, for example by weighing 10 ±0.1 g of sewage or sludge aseptically in an appropriate vessel and adding 90 ± 1.0 ml of maximum recovery diluent (MRD) or by adding 10 ± 0.1g of sewage or sludge to a vessel already containing 90 ± 1.0ml of MRD. Homogenise the diluted sample using an appropriate technique based on the characteristics of the matrix[(2)].

Prior to homogenising the diluted sample, lime treated samples need to be adjusted to a pH value of 7.0 $\pm$ 0.2 by the addition of hydrochloric acid.

pH the sample after homogenisation to a point whereby the highest concentration to have reagent added to it is at pH 7.0 $\pm$ 0.2.

Conventionally treated sludges may be homogenized by stomaching. Transfer the diluted sample into a sterile stomacher bag and homogenise at 200 $\pm$ 10 rpm for 2 mins ± 10 seconds. Adjustments made to the timing / speed are acceptable, provided

they are verified by the user. Transfer the homogenised sample back to the original container. Shake the suspension to ensure thorough mixing.

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 ± 1 min. Adjustments made to the timing / speed are acceptable, provided they are verified by the user.

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

In the event that sample processing cannot continue promptly, serial dilutions must be refrigerated at 5 ± 3 °C for not longer than 2 hours to prevent changes to the bacterial content of the sample.

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 ± 1.0 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 is 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 ± 1.0 °C for not less than 18 hours and not more than 22 hours.

Appropriate daily QC samples, including positive, negative and blank samples should be run alongside analysis.

C9.3 *Reading of results*

After incubation, the pouch is examined and the number of wells that exhibit a yellow coloration and exhibiting a blue-white fluorescence under ultraviolet light (365 nm) is recorded. The colour and fluorescence exhibited by samples are compared with manufacturer's scales to ensure correct interpretation of positive results.

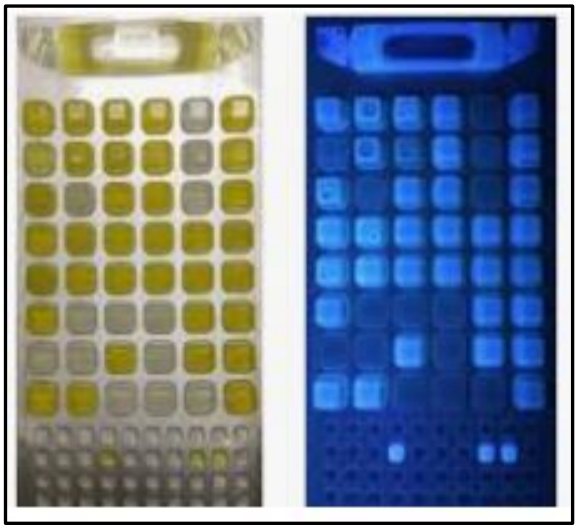

Figure C2 - Trays exhibiting presence of Coliforms (left) and *E. coli* (right)

C9.4 *Confirmation tests*

This method is validated to be highly specific for *E. coli* as demonstrated by the production of a yellow colour due to the enzymatic cleavage of ortho-nitrophenyl-β-D-galactopyranoside (ONPG) indicating the presence β-galactosidase, and a blue white fluorescence under UV light as a result of the enzymatic cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUG) indicating the presence β-glucuronidase – see figure C2. Hence, confirmation tests are not usually required. Should there be any doubt as to the type of response detected, then medium from those wells showing this type of response could be subcultured 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 only, yellow colouration only, and if necessary, wells displaying yellow colouration AND blue-white florescence. The medium should be subcultured as soon as practicable after the reaction pouch is removed from the incubator. Alternatively, pouches may be stored at 5 ± 3°C until sub-culture can be carried out.

C10 Calculations

The number of wells showing a positive reaction, (i.e. showing both yellow and blue-white fluorescence 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-well MPN pouch system, if there are 15 wells showing both positive yellow coloration and 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 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

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

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 tenfold, and a serial dilution of 10 ml is made, i.e. a twofold dilution, and 1 ml of the final dilution taken for addition to the medium, and 15 wells counted showing yellow colouration and blue-white fluorescence, an MPN of 18, then

$$C_w = \frac{18 \times 10 \times 10}{1 \times 5} = 360$$

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

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

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 Water and Associated Materials (2017) - 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 2002, Statutory Instrument 2002 No. 2677.

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

7. Standing Committee of Analysts, The Microbiology of Drinking Water (2016) - 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.

Appendix C1

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

No. of wells giving positive reaction	MPN per 100 ml sample	95% Confidence Limits Lower	Upper
0	<1.0	0.0	3.7
1	1.0	0.3	5.6
2	2.0	0.6	7.3
3	3.1	1.1	9.0
4	4.2	1.7	10.7
5	5.3	2.3	12.3
6	6.4	3.0	13.9
7	7.5	3.7	15.5
8	8.7	4.5	17.1
9	9.9	5.3	18.8
10	11.1	6.1	20.5
11	12.4	7.0	22.1
12	13.7	7.9	23.9
13	15.0	8.8	25.7
14	16.4	9.8	27.5
15	17.8	10.8	29.4
16	19.2	11.9	31.3
17	20.7	13.0	33.3
18	22.2	14.1	35.2
19	23.8	15.3	37.3
20	25.4	16.5	39.4
21	27.1	17.7	41.6
22	28.8	19.0	43.9
23	30.6	20.4	46.3
24	32.4	21.8	48.7
25	34.4	23.3	51.2
26	36.4	24.7	53.9
27	38.4	26.4	56.6
28	40.6	28.0	59.5
29	42.9	29.7	62.5
30	45.3	31.5	65.6
31	47.8	33.4	69.0
32	50.4	35.4	72.5
33	53.1	37.5	76.2
34	56.0	39.7	80.1
35	59.1	42.0	84.4
36	62.4	44.6	88.8
37	65.9	47.2	93.7
38	69.7	50.0	99.0
39	73.8	53.1	104.8
40	78.2	56.4	111.2
41	83.1	59.9	118.3
42	88.5	63.9	126.2
43	94.5	68.2	135.4
44	101.3	73.1	146.0
45	109.1	78.6	158.7
46	118.4	85.0	174.5
47	129.8	92.7	195.0
48	144.5	102.3	224.1
49	165.2	115.2	272.2
50	200.5	135.8	387.6
51	> 200.5	146.1	infinite

(Table courtesy of IDEXX Laboratories Inc.)

D The enumeration of *Escherichia coli* using a chromogenic agar plating technique

This method has not been subjected to widespread use within the UK or verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method, and similar methods, would be welcomed to assess their full capabilities.

D1 Introduction

Sewage sludge may contain pathogenic micro-organisms such as *Salmonella* species and *Escherichia coli* 0157 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[1]. Rapid information on numbers of *E. coli* removed through the sludge treatment process and the numbers remaining in the final product, are of great importance since it is not advisable to store the product for more than a few days before applying to land, as the bacteriostatic effect of some treatments (such as lime) can be reduced during storage. The significance of *E. coli* is described in more details elsewhere[2] in this series.

D2 Scope

The method is suitable for the examination of all types of untreated and conventionally treated sludges[3]. The preferred means of sample preparation prior to using this method is the use of a stomacher. Depending on the sludge matrix, different preparative techniques may be appropriate. These techniques are described elsewhere[4] in this series. A variety of initial dilutions may be required for plating.

Users wishing to employ this method should verify its performance under their own laboratory conditions[5].

D3 Definitions

In the context of this method, organisms which produce β-glucuronidase forming blue colonies on tryptone bile glucuronide (TBX) agar after incubation for 4 ± 0.25 hours at

30 °C ± 1.0 °C followed by 14 ± 1 hours at 44 °C ± 1.0 °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.

It has been reported that 98 – 99% of *E. coli* strains produce β-glucuronidase[6] as do some strains of Shigella and Salmonella.

D4 Principle

A sample of sludge is homogenised, serially diluted in maximum recovery diluent. A small volume of each dilution is spread onto the surface of selective agar medium. Colonies of *E. coli* are enumerated after incubating for 4 hours at 30 ± 1.0 °C followed by 14 hours at 44 ± 1.0 °C. The agar medium contains the chromogenic substrate, 5-bromo-4-chloro-3-indolyl- β-D-glucuronide (BCIG), which when hydrolysed, indicates the activity of β-glucuronidase. Colonies that are β-glucuronidase-positive, evidenced by a blue pigmentation, 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.

D5 Limitations

The TBX agar is selective against *E. coli* strains which are not thermotolerant or that do not express glucuronidase activity, therefore 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 cannot hydrolyse BCIG. This enzyme is expressed in most *E. coli* strains, however some strains of *Shigella*, *Yersinia* and *Salmonella* can also express this enzyme.

