SLOVENSKI STANDARD
SIST-TP CEN/TR 16193:2013

01-julij-2013

Blato, obdelani biološki odpadki in tla - Ugotavljanje prisotnosti in števila Escherichia coli

Sludge, treated biowaste and soil - Detection and enumeration of Escherichia coli

Schlamm, behandelter Bioabfall und Boden - Nachweis und Zählung von Escherichia coli

Boue, biodéchet traité et sol - Recherche et dénombrement des Escherichia coli

Ta slovenski standard je istoveten z: **CEN/TR 16193:2013**

ICS:

13.030.20	Tekoči odpadki. Blato	Liquid wastes. Sludge
13.080.30	Biološke lastnosti tal	Biological properties of soils

SIST-TP CEN/TR 16193:2013 **en,de**

TECHNICAL REPORT

RAPPORT TECHNIQUE

TECHNISCHER BERICHT

CEN/TR 16193

May 2013

ICS 13.030.01

English Version

Sludge, treated biowaste and soil - Detection and enumeration of Escherichia coli

Boue, biodéchet traité et sol - Recherche et dénombrement des Escherichia coli

Schlamm, behandelter Bioabfall und Boden - Nachweis und Zählung von Escherichia coli

This Technical Report was approved by CEN on 1 March 2011. It has been drawn up by the Technical Committee CEN/TC 400.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. CEN/TR 16193:2013: E

Contents

Page

Foreword

This document (CEN/TR 16193:2013) has been prepared by Technical Committee CEN/TC 400 “Project Committee - Horizontal standards in in the fields of sludge, biowaste and soil”, the secretariat of which is held by DIN.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

This document is part of a modular horizontal approach in which this document belongs to the analytical step.

The preparation of this document by CEN is based on a mandate by the European Commission (Mandate M/330). The mandate considers standards on sampling and analytical methods for hygienic and biological parameters as well as inorganic and organic determinants. It was the aim of the mandate to develop standards that are applicable to sludge, treated biowaste and soil and lead to equivalent results as far as this is technically feasible.

Until now, test methods determining properties of materials within the environmental area were prepared in Technical Committees (TCs) working on specific products/matrices (soil, waste, sludge etc). However, it is recognised that many steps in test procedures can be used in test procedures for other products/matrices. By careful determination of these steps and selection of specific questions within these steps, elements of the test procedure can be described in a way that can be used for more matrices and materials with certain specifications. This optimisation is in line with the development among end-users of standards. A majority of routine environmental analyses are carried out by institutions and laboratories working under a scope which is not limited to one single environmental matrix but covers a wide variety of matrices. Availability of standards covering more matrices contributes to the optimisation of laboratory procedures and standard maintenance costs, e.g. costs related to accreditation and recognition.

A horizontal modular approach was developed in the project 'Horizontal'. 'Modular' means that a test standard developed in this approach concerns a specific step in assessing a property and not the whole "chain of measurement” (from sampling to analyses). A beneficial feature of this approach is that “modules” can be replaced by better ones without jeopardising the standard “chain”.

The results of the desk study as well as the evaluation and validation studies have been subject to discussions with all parties concerned in the CEN structure during the development by project 'Horizontal'. The results of these consultations with interested parties in the CEN structure have been presented to and discussed in CEN/TC 400.

This Technical Report contains the most common detection and enumeration methods for the determination of *E. coli* consolidated in one document. The individual methods are specified in the following clauses:

- Clause 6: Method A - Membrane filtration method for quantification;
- Clause 7: Method B - Miniaturised method (Most Probable Number) by inoculation in liquid medium;
- Clause 8: Method C - Macromethod (Most Probable Number) in liquid medium.

Introduction

Escherichia coli is a non-pathogenic, Gram negative bacterium with a faecal origin. Consequently, it can be used as an indicator of faecal contamination. It can also be used to monitor the effectiveness of pasteurisation or disinfection treatments but it is comparatively sensitive (to heat, high pH) and cannot therefore reflect the behaviour of all pathogens in these materials.

This Technical Report contains three different methods for the detection and enumeration of *Escherichia coli* which were included in a validation trial in 2007.

The results achieved in this validation trial have been judged differently by experts. Consequently, it was decided by CEN/TC 400 to publish the methods as a Technical Report, aiming for further improvement of the methods and a later publication as European Standard.

