PD CEN/TR 16193:2013

BSI Standards Publication

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

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National foreword

This Published Document is the UK implementation of CEN/TR 16193:2013.

This technical report contains details of three methods evaluated for their suitability for 'horizontal' application for sludge, treated biowaste and soil. The UK committee believes that these methods are not fit for their intended purpose and are not appropriate to samples of all such materials, as the sample matrices are not sufficiently similar.

It should be noted that in merging three separate drafts to achieve the final text, incompatibilities and conflicting interpretation have been introduced:

— the document does not adhere to a recognised definition of *E. coli* as target organism (see SCA methods referred to below);

— the inter laboratory validation undertaken (as found in Tables 4, 9 and 14) is considered by UK technical experts as demonstrating poor reproducibility;

— soil matrices were not included in the validation.

The recommended methods for UK laboratories intending to analyze waste-water sludge can be found in 'The Microbiology of Sewage Sludge: Part 3 (2003) — Methods for the isolation and enumeration of *Escherichia coli,* including verocytotoxigenic *Escherichia coli*' published by the Environment Agency Standing Committee of Analysts (SCA) in the series 'Methods for the examination of water and associated materials'.

SCA methods are also considered suitable for determining *E. coli* in some treated biowaste applications such as compost, under PAS 100:2005, and digestates, under PAS 110:2010. BS ISO 16649-2:2001 provides a method suitable for enumeration of *E. coli* in food and animal feeding stuffs. These methods might also be applicable to soil but this would need to be verified using the approach described in DD ENV ISO/TR 13843:2001, *Water Quality — Guidance on Validation of Microbiological Methods*.

The UK participation in its preparation was entrusted to Technical Committee H/-/4, Environmental testing programmes.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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TECHNICAL REPORT

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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.

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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 $\pm$ 1) °C for (4,0 $\pm$ 0,5) h. Subsequently, increase the temperature to (44 $\pm$ 1) °C for (16 $\pm$ 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).

$$c = \frac{n}{V} \quad (1)$$

where

c is the *E. coli* concentration per gram (g) wet weight of original sample;

n is the total number of typical *E. coli* colonies on the selected filter membranes: $n = n_1 + n_2 + \ldots$;

V is the total volume filtered through the selected filter membranes (from 1 ml): $V = V_1 + V_2 + \ldots$;

The dilution factor of the dilution A taken for filtration step should not be forgotten in the final calculation.

EXAMPLE If the volume of the test dilution used (V_i) is 1 ml of dilution A and the following counts are obtained at the respective dilutions:

Dilution	Counts
10-2	81 colonies
10-3	15 colonies

Then:

n = 81 + 15 = 96

V = (0,1 × 1 × 0,01) + (0,1 × 1 × 0,001)

c =96/0,001 1 = 8,7 104 cfu/g ww

Numbers present per g dry weight of sample are calculated according to Formula (2):

$$c = \frac{n}{Ve} \times 100 \quad (2)$$

where

e is the dry residue of the original wet sample in percent, %.

6.8 Performance data of the interlaboratory comparison — Method A

6.8.1 Material used in the interlaboratory comparison study

The interlaboratory comparison of the membrane filtration method for quantification of *E. coli* in soil, sludge and treated biowaste took place from May to July 2007. It was carried out with 14 European laboratories on 7 different matrices. The matrices selected for the interlaboratory comparison were chosen to represent soil, sludge and biowaste as broadly as possible, because Method A will find general application across different types of soil and soil related materials (detailed information can be found in the final report on the interlaboratory comparison study).

Table 2 provides a list of the matrices chosen for *E. coli* detection.

Table 2 — Matrices tested in the interlaboratory comparison trial

Matrix type	Abbreviation
Mesophilic anaerobic digested sewage sludge	**MAD**
Anaerobic treated biowaste	**ATB**
Pelletised air-dried sludge	**PADS**
Digested sewage sludge presscake	**DSSP**
Composted sewage sludge	**CSS**
Composted green waste	**CGW**
Composted biowaste	**CBW**

In the interlaboratory comparison study, the following starting points were used:

The laboratory samples were all taken from a large batch of the different matrices according to the normal practice. The choice was made to analysed only spiked samples so as to obtain positive results. The spiking, the mixing and the sub-sampling were carried out as needed to prepare representative laboratory samples of approximately 150 g from the large batch sample. These were sent out by courier to each of the participating laboratories.