Competing organisms may obscure the blue coloured colonies although the choice of suitable dilutions should enable accurate counts of presumptive *E. coli* colonies to be made. The maximum number of colonies that should be counted on a single plate is 300, however this can be reduced by selecting an appropriate dilution. The size of the inoculum level onto the agar plate is dependent on the moisture content of the agar plates. Therefore to use high volumes of around 1ml, the agar plates require sufficient drying.

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 used, wherever possible[7].

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[8] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[5] in this series.

D7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[5]. Other items required include:

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

D7.2 Incubators capable of maintaining temperatures of 30 ± 1.0 °C and 44 ± 1.0 °C, or cycling incubators, fitted with timers capable of attaining these temperatures.

D7.3 Petri dishes, 90 mm diameter.

D7.4 Stomacher

D7.5 Sterile glass or plastic spreaders

D7.6 Calibrated pipette.

D7.7 Timer

D8 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. The effects of variations in the preparation and storage of media should also be verified. Unless otherwise stated chemical constituents should be added as anhydrous salts. Water should be distilled, deionised or similar grade quality.

D8.1 *Maximum recovery diluent*

Bacteriological peptone	1 g
Sodium chloride	8.5 g
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 5 ± 3 °C in the dark and used within three months.

Where small volumes (for example 9 ml) of diluent, are required to dilute samples, these volumes should ideally be dispensed aseptically into sterile containers after the diluent has been sterilised. They should ideally be used immediately, but may be stored, for example at 5 ± 3 °C in the dark, they should then be used as soon as possible due to the risk of contaminant growth and deterioration. Additional guidance on the production, dispensing, control and use of smaller volumes of diluents for the purposes of serial dilution can be found in section 6.8 of MWAM Practices and Procedures for Laboratories (2017).

D8.2 *Tryptone bile glucuronide agar* (TBX agar)[6,7]

Tryptone	20.0 g
Bile Salts No. 3	1.5 g
Agar	15.0 g
BCIG	75 mg
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 a solution consisting of 2.5 ml of 95% v/v aqueous ethanol and 0.5 ml of 1M sodium hydroxide solution. Add this solution to the medium. Alternatively, the sodium salt can be added directly to the medium. Mix the solution well and autoclave at 121 °C for 15 minutes. Allow the medium to cool to 50 °C, distribute in suitable volumes to Petri dishes, and allow the medium to solidify. Petri dishes containing the agar medium may be stored at a temperature in the range 5 ± 3 °C for up to one week, protected against dehydration. Storage beyond this period may result in a deterioration of the medium. The pH of the medium after sterilisation should be 7.2 ± 0.2.

D8.3 *Other media*

Standard and commercial formulations of the 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).

D9 Analytical procedure

D9.1 *Sample preparation*

Ideally, the dilutions of samples should be chosen so that the number of colonies to be counted on the agar lies between 30 and 300 on one plate. Prepare an initial tenfold dilution of the original sample by weighing 10 ± 0.1 g of sewage or sludge sample

aseptically and place in a stomacher bag. Add 90 ± 1.0 ml of MRD to make a 1:10 dilution. Prior to homogenising the diluted sample, lime treated samples need to be adjusted to a pH value of 7.0 ± 0.2 by the addition of 2M hydrochloric acid. Place the bag in the stomacher and homogenise at 200 ± 10 rpm for 2 mins ± 10 seconds. Adjustments made to the timing / speed are acceptable, provided they are verified by the user.

In the event that sample processing cannot continue promptly, serial dilutions must be refrigerated at 5 ± 3 °C for not longer than 2 hours to prevent changes to the bacterial content of the sample.

D9.2 *Sample processing*

It is usually important that several serial dilutions are prepared so that the number of colonies on at least one of the Petri dishes lies within the counting range. Prepare an appropriate set of serial dilutions by adding 1 ml of the diluted homogenised original sample to 9 ml of MRD. The range of dilutions required will depend on the type of sludge being examined. From each dilution pipette up to 0.5 ml onto a well-dried plate of TBX agar. Spread this inoculum evenly using a sterile glass or plastic spreader, being careful not to touch the sides of the plate and allow the surface of the agar to absorb all the liquid before placing the Petri dishes, inverted, in an incubator.

Alternatively, from each dilution pipette up to 1 ml into an empty petri dish and pour 15 – 20 ml of molten TBX agar – tempered to around 46 °C. Mix the agar and the inoculum thoroughly and allow the agar to solidify before inverting and incubating. Incubate for 4 ± 0.25 hours at 30 ± 1.0 °C followed by 14 ± 1 hours at 44 ± 1.0 °C.

D9.3 *Reading of results*

After incubation, select plates with less than 300 blue colonies. Count and record the number of blue colonies (i.e. that are β-glucuronidase-positive), together with the dilution. These are regarded as presumptive *E. coli.*

D9.4 *Confirmation tests*

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

If confirmation is deemed necessary, procedures described elsewhere[9] in this series should be used. Depending on the intended purpose of the analysis in terms of the required accuracy, sub-culture all, or a suitable number of, blue colonies.

If the aim of confirmation is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be subcultured if fewer than ten colonies are present or, at least ten colonies should be subcultured 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 colonies in a randomly chosen segment of appropriate size should be subcultured. 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 subcultured isolates should then be used to calculate the confirmed number of colonies of *E. coli*.

Colonies for confirmation tests should be subcultured 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 subculturing.

D10 Calculations

D10.1 Count of *E. coli*

The number, N, of 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 volume used on the spread plate. Where the result is to be expressed on a count per dry sludge basis, the percent dry solids content would need to be determined. 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 blue colonies counted on the agar;

a is the volume of sample inoculated onto the agar (typically 0.5 ml);

b is the initial dilution factor for the sludge in MRD; 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 dried sludge;

C_w is the number of *E. coli* in 1 g of the original (wet) sludge;

e is the percent dry solids content of the original (wet) sludge.

For example, if 10 g of original sludge are initially diluted tenfold, and a serial dilution of 1 ml to 100 ml (i.e. a hundredfold dilution is made) and 0.5 ml of the final dilution are taken for filtration, and that 20 blue colonies are counted on the agar, then

$$C_w = \frac{20 \times 10\ 100}{0.5} = 40000$$

If the percent dry solids content of the original (wet) sludge is 20%, then

$$C_d = \frac{C_w \times 100}{20} = 200000$$

The theoretical detection level using one petri dish is < 200 *E. coli* per g of original (wet) sludge. This can be improved if required by increasing the number of petri dishes used for each dilution, i.e. if two petri dishes are prepared and no *E. coli* are counted, then the detection level becomes < 100 per g (wet) sludge.

D10.2 *Confirmed E. coli*

Confirmation is not usually required, however, where it is deemed necessary, the number of confirmed *E. coli* is calculated as the count of blue colonies regarded as presumptive *E. coli* multiplied by the proportion of the isolates that confirm (before applying the calculations above). In this case, confirmed colonies are those that are lactose-positive (in lactose peptone water at 44 ± 1.0 °C) positive for the production of indole in tryptone water at 44 ± 1.0 °C, and oxidase-negative.

D11 Expression of results

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

D12 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*) to ensure consistent performance from batch to batch. Petri dishes should be incubated at a temperature and time appropriate to application of this method. Further details are given elsewhere[5].

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 can then be undertaken.

D13 References

1. *The Safe Sludge Matrix.* Guidelines for the Application of Sewage Sludge to Agricultural Land. 2001, Agricultural Development and Advisory Service, Water UK and the British Retail Consortium.

2. 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.

3. WRc NSF, Improving Methods for the Bacteriological Examination of Sludge from all Sources (2004). Collaborative Project CP112 Report No. P6679. (by permission)

4. 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.

5. Standing Committee of Analysts, The Microbiology of Water and Associated Materials (2017) - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

6. ISO 16649-1: 2001. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* . Part 1: Colony-count technique at 44°C using membranes and 5-bromo-4-chloro-3-indoyl-β-D-glucuronide.

7. ISO 16649-2: 2001. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* . Part 2: Colony-count technique a 44 °C using 5-bromo-4-chloro-3-indoyl-β-D-glucuronide.

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

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

10. Standing Committee of Analysts, The Microbiology of Drinking Water (2016) – 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.

11. 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.

Appendix D1

Performance characteristics of a chromogenic agar plating technique using Tryptone Bile Glucuronide agar medium (TBX) for the enumeration of *Escherichia coli* in sludge

1 Introduction

The information recorded here is for reference purposes only. It provides anyone wishing to use this medium with a starting point for primary validation and secondary verification[1,2] in keeping with ISO 13843[1]. Its primary purpose is to record data from an informal method comparison exercise undertaken in three laboratories. These comparisons were individual to the laboratories and were not performed according to ISO 17994[3].