Table 1 — Matrices for which the methods described in this Technical Report are applicable and tested in a validation trial

Matrix	Method A	Method B	Method C
Sludge	Mesophilic anaerobic digested sewage sludge	Mesophilic anaerobic digested sewage sludge	Mesophilic anaerobic digested sewage sludge
	Pelletised air dried sludge	Pelletised air dried sludge	Pelletised air-dried sludge
	Digested sewage sludge presscake	Digested sewage sludge presscake	Digested sewage sludge presscake
	Composted sewage sludge	Composted sewage sludge	Composted sewage sludge
Biowaste	Composted biowaste	Composted biowaste	Anaerobic treated biowaste
	Composted green waste	Composted green waste	Composted green waste
	Anaerobic treated biowaste	Anaerobic treated biowaste	Composted biowaste

WARNING — Persons using this Technical Report should be familiar with normal laboratory practice. This Technical Report does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

WARNING — Samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently, it is recommended that these samples be handled with special care. The gases which can be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. National regulations should be followed with respect to microbiological hazards associated with this method.

IMPORTANT — It is absolutely essential that tests conducted according to this Technical Report be carried out by suitably trained staff.

1 Scope

This Technical Report specifies three methods for the detection and enumeration of *Escherichia coli* in sludge, treated biowaste and soil:

— Method A - Membrane filtration method for quantification (see Clause 6);

— Method B - Miniaturised method (Most Probable Number, MPN) by inoculation in liquid medium (see Clause 7);

— Method C - Macromethod (Most Probable Number) in liquid medium (see Clause 8).

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 15934, *Sludge, treated biowaste, soil and waste — Calculation of dry matter fraction after determination of dry residue or water content*

EN ISO 9308-3:1998, *Water quality — Detection and enumeration of Escherichia coli and coliform bacteria in surface and wastewater — Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium (ISO 9308-3:1998)*

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
Escherichia coli
E.coli
β-D-glucuronidase-positive microorganism growing at an incubation temperature of 44 °C in the specified liquid medium containing 4-methylumbelliferyl-β-D-glucuronide (MUG)

[SOURCE: EN ISO 9308-3:1998]

Note 1 to entry: During growth, indole is produced from tryptophan and gas produced from lactose.

3.2
vegetative bacteria
bacteria which are capable of normal growth in broth or on agar media without pre-culture resuscitation

3.3
sub-lethally damaged bacteria
bacteria which have been stressed but not killed by storage or subsequent treatment by, e.g., mesophilic anaerobic digestion, lime stabilisation or composting, and therefore may not be recovered

3.4
resuscitation
recovery to vegetative growth of sub-lethally damaged bacteria previously incapable of growth on agar media

3.5
quantitative resuscitation
recovery to vegetative growth of sub-lethally damaged bacteria isolated discretely on a membrane filter, prior to transfer to chromogenic medium for growth of individual colonies

3.6
colony forming unit
cfu
growth of individual bacterial cells into visible colonies on agar media, including on membrane filters overlaying the agar media

3.7
Most probable number
MPN
every well whose inoculum contains even one viable organism will produce detectable growth or change

Note 1 to entry: The individual wells of the sample are independent.

4 Abbreviations

BCIG: 5-bromo-4-chloro-3-indolyl-β-glucuronide

CN: Characteristic number

DS: Dry solid

E. coli: *Escherichia coli*

MLGA: Membrane Lactose Glucuronide Agar

MPN: Most Probable Number

MUG: 4-methylumbelliferyl-β-D-glucuronide

SMD: Special Microplate Diluent

5 Quality assurance

Suitable quality control procedures, at least those described in ISO 8199, shall be applied.

6 Method A — Membrane filtration method for quantification

6.1 Scope

Method A specifies a membrane filtration procedure for the quantitative detection, by culture of individual colonies on chromogenic agar media. It is not suitable for materials whose treatment will significantly reduce bacterial levels to less than 10 viable *E. coli* per g wet weight, such as lime addition, drying or pasteurisation.

This membrane filtration method is not appropriate for enumeration and detection of other coliform bacteria without modifications to the chromogenic agar medium.

It is suitable to evaluate the log reduction of *E. coli* through treatment, as well as the quality of the end product.

Method A has a limit of detection of approximately 27 *E. coli* cfu · g^{-1} wet weight according to ENV ISO 13843, dependent on the solids content which at high concentrations (> 0,1 g/ml) may restrict filtration of the sample volume through the membrane if not first diluted.

6.2 Principle

The homogenised diluted sample is filtered, the membrane filter recovered aseptically and incubated on membrane lactose glucuronide agar (MLGA), initially at (30 ± 1) °C for (4,0 ± 0,5) h. Subsequently, the temperature is increased to (44 ± 1) °C for (16 ± 2) h. The presence of *E. coli* is indicated by green colonies resulting from the hydrolysis of BCIG.