The experimental plan requested information on the basis of each laboratory being given 3 laboratory samples of each of the 2 batches of the seven matrices to be tested.

6.8.2 First assessment of the precision of the method

The statistical evaluation was conducted according to ENV ISO 13843. The limit of detection, the upper limit of quantification, the range of quantification and the results of dispersion U^2 were obtained (Table 3).

The limit of detection corresponds to the number of particles (germs per test portion) when the probability of a negative result is 5 % (superior limit of the confidence interval of the null result).

Poisson distribution corresponds to the random distribution of the number of particles at the moment of sampling a perfectly homogenised suspension.

The relative variance U^2 corresponds to the relative standard deviation squared ratio of the standard deviation squared and the mean squared as $U^2 = s^2 / m^2$.

NOTE 1 This statistic is commonly used to express dispersion or uncertainty of microbiological test results.

Table 3 — Summary of components of the *E. coli* Membrane filtration method precision

Limit of detection 5 % *E. coli*/g wet weight	Upper limit of quantification 5 % *E. coli*/g wet weight	Range of quantification Log10 unit	Results of dispersion U^2
26,96	$1{,}32\ 10^{11}$	9,7	Less than 0,05

NOTE 2 In judging the results it is important to consider that they do not depend on the experimental data but only on the design of the measurement protocol (random variation).

6.8.3 Interlaboratory comparison results

The statistical evaluation was conducted according to ISO 5725-2. The average values, the repeatability (r) and the reproducibility (R) were obtained (Table 4).

The repeatability corresponds to the maximum difference that can be expected (with a 95 % statistical confidence) between one test result and another, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the method by the same laboratory using its own facilities and testing laboratories samples obtained from the same primary field sample and prepared under identical procedures.

The repeatability limit was calculated using the relationship: $r_{\text{test}} = f \cdot \sqrt{2} \cdot s_{r.\text{test}}$ with the critical range factor $f = 2$.

NOTE 1 The above relationship refers to the difference that may be found between two measurements results performed each on two laboratory samples obtained under the same conditions. The value f = 2 used in the factor $f \cdot \sqrt{2}$ corresponds to the theoretical factor of 1,96 for a pure normal distribution at 95 % statistical confidence. Also, this value $f = 2$ corresponds to the usual value $k = 2$ of the coverage factor. However, it may be necessary to a use a larger value for f in situations as described Clause 12.

The reproducibility, like repeatability corresponds to the maximum difference that can be expected (with a 95 % statistical confidence) between one test result and another test result obtained by another laboratory, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present method by two different laboratories using their own facilities and testing laboratory samples obtained from the same primary field sample and prepared under identical procedures.

This reproducibility limit was calculated using the relationship: $R = 2 f \cdot \sqrt{2} \cdot s_R$ with the critical range factor $f = 2$.

NOTE 2 The above relationship refers to the difference that may be found between two measurements results performed each on two laboratory samples obtained under the same conditions. The value $f = 2$ used in the factor $f \cdot \sqrt{2}$ corresponds to the theoretical factor of 1,96 for a pure normal distribution at 95 % statistical confidence. Also, this value $f = 2$ corresponds to the usual value $k = 2$ of the coverage factor. In the case when reference is made to the dispersion of the values that could reasonably be attributed to the parameter being measured, the dispersion limit is equal to $k \cdot s_R$ with the usual value $k = 2$, resulting in a dispersion limit lower than the reproducibility limit (i.e. a ration of $\sqrt{2}$). However, it may be necessary to use a larger value $f \cdot \sqrt{2}$ (or k) in situations as described in Clause 9.