2 Primary Validation

Formal primary validation for application to sludge has not been undertaken. The medium is a modification of Tryptone Bile Agar (TBA), a well characterised medium which has been widely used for *E. coli* isolation and enumeration from a range of matrices. It contains Tryptone to promote growth and facilitate the detection of Indole production and bile salts as a selective agent for enteric bacteria. Modification by the inclusion of the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (BCIG) increases its specificity for *E. coli*. Glucuronidase activity causes positive colonies to be a distinctive blue colour.
Further information on the characteristics of the medium can be derived from published literature and standards[4,5] and manufacturers' product information.

3 Verification

The available verification data for application of the method to sludge is limited to the analysis of 8 separate sludge samples consisting of 4 duplicates where results can be compared to those obtained on MLGA in one laboratory[6].

In addition, data for the analysis of a proprietary standardised material, Vitroid, of known *E. coli* content, also in duplicate, is available for one laboratory together with contemporary comparative results for the same material on MLGA in 3 other laboratories.

Paired results were tested using a Sign test. In all cases the TBX results were higher than those on MLGA. The probability value of P=0.0078 was significant at the 99% confidence level. In this trial the median colony count using TBX was 2.5 times greater than that on MLGA.

Table 1 Paired sample Sign test: MLGA versus TBX

Number of samples	Below	Equal	Above	P
8	8	0	0	0.0078

4 Informal laboratory method comparisons

Informal comparisons between the TBX method and in-house application of methods A (MLGA by membrane filtration), B (MPN) and C (defined substrate MPN), described in this document, were undertaken in 3 laboratories. A total of 91 sludge samples were analysed. This included samples from 5 different sludge matrices: Mesophilic anaerobic digested (MAD), Raw, Limed Cake, Pressed Cake and Thickened.

Laboratory 1 compared TBX with MLGA only and analysed 17 samples of 2 matrices. Laboratory 2 analysed 29 samples of 3 matrices comparing TBX with Colilert only and laboratory 3 analysed 45 samples of 4 matrices comparing TBX with a mixture of MLGA (22 samples) and MPN (23 samples) methods. These comparisons are summarised in the table below.

Table 2 Sludge matrices analysed by laboratories

Laboratory	MAD	Limed cake	Pressed cake	Raw sludge	Thickened	All sludges
1			15	2		17
2	16	5	8			29
3a		25	5	9	6	45
Totals	16	30	28	11	6	91

The tables below present the results obtained comparing by method used and by sludge type.

Table 3 Comparison of TBX result against in house method

SCA method used in house	No. of samples tested	TBX result greater	TBX and Lab equivalent	Laboratory method greater than TBX
A - MLGA	39	27	3	9
B - MPN	23	2	0	21
C – Defined substrate	29	20	3	6
All methods	91	49	6	36

Table 4 Comparison of TBX result against in house method by sludge type

SCA Method used by sludge type	TBX result greater	TBX and Lab equivalent	Laboratory method greater than TBX
A – Pressed cake	14	2	4
A – Raw sludge	9	1	1
A – Thickened sludge	2	0	4
A – Limed sludge	2	0	0
B – Limed cake	2	0	21
C – MAD	13	0	3
C – Pressed cake	4	1	3
C – Limed cake	3	2	0
All sludges	49	6	36

Additional results were provided by a fourth laboratory analysing 10 sludge samples by Method A (MLGA). The sludge types analysed were Raw (1), MAD (4), Limed (1) and Cake (4). TBX gave higher counts for all 10 samples.

5 Conclusions

Two UK laboratories already use this method having demonstrated satisfactory performance for application to their sludge matrices.

The results from this informal evaluation indicate that the method is suitable for use with some but perhaps not all types of sludge. Those wishing to use this method are advised that they should verify its performance under their own laboratory conditions and are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method would be welcomed to assess its full capabilities.

6 Acknowledgements

The Standing Committee of Analysts is indebted to WRc and Anglian Water for access to data from a collaborative project also to the managers and analysts of the following laboratories for providing data from informal studies:

Alcontrol Laboratories (Rotherham)
Severn Trent Laboratories (Runcorn)
Thames Water (Reading)
Wessex Water (Bath)

7 References

1. ISO/TR 13843 (2000) Water Quality – Guidance on validation of microbiological methods

2. ISO 17994 (2004) Water Quality – Criteria for the Establishment of Equivalency between Microbiological Methods

3. David P Sartory (2005) Review – Validation, verification and comparison: Adopting new methods in water microbiology. Water SA **31** pp 393-396

4. R A E Barrell (1992) A Comparison between Tryptone Bile Agar and Membrane Lauryl Sulphate Broth for the Enumeration of Presumptive *Escherichia coli* in water. Water Res. **26** pp 677-681

5. ISO 16649-2:2001. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of ß-glucuronidase- positive *Escherichia coli* – Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl ß-D-glucuronide.

6. WRc NSF, Improving Methods for the Bacteriological Examination of Sludge from all Sources (2004). Collaborative Project CP112 Report No. P6679 (by permission)

E The enumeration of *Escherichia coli* using an alternative chromogenic agar plating technique

This method has not been subjected to widespread use within the UK or verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method, and similar methods, would be welcomed to assess their full capabilities.

E1 Introduction

Sewage sludge may contain pathogenic micro-organisms such as *Salmonella* species and *Escherichia coli* 0157 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 important routine microbiological examinations 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 potential use on agricultural land and to demonstrate compliance with standards[1]. Rapid information on numbers of *E. coli* removed through the sludge treatment process and the numbers remaining in the final product, are of great importance since it is not advisable to store the product for more than a few days before applying to land, as the bacteriostatic effect of some treatments (such as lime) can be reduced during storage. The significance of *E. coli* is described in more details elsewhere[2] in this series.

E2 Scope

The use of this method in comparison with other methods is described elsewhere[3]. The method is suitable for the examination of all types of untreated and conventionally treated sludges. Depending on the sludge matrix, different preparative techniques may be appropriate. These techniques are described elsewhere[4] in this series. A variety of initial dilutions may be required for plating.
Users wishing to employ this method should verify its performance under their own laboratory conditions[5].

E3 Definitions

In the context of this method, organisms which produce acid from lactose and produce β-glucuronidase forming green colonies on membrane lactose glucuronide agar 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, produce indole from tryptophan and give an oxidase-negative reaction. Most strains produce β-glucuronidase[(6)].

E4 Principle

A sample of sludge, usually 10 ± 0.1 g, is initially mixed with 90 ± 1.0 ml of maximum recovery diluent (MRD) and homogenised using a stomacher. The homogenised sample is then serially diluted using MRD. A known, small volume of each dilution is inoculated and spread onto the surface of selective agar medium contained in a Petri dish. Colonies of *E. coli* are enumerated after incubating for 4 hours at 30 ± 1.0 °C followed by 14 hours at 44 ± 1.0 °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 are regarded as *E. coli*. No further confirmation should be required, but if necessary, confirmation tests may be carried out.

E5 Limitations

Enumeration of colonies by this method will exclude a small proportion of strains of *E. coli* that are unable to grow at 44 °C or that cannot express β-glucuronidase activity.

Competing organisms may obscure the green coloured colonies although the choice of suitable dilutions should enable accurate counts of presumptive *E. coli* colonies to be made. The maximum number of colonies that should be counted on a single plate is 300, however this can be reduced by selecting an appropriate dilution. The size of the inoculum level onto the agar plate is dependent on the moisture content of the agar plates. Therefore, to use high volumes of around 1 ml, the agar plates require sufficient drying.

E6 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 be pressurised, and

if made of glass to explode. Infectious material and/or pathogenic aerosols may, therefore, be of concern and may be potentially hazardous. Glass bottles should not therefore be used.

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[5] in this series.

E7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[5]. Items required may include:

E7.1 Sterile sample containers of appropriate volume, made of suitable material.

E7.2 Incubators capable of maintaining temperatures of 30 ± 1.0 °C and 44 ± 0.5 °C, or cycling incubators, fitted with timers capable of attaining these temperatures.

E7.3 Petri dishes, 90 mm diameter.

E7.4 Stomacher with appropriate bags

E7.5 Sterile glass or plastic spreaders

E7.6 Calibrated pipette.

E7.7 2N hydrochloric acid, 2N Sodium hydroxide where pH adjustment is necessary

E7.8 Timer

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. The effects of variations in the preparation and storage of media should also be verified. Unless otherwise stated chemical constituents should be added as anhydrous salts. Water should be distilled, deionised or of similar grade quality.