6.3 Reagents, diluents and culture media

6.3.1 General instructions

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with demineralised or distilled water free from substances capable of inhibiting growth under the test conditions (see ISO 8199).

The use of chemicals of other grades is permissible provided that they are shown to be of equivalent performance in the test.

6.3.2 Peptone saline solution

Bacteriological peptone 1,0 g

Sodium chloride 8,5 g

Distilled water 1 000 ml

Sodium hydroxide solution

Hydrochloric acid, 1mol/l

Dissolve the bacteriological peptone and sodium chloride into distilled water. Adjust the pH by adding sodium hydroxide solution or hydrochloric acid so that, after sterilisation, it will correspond to (7,0 ± 0,5) at 25 °C.

Sterilise in the autoclave (6.4.1) at (121 ± 3) °C for (15 ± 1) min. Store at (5 ± 3) °C for a maximum of 3 months.

6.3.3 Membrane Lactose Glucuronide Agar (MLGA)

6.3.3.1 5-bromo-4-chloro-3-indolyl-β-glucuronide (BCIG) suspension

BCIG, monohexylammonium salt 0,2 g

Aqueous ethanol, 95 % 2,5 ml

Sodium hydroxide, 1 mol/l 0,5 ml

Dissolve 200 mg BCIG in a combined solution of 95 % aqueous ethanol and 1 mol/l sodium hydroxide.

6.3.3.2 MLGA

Peptone 40,0 g

Yeast extract	6,0 g
Lactose	30,0 g
Sodium lauryl sulphate	1,0 g
Phenol red	0,2 g
Sodium pyruvate	0,5 g
Bacteriological agar	10,0 g
Demineralised or distilled water	1 000 ml

Mix all ingredients and bring to the boil whilst stirring continuously.

Add the BCIG suspension to the molten base agar medium and mix thoroughly. Adjust the pH to (7,0 ± 0,5).

Sterilise by autoclaving at (121 ± 3) °C for (15 ± 1) min. Pour into 55 mm Petri dishes in volumes of approximately 10 ml. Allow setting and store refrigerated at (5 ± 3) °C in the dark. Use within 7 days.

6.3.4 MacConkey Agar

Peptone	20,0 g
Lactose	10,0 g
Bile salts	5,0 g
Sodium chloride	5,0 g
Neutral red	0,075 g
Agar	12,0 g
Distilled water	1 000 ml

Suspend the ingredients in 1 000 ml of distilled water. Bring to the boil whilst stirring continuously to dissolve all ingredients completely. Adjust the pH to (7,0 ± 0,5).

Sterilise in the autoclave (6.4.1) at (121 ± 3) °C for (15 ± 1) min. Store at (5 ± 3) °C for a maximum of 1 month. Dry the surface of the agar before inoculation.

6.4 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199.

Usual microbiological laboratory equipment and in particular:

6.4.1 Apparatus for sterilisation – autoclave.

6.4.2 Thermostatic incubator(s) adjustable to (30 ± 1) °C and/or (44 ± 1) °C.

6.4.3 Homogeniser

6.4.4 Centrifuge, capable of centrifuging 50 ml at 200 g[1)] to 300 g.

6.4.5 Membrane filters, 0,45 µm gridded, cellulose nitrate.

6.4.6 Glass fibre pre-filter discs, 47 mm diameter, pore size 2,7 µm.

6.4.7 Vacuum pump

6.4.8 Vacuum manifold – magnetic filter bases and cups.

6.4.9 Sterile homogeniser bags, 250 ml volume, with or without integrated mesh to exclude large particulate matter.

6.4.10 Sterile Petri dishes, 50 mm in diameter, for holding MLGA medium.

6.4.11 Sterile universals of 20 ml volume, or containers with similar capacity.

6.4.12 Sterile pipettes, glass or disposable plastic ware, capable of dispensing 1 ml and 10 ml volumes.

6.4.13 Sterile conical centrifuge tubes, 50 ml volume, disposable plastic.

6.4.14 Tweezers, capable of sterilisation by immersion in ethanol and subsequent flaming.

6.4.15 Analytical balance

6.4.16 Refrigerator, capable of maintaining (5 ± 3) °C.

6.4.17 Vortex mixer

6.4.18 pH meter with an accuracy of ± 0,1.

6.4.19 Beakers or containers, 100 ml, 250 ml and 1 000 ml.

6.4.20 Laboratory spatula

6.4.21 Boiling bath

6.4.22 Bunsen burner

6.4.23 Sterile forceps

6.4.24 Filter funnels

6.5 Sampling

6.5.1 General

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 h). In order to prevent propagation or inactivation of *E. coli* during transport to the laboratory and subsequent storage, refrigerate the sample at (5 ± 3) °C.