In case of relatively heterogeneous materials, the repeatability and the reproducibility limits may be larger than the values given in Table 4 (this means that the value chosen for the critical range factor f is larger than 2 as well as for the coverage factor k for dispersion). This is because the extreme results may have been obtained in accordance with the present method and/or be caused by the variability within, or in between, the laboratory samples.

For the calculations, as the test results were expressed on a log scale, the standard deviations in repeatability and Reproducibility conditions, respectively s_r and s_R were also expressed on a log scale. The expression of repeatability and Reproducibility in terms of maximum difference that can be expected between one test and another is given then by the limit of repeatability and Reproducibility respectively $r = 2\sqrt{2} \cdot s_r$ and $R = 2\sqrt{2} \cdot s_R$.

In order to make easier the interpretation, the values of repeatability and reproducibility are expressed in terms of maximal difference between two independent measurements on log scale, with a confidence level of 95 %:

EXAMPLE 1 Assuming r_1 and r_2 two independent measurements observed for a given method in repeatability conditions with $r_1 > r_2$:

$\log(r_1) - \log(r_2) \leq 0{,}9$ (95 % of the cases), corresponding to almost 1 log of difference between results.

The deviations between test results obtained under repeatability and reproducibility conditions can also be expressed by the maximal ratio between two independent measurements on natural scale (number of germs), with a confidence level of 95 %.

EXAMPLE 2 $\log(r_1) - \log(r_2) \leq 0{,}9$ (95 % of the cases)

then $r_1 / r_2 \leq 10^{0,9}$

then $r_1 / r_2 \leq 7{,}9$

thus r_1 is significantly higher than r_2 if $r_1 / r_2 > 7{,}9$.

Table 4 — Summary of *E. coli* membrane filtration method results of inter-laboratory comparison

Matrix	Overall mean (*E. coli* /g wet weight)	Repeat-ability (Ratio)	Reproduc-ibility (Ratio)	Discarded outliers (Statistical fitness)	Removed data (Other reasons)	Total number of data	Total number of labo-ratories
Mesophilic anaerobic digested sewage sludge	< 26,96[a]	–	–	–	–	0	13
	< 26,96[a]	–	–	–	–	3	13
Anaerobic treated biowaste	1 784 260	2,8	59,4	2	–	33	13
	2 898 637	3,5	72	–	–	36	12
Pelletised air dried sludge	< 26,96[a]	–	–	–	–	0	13
	385 222	7,1	29 465,7	–	1	36	13
Digested sewage sludge presscake	6 632	3,2	7 499,5	–	–	35	12
	3 651	4,1	655,8	–	–	36	12
Composted sewage sludge	94 944 250	11,9	34,8	1	–	33	12
	968 981	5,9	88,1	1	–	36	13
Composted green waste	824 211	3,6	51,7	1	–	36	13
	433 007	2,5	32,3	–	–	39	13
Composted biowaste	7 883[b]	262,7[b]	262,7[b]	–	–	12	13
	16 967 925	4,3	33,6	–	–	39	13

[a] Theoretical limit of detection with a probability of 95 % calculated for the method.

[b] Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.

NOTE 3 In judging the results it is important to consider the concentrations levels, at which measurements have been carried out.