E8.1 *Maximum recovery diluent*

Bacteriological peptone	1 g

Sodium chloride	8.5 g
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 5 ± 3 °C in the dark and used within three months.

Where small volumes (for example 9 ml) of diluent, are required to dilute samples, these volumes should ideally be dispensed aseptically into sterile containers after the diluent has been sterilised. They should ideally be used immediately, but may be stored, for example at 5 ± 3 °C in the dark, they should then be used as soon as possible due to the risk of contaminant growth and deterioration. Additional guidance on the production, dispensing, control and use of smaller volumes of diluents for the purposes of serial dilution can be found in section 6.8 of MWAM Practices and Procedures for Laboratories (2017).

E8.2 *Membrane lactose glucuronide agar*[8]

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
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 in Petri dishes, and allow the medium to solidify. Petri dishes containing the agar medium may be stored at a temperature in the range 5 ± 3°C for up to one week, protected against dehydration. Storage beyond this period may result in a deterioration of 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.

E8.3 *Other media*

Standard and commercial formulations of the 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).

E9 Analytical procedure

E9.1 *Sample preparation*

Ideally, the dilutions of samples should be chosen so that the number of colonies to be counted on the agar lies between 30 and 300 on one Petri dish. Prepare an initial tenfold dilution of the original sample by aseptically weighing and transferring to a stomacher bag 10 ± 0.1 g of sewage or sludge sample. Add 90 ± 1.0 ml of MRD to the bag and mix well. Place the bag in the stomacher and homogenise at 200 ± 10 rpm for 2 mins + 10 seconds. Adjustments made to the timing / speed are acceptable, provided they are verified by the user.

In the event that sample processing cannot continue promptly, serial dilutions must be refrigerated at 5 ± 3 °C for not longer than 2 hours to prevent changes to the bacterial content of the sample.

E9.2 *Sample processing*

It is usually important that several serial dilutions are prepared so that the number of colonies on at least one of the Petri dishes lies within the counting range (E9.1). Prepare an appropriate set of serial dilutions by adding 1 ml of the diluted homogenised sample (E9.1) to 9 ml of MRD. The range of dilutions required depends upon the type of sludge being examined and the number of organisms expected. From each dilution prepared, pipette 0.5 ml onto a well-dried plate of membrane lactose glucuronide agar. Spread this inoculum evenly using a sterile glass or plastic spreader and allow the surface of the agar to absorb all the liquid before placing the Petri dishes, inverted, in an incubator. Incubate for 4 hours at 30 ± 1.0 °C followed by 14 hours at 44 ± 1.0 °C.

E9.3 *Reading of results*

After incubation, select those Petri dishes containing the appropriate range of colonies, i.e. 30 - 300 green colonies. Count and record the number of green colonies (i.e. that are β-glucuronidase-positive), however faint, and note the associated dilution. These colonies are regarded as presumptive *E. coli.* In addition, any blue colonies (i.e. possibly lactose-negative *E. coli*) should be regarded as *E. coli*.

E9.4 *Confirmation tests*

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*

should not be required. The combination of media selectivity, temperature and the specificity of β-glucuronidase are sufficient for most practical purposes.

If confirmation is deemed necessary, procedures described elsewhere[9] in this series should be used. Depending on the intended purpose of the analysis in terms of the required accuracy, sub-culture all, or a suitable number of green colonies. 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 subcultured if fewer than ten colonies are present or, at least ten colonies should be subcultured 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 colonies in a randomly chosen segment of appropriate size should be subcultured. 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 subcultured isolates should then be used to calculate the confirmed number of colonies of *E. coli*.

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

E10 Calculations

E10.1 Count of *E. coli*

The number of green, and if present blue, colonies (N) 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 volume used. Where the result is to be expressed on a count per dry sludge basis, the percent dry solids content would need to be determined[10]. 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 agar;

a is the volume of sample spread on the agar (typically 0.5 ml);

b is the initial dilution factor for the sludge in MRD (typically tenfold); and

d is the dilution factor for the serial dilutions in MRD.

b) For count, C_d per g of 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 dried sludge;

C_w is the number of *E. coli* in 1 g of the original (wet) sludge;

e is the percent dry solids content of the original (wet) sludge.

For example, if 10 g of original sludge are initially diluted tenfold, and a serial dilution of 1 ml to 100 ml (i.e. a hundredfold dilution) is made, and 0.5 ml of the final dilution are taken for incubation, and 20 green colonies are counted on the agar, then

$$C_w = \frac{20 \times 10 \times 100}{0.5} = 40000$$

If the percent dry solids content of the original (wet) sludge is 20 %, then

$$C_d = \frac{C_w \times 100}{20} = 200000$$

Using one Petri dish and the values of the above example, the theoretical detection level is < 200 *E. coli* per g of original (wet) sludge. This can be improved if required by increasing the number of Petri dishes used for each dilution, i.e. if two Petri dishes are prepared for each dilution and the values of the above example used but no *E. coli* are counted, then the detection level becomes < 100 per g (wet) sludge.

E10.2 *Confirmed E. coli*

Confirmation is not usually required, however, where it is deemed necessary, the number of confirmed *E. coli* is calculated as the count of green and blue colonies regarded as presumptive *E. coli* multiplied by the proportion of the isolates that confirm (before applying the calculations above). In this case, confirmed colonies are those that are lactose-positive (in lactose peptone water at 44 ± 1.0 °C), positive for the production of indole in tryptone water at 44 ± 1.0 °C ,and oxidase-negative.

E11 Expression of results

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

E12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of the target bacteria *E. coli* and non-target bacteria (for example *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) to ensure consistent performance. Petri dishes should be incubated for 4 hours at 30.0 ± 1.0 °C followed by 14 hours at 44.0 ± 1.0 °C. Further details, including suitable strains, are given elsewhere[5].

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 can then be undertaken.

E13 References

1. *The Safe Sludge Matrix.* Guidelines for the Application of Sewage Sludge to Agricultural Land. 2001, Agricultural Development and Advisory Service, Water UK and the British Retail Consortium.

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

7. 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.

8. Standing Committee of Analysts, The Microbiology of Drinking Water (2016) – 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.

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.

F The detection of Escherichia coli O157 by selective enrichment and immuno-magnetic separation

This method has not been subjected to widespread use within the UK or verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method, and similar methods, would be welcomed to assess their full capabilities.

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* O157:H7 in wastewater sludge is described elsewhere[1] in this series.

E. coli O157 is a recognised cause of haemorrhagic colitis in humans, an illness characterised by symptoms of bloody diarrhoea with 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, contaminated water and recreational 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.

These pathogenic strains of *E. coli* also cause illness in 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.

This method may be subject to cross-contamination and aerosol production during the immuno-magnetic separation (IMS) procedure. Any laboratory that does not have access to automated IMS beads retrieval, for example a “Dynal Beadretriever” or equivalent equipment, should consider whether to engage in the detection for *E. coli* O157:H7.

E. coli O157:H7 has been classified as a hazard Group 3 organism. Presumptive isolates and waters for which epidemiology strongly suggests the presence of *E. coli* O157, should be handled under containment level 3 conditions (as for *Salmonella typhi*).

The automated IMS procedure should be performed in a class 1 safety cabinet. Access to the room during this procedure should be restricted. Presumptive colonies on agar plates should be transferred to containment level 3 (CL3) facilities. All confirmatory tests should be performed in a CL3 laboratory.

F2 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].

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 straw-coloured colonies on cefixime tellurite sorbitol MacConkey agar, and which subsequently confirm by biochemical and serological tests.

F4 Principle

A sample of sludge is initially homogenised[2] and then serially diluted. The diluted sludge is filtered through a membrane filter and then subjected to 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. Alternatively, samples of some less filterable sludge matrices may be analysed by adding an equal volume of homogenised diluted sludge to double-strength medium as a presence/absence test or as an MPN test. Isolation of colonies is followed by selection of typical non-sorbitol-fermenting colonies for identification by biochemical or MALDI-TOF and serological tests.

F5 Limitations

This method does not identify atypical sorbitol-fermenting strains of *E. coli* O157 or other serotypes of *E. coli* that produce Shiga toxins.

Sludge with high solids content (greater than 20% m/v) tends 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. In these instances, after homogenisation, diluted samples can be inoculated directly into double or single strength medium.

When low numbers of *E. coli* O157 are present, the presence of high numbers of competing organisms may inhibit the growth or detection of *E. coli* O157.

F6 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 be avoided, 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.