Samples are liable to ferment and may contain pathogenic micro-organisms. It is essential to keep them away from any food or drink, and to protect any cuts. When transporting and handling samples, it is essential that national and international regulations relating to bio-hazardous samples are followed.

1) g = 9,81 m·s^{-2}

See also the Warning note in the introduction.

6.5.2 Storage

Do not store samples in the open laboratory. If samples are to be stored, store them at (5 ± 3) °C for no longer than 72 h after receipt.

6.5.3 Handling

Cleanliness when working is essential. When handling sludge samples, it is necessary to wear gloves, face and eye protection, and sufficient body protection to guard against bottles bursting. The gas evolved is usually flammable, so all equipment used in the vicinity shall be flame proof to avoid any source of ignition.

6.6 Procedure

6.6.1 Sample preparation

6.6.1.1 General

Weigh a representative 10 g (wet weight) of the sample as received into a 250 ml container (6.4.19).

Add an appropriate volume of peptone saline solution (6.3.2) so that the final weight is 100 g and mix thoroughly using a vortex mixer (6.4.17).

Place in homogeniser bag (6.4.9) and place in the homogeniser (6.4.3) and homogenise for 2 min to obtain the sample suspension (dilution A). For samples with a dry solid content > 20 % a homogeniser bag with an integrated mesh should be used. For samples with dry solid content < 20 % a homogeniser bag without integrated mesh can be used.

6.6.1.2 For lime-treated materials

Adjust the pH to (7,0 ± 0,5) with 1 mol/l hydrochloric acid (6.3.2). The sample is mixed by shaking between each addition of hydrochloric acid to ensure the correct pH is achieved. The sample is transferred to a sterile 250 ml container and tested using a pH meter (6.4.18).

If the pH drops below 4,5 during the neutralisation process, start a new analysis with a fresh test portion.

For other relevant treatment chemicals (e.g. peracetic acid), a suitable oxidant neutralisation procedure shall be used (e.g. EN 1040).

Centrifugation and pre-filtration: The variation in the level of solid material contained within the matrices applicable to Method A means that some samples will require centrifugation and pre-filtration before they can be processed by membrane filtration without blocking the membrane. Not all samples will require these optional steps; if necessary then centrifugation and pre-filtration should be applied.

6.6.1.3 Centrifugation (optional)

Transfer the homogeniser bag contents to two disposable centrifuge tubes (6.4.13) and centrifuge the two 50 ml aliquots at 200 g to 300 g for 3 min.

6.6.1.4 Pre-filtration (optional)

Decant the supernatant from the tubes in a beaker and filter through a glass-fibre pre-filter (6.6) using a filter funnel with receiver (6.4.24) to remove fine debris.

The filter funnels should have been sterilised in a boiling bath (6.4.21) prior to analysis, the filter funnels are removed from the boiling bath using sterile forceps (6.4.23) and attached to the vacuum pump (6.4.7).

The glass fibre filter (6.4.6) is placed on the filter funnel using sterile tweezers (6.4.14) before the filter funnel cup is secured in position. The vacuum may now be used to draw the sample through the glass fibre filter; it is recommended that the sample is not all introduced to the filter funnel cup at the same time because blockages may occur.

The filter funnels should be returned to the boiling bath and be sterilised for a minimum of 5 min before being used again.

6.6.2 Sample dilution

The number of dilutions to subsequently filter varies according to the presumed level of contamination of the material to be tested. Typically, dilution A (the filtrate) should be serially diluted 10^{-1} to 10^{-3} with peptone saline solution (6.3.2). This will permit the enumeration of up to 104 *E. coli* per g wet weight sample. Samples with greater concentrations or counts of bacteria will require additional dilutions of the filtrate to 10^{-8} (e.g. untreated sludge may contain 10^{8} to 10^{9} *E. coli* per g wet weight).

Prepare the relevant number of sterile universals (6.4.11) according to the number of selected dilutions. Add 9 ml of sterile peptone saline solution (6.3.2) to each.

Using a sterile pipette (6.4.12), transfer 1 ml of the filtrate to the first universal containing 9 ml of peptone saline solution (6.3.2) and mix thoroughly using a vortex mixer (6.4.17).

Using a fresh pipette (6.4.12), transfer 1 ml of the diluted sample to the second universal containing 9 ml of peptone saline solution (6.4.2) and mix thoroughly using a vortex mixer (6.4.17).

Continue as above until all the dilutions have been prepared.