6.9 Pre-filtration and centrifugation — Comparison tests

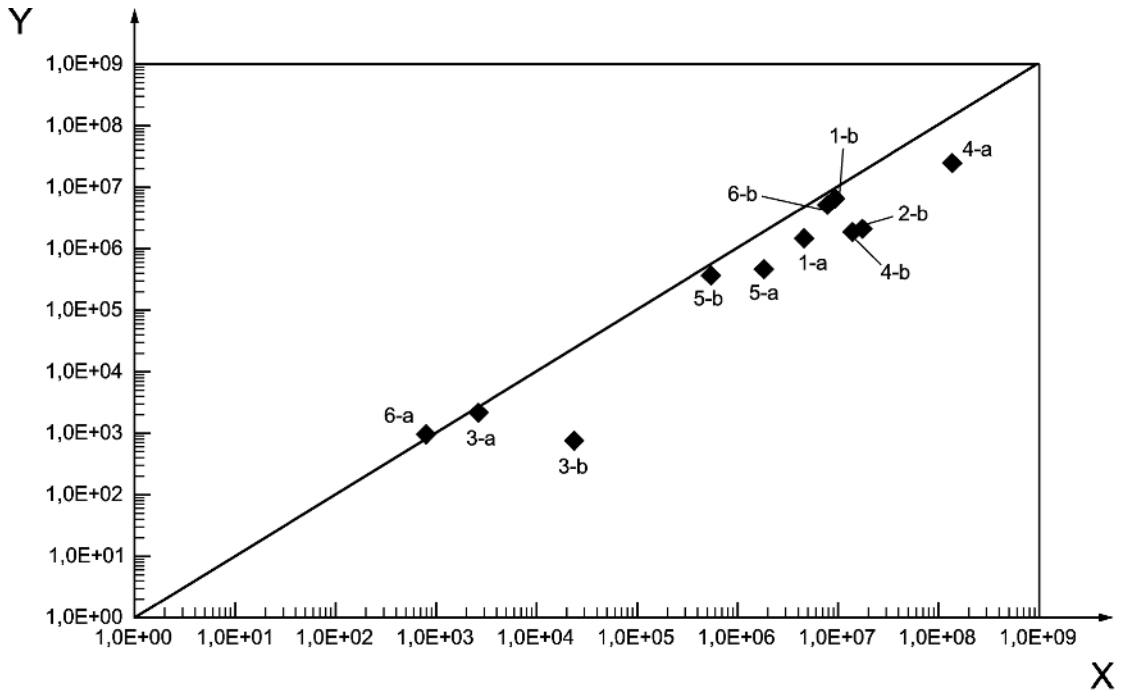
In parallel with the validation study, a short comparative study was performed by two laboratories to evaluate the effect of the pre-filtration and centrifugation step according to various matrices to be analysed (see Figure 1).

Following the experimental plan of the validation study, two batches of three samples of each matrix were analysed by each of the two laboratories.

The results of the method applied with and without pre-filtration and centrifugation step were plotted on the same graph. The statistical processing was carried out with all data using paired comparisons. Any dot represents the results of the six analyses of the same matrix batch with and without the pre-filtration and centrifugation step. The position of the dots on the biplot related to the straight line of equivalence allows the detection of any trend.

A non-parametric statistical test, Wilcoxon test – paired signed rank test, was used to determine whether the trend was statistically significant.

The paired comparisons of results with and without pre-filtration and centrifugation step of each set of matrix are shown in Figure 1.



Key

1-a Anaerobic treated biowaste, first batch
1-b Anaerobic treated biowaste; second batch
2-b Pelletised air-dried sludge; second batch
3-a Digested sewage sludge presscake; first batch
3-b Digested sewage sludge presscake; second batch
4-a Composted sewage sludge; first batch
4-b Composted sewage sludge; second batch
5-a Composted green waste; first batch
5-b Composted green waste; second batch
6-a Composted biowaste; first batch
6-b Composted biowaste; second batch
X Method with prefiltration
Y Method without prefiltration

Figure 1 — Biplot representations of *E. coli* filtration method results, with and without pre-filtration

Wilcoxon test p-value = 0,004 4

The comparison of the paired results for the *E. coli* filtration method with and without the pre-filtration and centrifugation showed a significant trend through the different matrices batches. The method applied with the pre-filtration and centrifugation step gave higher results than the one followed without the addition of the pre-filtration and centrifugation step.

7 Method B — Miniaturised method (Most Probable Number) by inoculation in liquid medium

7.1 Scope

Method B specifies a miniaturised most probable number (MPN) method for the semi-quantitative detection of *Escherichia coli* in sludge, soils and organic fertilisers of similar consistency to the matrices validated. It is suitable to evaluate the Log reduction of *E. coli* through treatment as well as the quality of the end product. Method B has a limit of detection (5 %) of approximately 67 *E. coli* MPN per g of wet weight, and a quantification range of 6 Log (see ENV ISO 13843).

7.2 Principle

This method is based on the method specified in EN ISO 9308-3.