Strains of *E. coli* O157:H7 which produce Shiga toxin have been reclassified from "Hazard Group 2" to "Hazard Group 3" [6]. Where samples are not expected to contain *E. coli* O157:H7, routine processing may be undertaken in "Hazard Group 2" containment facilities, however they must be incubated within a Hazard Group 3" containment facilities. Where samples are expected to contain *E. coli* O157:H7, routine processing 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, WDCM 00014). 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[3] in this series. Principally appropriate membrane filtration apparatus, equipment for IMS and fan-assisted incubators are required. Other items may include:

F7.1 Sterile sample containers of appropriate volume and made of suitable material as outlined elsewhere[3] in this series.

F7.2 Incubators (or water baths) capable of maintaining a temperature of 37 ± 1.0 °C and 41.5 ± 1.0 °C.

F7.3 Stomacher and stomacher bags with or without integral mesh filter.

F7.4 Centrifuge capable of maintaining 200 – 300 g for 1 minute.

F7.5 Filtration apparatus, sterile or sterilisable, filter funnels and vacuum source

F7.6 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based, 0.45 mm nominal pore size.

F7.7 Glass fibre filters, for example 47 mm diameter

F7.8 Smooth-tipped forceps.

F7.9 Vortex mixer

F7.10 Rotary sample mixer for IMS mixing (for example, Dynal or equivalent) suitable for use with Eppendorf or screw-capped tubes

F7.11 Magnetic particle concentrator (for example, Dynal MPC-m or equivalent) suitable for use with Eppendorf or screw-capped tubes

F7.12 Eppendorf tubes and tube opener or suitable screw-capped tubes

F7.13 2N hydrochloric acid, 2N Sodium hydroxide where pH adjustment is necessary

F7.14 Timer

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[3]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated, chemical constituents should be added as the anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator, they should be allowed to reach room temperature before use.

F8.1 *Modified tryptone soya broth*[7]

Tryptone soya broth	30 g

Bile salts number 3	1.5 g
Dipotassium hydrogen phosphate	1.5 g
Novobiocin	20 mg
Water	1 litre

Dissolve the ingredients in the water and adjust the pH to 7.4 ± 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 ± 0.2. The sterilised medium may be stored at room temperature in the dark for up to one month, if protected from dehydration.

F8.2 *Buffered peptone water*[8]

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1 litre

Dissolve the ingredients in the water. Dispense the resulting solution in 90 ± 1.0 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 ± 0.2. Autoclaved media may be stored in the dark at room temperature for up to one month, if protected from dehydration.

F8.3 *Cefixime tellurite sorbitol MacConkey agar*[9]

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
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 ± 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 5 ± 3 °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 to1 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 5 ± 3 °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
Water	1 litre

Dissolve the ingredients in the water and check that the pH is 7.4 ± 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 ± 0.2. Allow the solution to cool. This solution can be stored in the dark at room temperature for up to one month.

F8.5 *Magnetic beads*[10]

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

F8.6 *Other media*

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

F9 Analytical procedure

F9.1 *Sample preparation*

Add 225 ml of modified tryptone soya broth to 25 g of sewage sludge in a suitable container. Mix thoroughly. Alternatively, add 225 ml of buffered peptone water 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].

Prior to homogenising the diluted sample, lime-stabilised samples should be adjusted to pH 7.0 ± 0.2 by the addition of 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 at 200 ± 10 rpm for 2 mins ± 10 seconds. Adjustments made to the timing / speed are acceptable, provided they are verified by the user.

Transfer the homogenised sample to suitable centrifuge tubes and centrifuge at 200-300 g for 1 minute. Decant the supernatant liquid from the centrifuge tubes to use for further analysis. Alternatively, if the sample contains material that may interfere with subsequent analysis, filter through a glass-fibre filter to remove fine debris.

In the event that sample processing cannot continue promptly, serial dilutions must be refrigerated at 5 ± 3 °C for not longer than 2 hours to prevent changes to the bacterial content of the sample.

F9.2 *Sample processing - Enrichment, immuno-magnetic separation and sub-culture to selective agar*

Thoroughly mix the modified tryptone soya broth or buffered peptone water from section F9.1 Incubate the modified tryptone soya broth at 41.5°C+/- 1°C for 21 hours and the buffered peptone water at 37 ± 1.0 °C for 21 hours[11, 12]. Enrichment broths should be subjected to IMS, firstly, after incubation for 6 - 7 hours, and then again at 21 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 ± 1.0 °C for 21 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 (see Figure F1). Strains of *E. coli* which ferment sorbitol are pink in colour (see Figure F1).

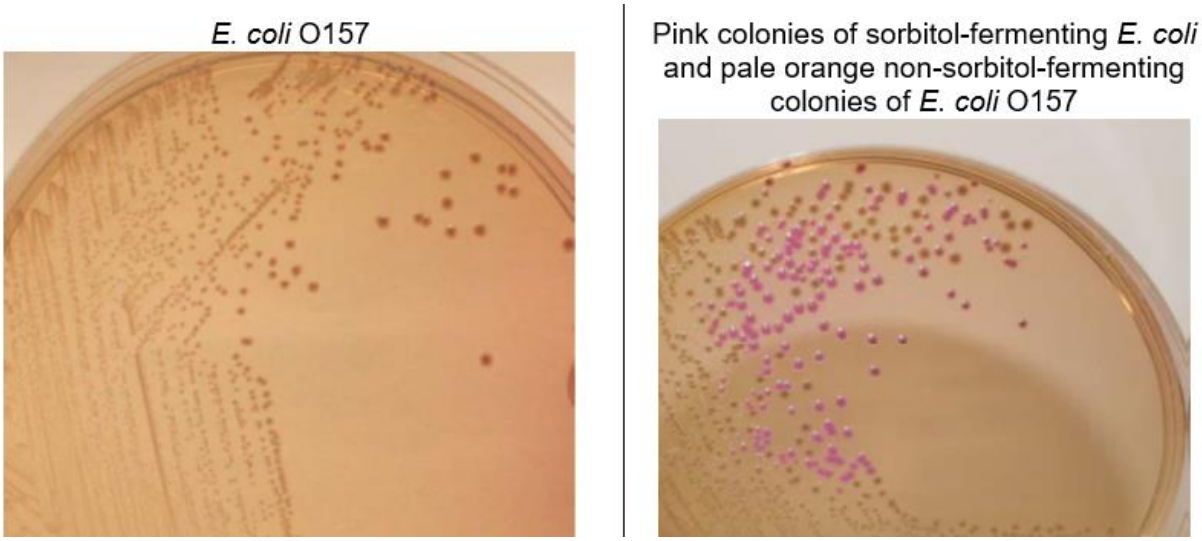

Figure F1 - Colonies on cefixime tellurite sorbitol MacConkey agar

F9.4 *Confirmation tests*

Inoculate typical colonies onto NA or similar non-selective agar (and MA if isolate purity needs to be checked) and incubate at 37 ± 1.0 °C for 21 hours ± 3 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, see Figure F2. Some isolates may require further identification by biochemical testing as some non-sorbitol-fermenting coliform bacteria (for example, *E. hermanii*) 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.

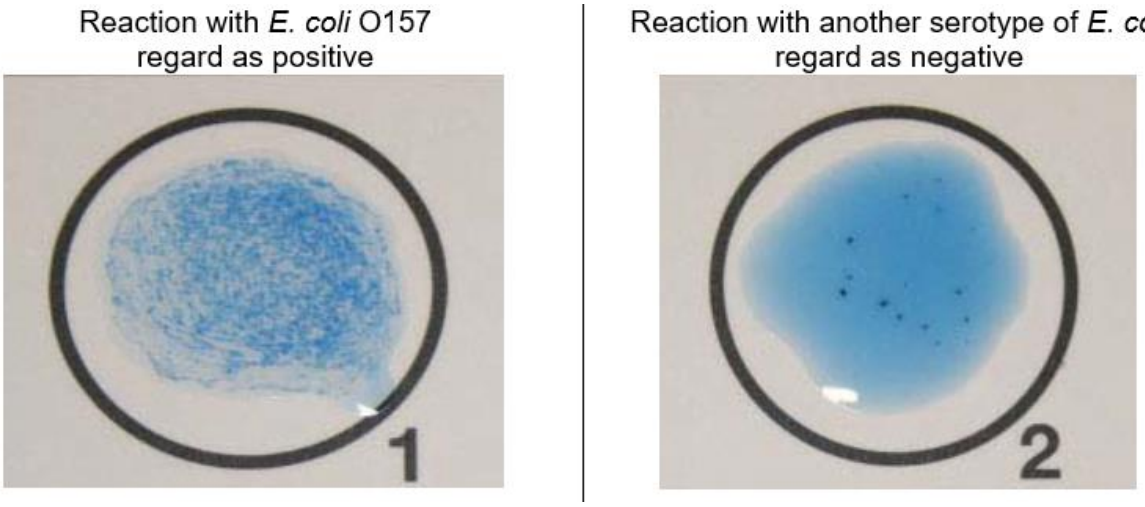

Figure F2 - Agglutination test

F10 Calculations

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

F11 Expression of results

E. coli O157 are reported as being detected or not detected in the wet or dry weight equivalent[13] of sludge analysed.

F12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, non- toxigenic *E. coli* O157) and non-target bacteria (for example, other strain of *E. coli*). Further details are given elsewhere[3] in this series.