6.6.3 Membrane filtration

Transfer the magnetic filter base (6.4.8) from the boiling water bath (6.4.21) to the manifold using sterilised forceps (6.4.23).

Membranes (6.4.5) are removed from their packets with sterilised tweezers (6.4.14) and placed grid side up onto the magnetic filter base. Membranes that have torn are dropped or which touch any object shall be discarded. Membranes should only be handled by the edge with tweezers designed for that purpose. The tweezers shall be sterilised first by immersing them in ethanol which is subsequently flamed off using a Bunsen burner (6.4.22), and then dipping directly into boiling water.

The magnetic filter cup is then removed from the boiling bath and attached to the magnetic base taking care not to wrinkle the membrane. Magnetic filter cups should only be removed from the boiling bath with disinfected forceps and placed directly onto magnetic bases. Hands may be used to transfer magnetic filter cups back to the boiling bath. Neither forceps nor tweezers should be placed directly onto the bench. If the filtration equipment is left for any significant length of time, the magnetic bases shall be returned to the boiling bath. Magnetic bases left unused for short periods can be covered with the base or lid of a sterile Petri dish until filtration recommences.

Add a sufficient volume of peptone saline solution (15 ± 5) ml into the filter cup, pipette 1 ml of the diluted sample into the filter cup. Replace the top on the universal. Place the used universal back into the rack. The universal shall not be placed on the filtration bench.

The sample may now be drawn through the filter by vacuum and only when filtration is complete should the vacuum be turned off. The magnetic filter cup is lifted off, and returned to the boiling bath.

The membrane is carefully removed using sterile tweezers and transferred to the MLGA 55 mm Petri dish (6.4.10). The membrane should be ‘rolled’ into the plate to prevent air bubbles becoming trapped between the

growth medium and the membrane, and the lid of the Petri dish is replaced. Bubbles should be excluded so that the membranes are in intimate contact with the agar surface allowing unrestricted growth of viable bacteria present on the membrane surface.

Any wrinkled or torn membranes discovered after filtration shall be discarded. The magnetic filter base shall then be re-sterilised and the dilution filtered again.

Once filtration of samples is complete, the filter funnels are placed in to the boiling water bath for disinfection. They shall be totally immersed in boiling water for at least 2 min before being removed to continue filtration.

A positive control suspension containing 10^2 to 10^3 target organisms is prepared using stock cultures (i.e. reference material). The positive control sample should be analysed as the last sample in the analytical run. A blank control suspension is prepared using peptone saline solution. The blank control should be analysed at the beginning and as the penultimate sample of the analytical run.

6.6.4 Resuscitation and enumeration of colonies on chromogenic agar

Remove the filter from the housing using sterile tweezers (6.4.13) and transfer to the surface of a 55 mm diameter MLGA plate (6.3.3). Incubate plates initially at (30 ± 1) °C for (4,0 ± 0,5) h. Subsequently, increase the temperature to (44 ± 1) °C for (16 ± 2) h.

Enumerate typical green colonies by eye, only plates within the range 10 to 100 colonies should be considered for the expression of results. If no counts are in this range it may be appropriate to consider counts outside this range provided that an accurate enumeration is possible. The number of typical colonies that are identified for confirmation is determined by the experience of the analyst.

When enumerating typical colonies, be aware that strains in environmental samples can give pale green colonies on initial isolation and these should be considered for confirmation.

6.6.5 Confirmation of colony identity

The typical colonies are sub-cultured onto selective MacConkey agar (6.3.4). The MacConkey subculture plates are incubated at (36 ± 2) °C for (21 ± 3) h.

It is important to subculture any green colonies suspected of being *E. coli* regardless of colour alone: a minimum of two colonies per plate, per sample; and a maximum of each morphological type per plate and per sample should be taken for subculture.

Typical green colonies on MLGA plates corresponding to typical red colonies observed by eye on MacConkey plates should be considered as confirmed *E. coli* colonies for the expression of results.

For further confirmation additional biochemical tests can be performed.

6.6.6 Determination of the dry residue content

The numbers of *E. coli* may be calculated per wet weight or dry weight. For the latter, it is necessary to determine the dry residue of the sample using the method specified in EN 15934. This shall be performed in parallel with the microbiological analysis.

6.7 Calculation and expression of results

Calculation of the number of *E. coli* (present per g wet weight of the original sample) is by dividing the total number of typical colonies (n) on the filter membrane of the selected plates (9.4) by the total volume filtered of the initial sample. The result of the confirmation step shall be taken into account to estimate the total number of typical colonies to calculate the final result (see ISO 8199).