F13 References

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Appendix 1

Evaluation of the storage time for prepared plates of membrane lactose glucuronide agar (MLGA)

1 Introduction

Membrane lactose glucuronide agar (MLGA) is widely used in the UK for the enumeration of *E. coli* and coliform bacteria from drinking water. In the original description of the medium it was stated that poured plates could be stored at 5 °C for up to one month (Sartory & Howard, 1992). Subsequent studies by Walter *et al.* (1994) indicated poorer recovery of *E. coli* and coliforms enumerated on MLGA stored aerobically for 14 days compared to recoveries on freshly prepared medium. They assumed that this may not have been noticed in the Sartory & Howard (1992) study as the number of samples analysed was very large and that it was unlikely that the MLGA used had been stored for an extended period. Further studies (Sartory, unpublished observations), however, also indicated poorer recovery with medium stored for one month compared to MLGA that was stored for less than seven days. As a result, the method utilising MLGA as described in *The Microbiology of Drinking Water Part 4* (SCA, 2002) and its subsequent editions limit the storage time for MLGA prior to use to one week.

Over the intervening 13 years anecdotal evidence has indicated that a longer acceptable storage time may be appropriate. Additionally, one major manufacturer of MLGA has stated that the medium can be stored for up to six weeks. Consequently, the SCA Working Group 2 decided to conduct a multi-laboratory study on the impact of storage on the performance of MLGA on the recovery of *E. coli* and coliform bacteria from water.

2 Study design

The aim of the multi-laboratory study was to investigate the shelf life of poured plates of MLGA in terms of:

its ability to isolate coliforms and *E. coli* from water at 37 °C
its ability to isolate *E. coli* at 44 °C from water and related materials

Additionally, data on the following colony characteristics were gathered:

the typical size, colour and number of coliform colonies obtained at 37 °C
the typical size, colour and number of *E. coli* colonies at 37 °C
the typical size, colour and number of *E. coli* colonies at 44 °C

The participating laboratories were asked to prepare a single batch of plates of MLGA sufficient for the analysis of contaminated water samples for up to six weeks. These were stored at 5 ± 3 °C for the duration of the study. These were to be used to compare

recoveries of *E. coli* and coliforms against “freshly prepared” MLGA (i.e. medium not more than seven days old).

For each sample four filtrations were performed and one membrane was placed on each of a “fresh” MLGA plate for incubation at 37 °C (for *E. coli* and coliforms), a “fresh” MLGA plate for incubation at 44 °C (for *E. coli*), a “stored” MLGA plate for incubation at 37 °C (for *E. coli* and coliforms) and a “stored” MLGA plate for incubation at 44 °C (for *E. coli*). All plates were incubated at 30 °C for four hours prior to incubation at their final temperature for 14 – 20 hours.

After incubation colonies were counted separately that were yellow or green and the number within these colours that were large or small and bright or pale in appearance was recorded. The results were recorded on a supplied pro-forma Excel spreadsheet.

3 Data analysis

Data were received from eleven laboratories and analysed according to ISO 17994 (ISO, 2014) by assigning counts from stored MLGA medium as the Trial Method and corresponding counts from fresh MLGA medium as the Reference Method. Data from the three sets of comparisons (coliform bacteria incubated at 37 °C, *E. coli* incubated at 37 °C and *E. coli* incubated at 44°C) were censored by the removal of pairs of data where both counts were zero or where at least one count of a paired count was recorded as either > 100 or TNTC (too numerous to count). The paired count data were transferred to an Excel 2007 spreadsheet and analysed according to the mean relative difference approach of ISO 17994. Briefly, the relative difference (x) of each pair of counts was calculated using the equation $x = 100(\ln(a) - \ln(b))$, where ln(a) is the natural logarithm of the count by the trial method (stored MLGA), and ln(b) is the natural logarithm of the count by the reference method (fresh MLGA). Data with a zero count by one method had plus one (i.e. count +1) added to each pair of the counts prior to log-transformation. As the objective of the study was to show there was no difference between the trial method with an established reference method, it was considered that the ‘two-sided’ comparison according to ISO 17994 was appropriate. The percentage value of the upper and lower limits was set at +10% and -10% as suggested by ISO 17994. Initially the combined data for all the three data sets was analysed, not taking into account the length of storage time for the stored MLGA medium. The effect of length of storage time of MLGA on recoveries of coliform bacteria and *E. coli* was then studied for each parameter.

4 Coliform bacteria comparison

For this study the term coliform bacteria refers to those isolates that produced yellow colonies on MLGA after incubation at 30 °C for four hours followed by incubation at 37 °C to give a total incubation time of 21 ± 3 hrs (i.e. non-*E. coli* coliforms). There were 443 paired counts for these bacteria and the result of the ISO 17994 analysis of the data is presented in Table 1. Unfortunately, despite the large number of results, the outcome of the analysis is inconclusive (i.e. more samples are needed). Using the

number of samples formula in ISO 17994 the total number of paired counts needed to achieve a conclusive outcome is 850 (i.e. 407 more). The “confidence limits” in Table 1 do, however, indicate a tendency for lower counts to be obtained on stored MLGA and further samples may well confirm this.

Table 1 Outcome of mean relative difference analysis of the paired counts of coliform bacteria on stored MLGA and on fresh MLGA incubated at 37 °C from 443 samples according to ISO 17994

Number of paired counts	Mean relative difference	Standard deviation	W*	X_L†	X_U‡	Outcome
443	- 4.46	65.03	6.18	- 10.64	1.72	Inconclusive

* Half width of the ‘confidence interval’ around the mean relative difference.
† Value of the relative difference at the lower ‘confidence limit’.
‡ Value of the relative difference at the upper ‘confidence limit’.

To ascertain the impact of storage time the data were simply plotted as the difference in counts against age of stored MLGA. The plot is presented in Figure 1. The visual data indicated little difference in counts up to about storage day 30 after which it appears that the stored MLGA tends to produce lower counts.

In order to assess the above observation the data were grouped into week lots for stored MLGA (i.e. 0 – 7 days, 8 – 14 days, 15 – 21 days, 22 – 28 days, 29 – 35 days and 36 – 49 days) and reanalysed according to ISO 17994. The results are presented in Table 2. Although the individual data sets are small for ISO 17994 analysis the outcomes do indicate probable comparable performance for MLGA stored up to 28 days compared to fresh MLGA. After that there is an indication of deterioration of performance in recoveries for stored MLGA which becomes significant after 35 days storage.

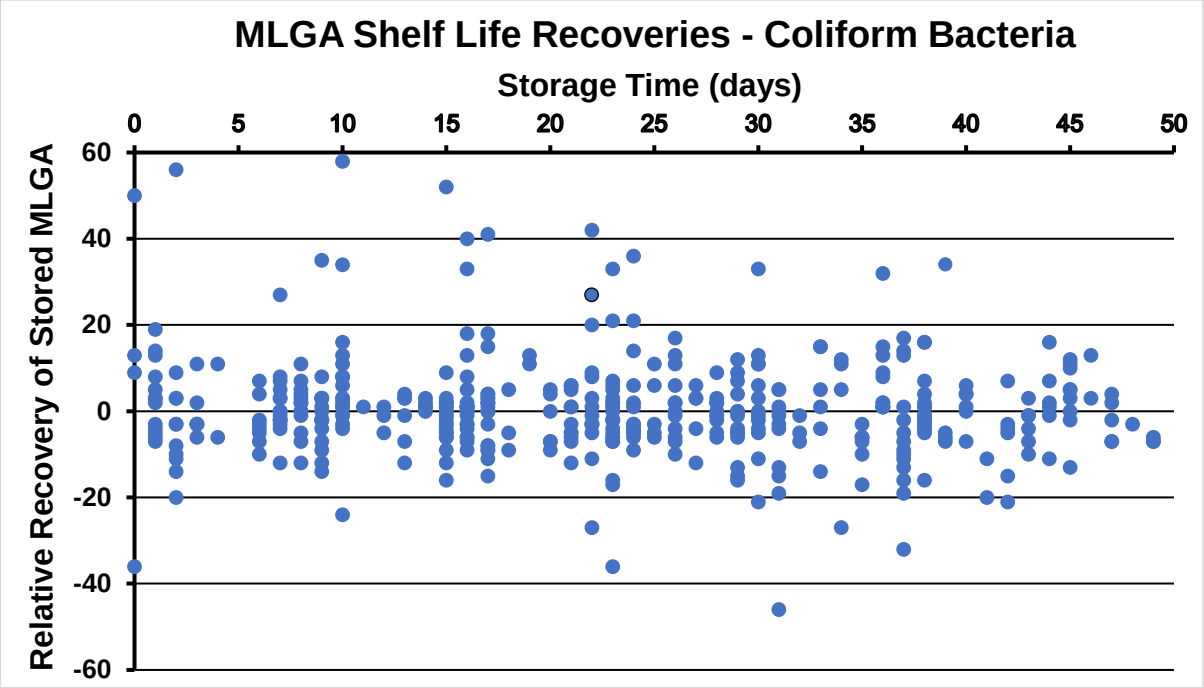

Figure 1 Plot of the difference in counts of non-*E. coli* coliforms on stored MLGA and fresh MLGA against storage time of stored MLGA before use

Table 2 Outcome of mean relative difference analysis of the paired counts of coliform bacteria on stored MLGA grouped into weekly lots compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 7	59	4.08	61.62	16.05	- 11.97	20.12	Inconclusive
8 – 14	62	10.51	88.70	22.53	- 12.02	33.04	Inconclusive
15 – 21	88	0.57	46.49	9.93	- 9.37	10.50	Inconclusive
22 – 28	84	- 0.91	46.07	10.05	-10.96	9.14	Inconclusive
29 – 35	57	- 17.44	73.00	19.34	- 36.78	1.89	Inconclusive
36 – 49	93	- 19.85	70.13	14.54	- 34.39	- 5.30	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To verify the acceptability of MLGA plates stored for up to 28 days for the enumeration of coliform bacteria the data were reanalysed to compare four week storage data against longer storage (Table 3). Again, although the data sets are small the outcomes indicate equivalent performance for MLGA stored for up to 28 days, but there was a significantly lower recovery after that period.

The results of these analyses indicate that MLGA plates stored for up to 28 days recover coliform bacteria to the same extent as MLGA stored for less than seven days.

Table 3 Outcome of mean relative difference analysis of the paired counts of coliform bacteria on MLGA stored for up to 28 days and for more than four weeks compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 28	293	2.95	60.55	7.07	- 4.12	10.07	Inconclusive
29 – 49	150	- 18.93	71.00	11.59	- 30.53	- 7.34	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

5 E. coli incubated at 37 °C comparison

There were 387 paired counts for *E. coli* enumerated after incubation at 37 °C and the result of the ISO 17994 analysis of the data is presented in Table 4. The statistical outcome for these samples is that there was no significant difference in recovery of *E. coli* between stored and fresh MLGA with the "confidence limits" of the analysis being well within those of +10% and -10% suggested by ISO 17994 for this type of comparison of performance.

Table 4 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA and on fresh MLGA incubated at 37 °C from 387 samples according to ISO 17994

Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
387	- 1.07	75.11	7.64	- 8.70	6.57	Methods not different

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To ascertain the impact of storage time the data was simply plotted as the difference in counts against age of stored MLGA. The plot is presented in Figure 2. The visual data indicate little difference in counts throughout the trial period.

Again, the data were grouped into week lots for stored MLGA (i.e. 0 – 7 days, 8 – 14 days, 15 – 21 days, 22 – 28 days, 29 – 35 days and 36 – 49 days) and reanalysed according to ISO 17994. The results are presented in Table 5. As previously the individual data sets are small for ISO 17994 analysis, but the outcomes do indicate probable comparable performance for MLGA stored up to 28 days compared to fresh MLGA. However, the data for 29 – 35 days stored plates indicate under-recovery by stored MLGA, but this is not reflected in the data for plates stored for more than 35 days which indicate a tendency for equivalent performance with fresh MLGA plates.

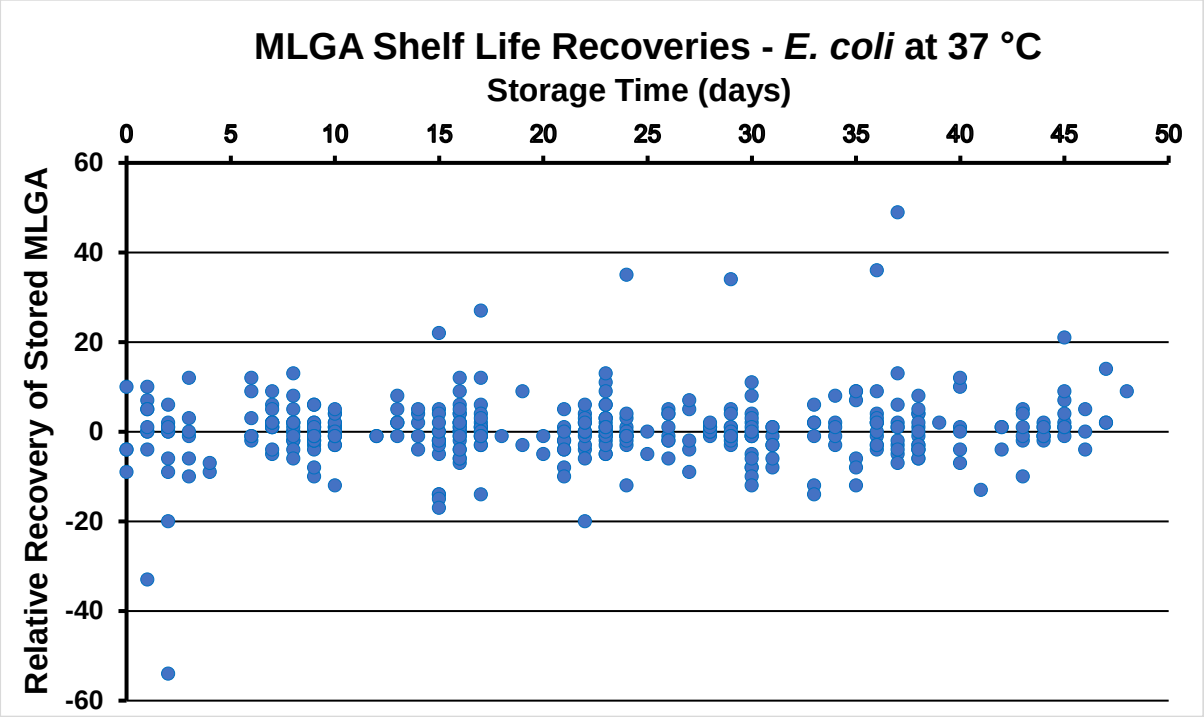

Figure 2 Plot of the difference in counts of *E. coli* incubated at 37 °C on stored MLGA and fresh MLGA against storage time of stored MLGA before use

Table 5 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA grouped into weekly lots compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 7	53	8.84	56.26	15.45	- 6.61	24.30	Inconclusive
8 – 14	62	7.12	70.52	17.91	- 10.79	25.03	Inconclusive
15 – 21	69	0.48	56.68	13.65	- 13.17	14.13	Inconclusive
22 – 28	73	1.29	51.12	11.97	- 10.68	13.25	Inconclusive
29 – 35	52	- 32.95	111.41	30.90	- 63.85	- 2.05	Stored MLGA lower recovery
36 – 49	78	3.38	88.67	20.08	- 16.70	23.46	Inconclusive

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To verify the acceptability of MLGA stored for up to 28 days for the enumeration of *E. coli* at 37 °C the data were reanalysed to compare four week storage data against longer storage (Table 6). Again, as the data sets may be small the outcomes indicate equivalent performance for MLGA stored for up to 28 days, but there is an indication of lower recovery after that period.

Table 6 Outcome of mean relative difference analysis of the paired counts of *E. coli* on MLGA stored for up to 28 days and for more than four weeks compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	W *	X_L†	X_U‡	Outcome
0 – 28	257	4.04	58.56	7.31	- 3.27	11.34	Inconclusive
29 – 49	130	-11.15	99.60	17.47	- 28.62	6.32	Inconclusive

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

The results of these analyses indicate that MLGA plates stored for up to 28 days and incubated at 37 °C recover *E. coli* to the same extent as MLGA stored for less than seven days.

6 *E. coli* incubated at 44 °C comparison

There were 367 paired counts for *E. coli* enumerated after incubation at 44 °C and the result of the ISO 17994 analysis of the data is presented in Table 7. The statistical outcome for these samples is that there was a significant under-recovery of *E. coli* by stored MLGA when incubated at 44 °C with the lower "confidence limit" being less than zero.

Table 7 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA and on fresh MLGA incubated at 44 °C from 367 samples according to ISO 17994

Number of paired counts	Mean relative difference	Standard deviation	W *	X_L†	X_U‡	Outcome
367	- 12.90	70.17	7.34	- 20.24	- 5.57	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To ascertain the impact of storage time the data were simply plotted as the difference in counts against age of stored MLGA. The plot is presented in Figure 3. The visual data indicate little difference in counts up to about storage day 25 after which it appears that the stored MLGA tends to produce lower counts.

To confirm the above observation the data were grouped into week lots for stored MLGA (i.e. 0 – 7 days, 8 – 14 days, 15 – 21 days, 22 – 28 days, 29 – 35 days and 36 – 49 days) and reanalysed according to ISO 17994. The results are presented in Table 8. As previously the individual data sets are small for ISO 17994 analysis but the outcomes do indicate probable comparable performance for MLGA stored up to 21 days compared to fresh MLGA. After that there is an indication of deterioration of performance in recoveries for stored MLGA which becomes significant after 28 days storage.

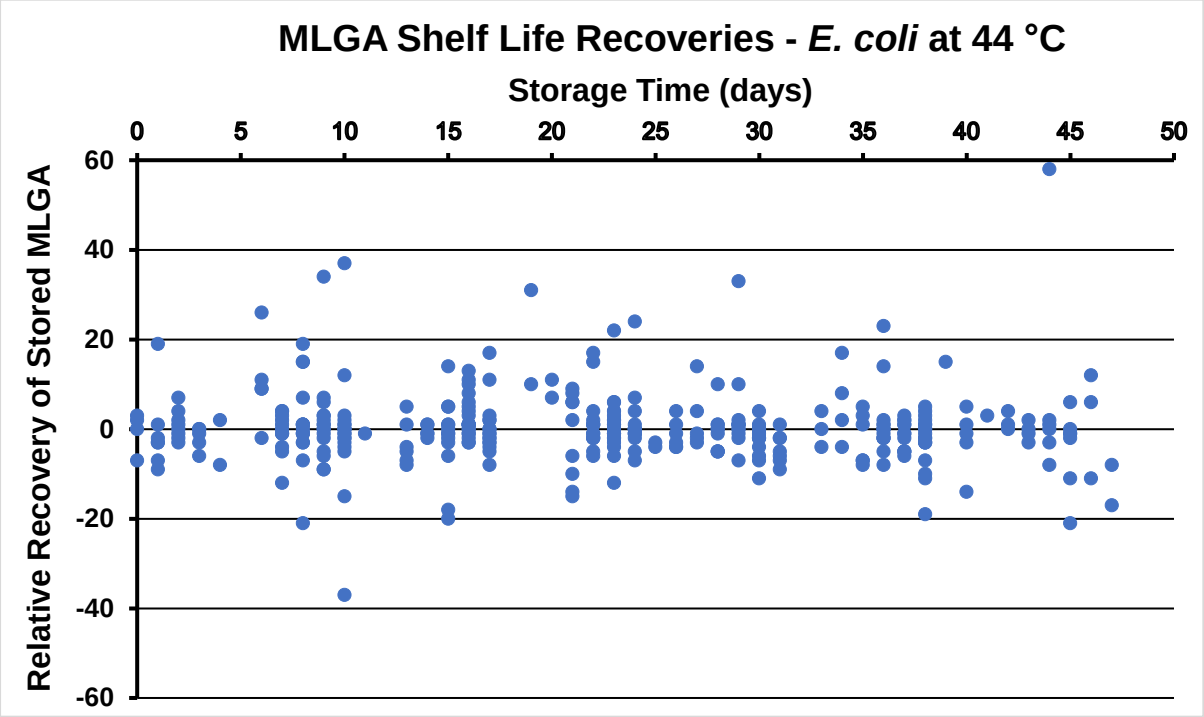

Figure 3 Plot of the difference in counts of *E. coli* incubated at 44 °C on stored MLGA and fresh MLGA against storage time of stored MLGA before use

Table 8 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA grouped into weekly lots compared to fresh MLGA incubated at 44 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	W *	X_L †	X_U ‡	Outcome
0 – 7	43	0.24	41.47	12.65	- 12.41	12.89	Inconclusive
8 – 14	62	- 4.75	71.24	18.09	- 22.85	13.34	Inconclusive
15 – 21	64	- 1.63	58.29	14.57	- 16.20	12.95	Inconclusive
22 – 28	75	- 8.56	51.55	11.91	- 20.46	3.35	Inconclusive
29 – 35	47	- 41.66	90.41	26.38	- 68.03	- 15.28	Stored MLGA lower recovery
36 – 49	76	- 22.98	86.85	19.92	- 42.90	- 3.05	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To verify the acceptability of MLGA stored for up to 21 or 28 days for the enumeration of *E. coli* at 44 °C the data were reanalysed to compare three and four week storage data against longer storage (Table 9). Again, although the data sets may be small the outcomes indicate equivalent performance for MLGA stored for up to 21 days, but there is an indication of lower recovery after that period with significant under-recovery on MLGA stored for more than 28 days.

Table 9 Outcome of mean relative difference analysis of the paired counts of *E. coli* on MLGA stored for up to 21 days for up to 28 days and for more than four weeks compared to fresh MLGA incubated at 44 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	W *	X_L†	X_U‡	Outcome
0 – 21	169	- 2.30	59.59	9.17	- 11.47	6.87	Inconclusive
0 – 28	244	- 4.22	57.21	7.32	- 11.55	3.10	Inconclusive
29 – 49	123	- 30.21	88.33	15.93	- 46.04	- 14.19	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

The results of these analyses indicate that MLGA plates stored for up to 21 days, and possibly 28 days, and incubated at 44 °C recover *E. coli* to the same extent as MLGA stored for less than seven days.

7 Impact of storage of MLGA on colony size and colouration

The data from the eleven laboratories contained information on the size and brightness of colouration of coliform and *E. coli* colonies on stored and fresh MLGA. The data, however, were not amenable for robust statistical analysis and so were examined visually. This did not reveal any obvious difference in the proportions of large or small colonies or in brightness of colonies for both coliforms and *E. coli* recovered on stored and fresh MLGA over the study period, regardless of the incubation temperature.

Two laboratories submitted observations on medium quality and performance for stored MLGA. One noted that stored MLGA became thinner in the Petri dish after three weeks storage with the Petri dish lids having excess condensation. This probably indicates loss of water during storage leading to shrinkage of the agar. Additionally, the laboratory reported reduction in "structural integrity" compared to fresh MLGA after approximately 17 – 18 days storage. The other laboratory reported an increase in the number of non-lactose fermenting "pink" colonies on MLGA stored for more than 40 days.

8 Conclusions and recommendations

This study assessed the recovery of coliform bacteria and *E. coli* on stored MLGA compared to recovery on fresh MLGA. The outcomes of ISO 17994 analyses of the data indicate that MLGA plates stored for up to 28 days provide equivalent recovery of coliforms and *E. coli* for plates incubated at 37 °C. For *E. coli* determinations at 44 °C the maximum storage time appears to be 21 days as the recovery of *E. coli* deteriorates after that storage time.

Thus, it is recommended that *The Microbiology of Drinking Water Part 4* is amended to allow storage of MLGA plates at 5 ± 3 °C for up to four weeks. Additionally, it is recommended that *The Microbiology of Recreational and Environmental Waters* is amended to allow storage of MLGA plates at 5 ± 3 °C for up to three weeks.

However, despite these findings, good practice would still be to use prepared media as fresh as practicable and that extended storage is dependent on correct storage of prepared media

9 Acknowledgements

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10 References

ISO (2014) *Water quality – Requirements for the comparison of the relative recovery of microorganisms by two quantitative methods*. ISO 17994:2014. International Organization for Standardization, Geneva, Switzerland.

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Walter KS, Fricker EJ & Fricker CR (1994) Observations on the use of a medium detecting β-glucuronidase activity and lactose fermentation for the simultaneous detection of *Escherichia coli* and coliforms. *Letters in Applied Microbiology*, **19**, 47-49.

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:

secretary@standingcommitteeofanalysts.co.uk

Members assisting with these methods.

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

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Amendment History

This bluebook has undergone a comprehensive review. Therefore, it is advised that users refamiliarise themselves with the entire publication to understand the changes, their significance and how this impacts them.

Due to the substantial number of revisions made, the amendment table below has not been used to record every change.

Page	Section(s)	Amendment
Full	Review	This bluebook underwent a substantial revision.

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analysts