The following text describes the sample preparation to obtain a liquid suspension, then the analysis is performed following EN ISO 9308-3, reaching Most Probable Number results in 100 ml. The final result is calculated to express the *E. coli* MPN per g of sample. The whole procedure described in the EN ISO 9308-3 is adapted to perform the analysis of *E. coli* in sludge, soils and organic fertilisers of similar consistency to the matrices validated.

The detection and enumeration of *E. coli* from sludge, soils and organic fertilisers require the following stages:

a) preparation of a homogenised sample suspension in peptone saline solution;

b) inoculation of the diluted homogenised sample in a row of microtitre plate wells containing dehydrated culture medium;

c) examination of the microtitre plate under ultraviolet light at 366 nm in the dark after an incubation period at (44 ± 1) °C during 48 h. The presence of *E. coli* is indicated by a blue fluorescence resulting from hydrolysis of MUG;

d) confirmation of the presence of *E. coli* by addition of Kovac's reagent, in each blue fluorescent well;

e) results are given as the *E. coli* Most Probable Number per g of sample (wet weight).

7.3 Reagents, diluents and culture media

7.3.1 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.

7.3.2 Peptone saline solution

Casein peptone	1,0 g
Sodium chloride	8,5 g
Distilled water	1 000 ml

Dissolve the compounds in the water, if necessary by heating. Adjust the pH (7,8) by adding sodium hydroxide solution or hydrochloric acid so that, after sterilisation, it will correspond to (7,0 ± 0,5) at 25 °C.

Sterilise the solution in a steam steriliser (7.4.1) at (121 ± 3) °C for (15 ± 1) min.

7.3.3 Special Microplate Diluent (SMD)

7.3.3.1 Bromophenol blue solution (optional)

Bromophenol blue	0,04 g
50 % ethanol	100 ml

Dissolve bromophenol blue in 100 ml ethanol.

NOTE It is only used to colour the SMD in blue and to avoid confusing with demineralised or distilled water.

7.3.3.2 SMD

Synthetic sea salt	22,5 g
Bromophenol blue solution (optional)	10 ml
Distilled water	1 000 ml

Dissolve the ingredients in the water. Pour 18 ml fraction into sterile tubes.

Sterilise in the autoclave (7.4.1) at (121 ± 3) °C for (15 ± 1) min.

NOTE A typical analysis of a commercially available and suitable synthetic sea salt is given in Annex C.

7.3.4 Culture medium: MUG/EC medium

7.3.4.1 MUG solution

MUG* (4-methyl-umbelliferyl-β-D-glucuronide)	100 mg
N-N-dimethylformamide	2 ml

Dissolve MUG, the fluorogenic constituent, in N-N-dimethylformamide.

WARNING — N-N dimethylformamide is toxic. Harmful by inhalation, in contact with skin and if swallowed. May cause cancer. Use in a chemical fume hood.

7.3.4.2 MUG/EC medium

Tryptone	40 g
Salicin	1 g
Triton x100	1 g
MUG solution	2 ml
Distilled water	1 000 ml

Successively add tryptone, salicin and triton to 1 l of distilled water while maintaining a gentle heat and magnetic stirring. Bring to the boil until complete dissolution. Allow to cool and add the MUG solution.

Adjust the pH to (6,9 ± 0,2).

Sterilise by filtration with membranes of average pore size 0,2 µm (7.4.14).

Distribute in 96-well microtitre plates (7.4.15) with a volume of 100 µl of media in each well (maximum capacity 350 µl) and dehydrate immediately in a tunnel drier or laminar airflow cabinet (7.4.3).

7.3.5 Kovac's reagent

4-di-methylaminobenzaldehyde, $C_9H_{11}NO$	5,0 g
Isoamyl alcohol, $C_5H_{12}O$	75,0 ml
Hydrochloric acid ($p = 1,18\ g.ml^{-1}$)	25,0 ml

Dissolve 4-di-methylaminobenzaldehyde in isoamyl alcohol and heat in a water bath at 60 °C for 5 min. Then add slowly 25 ml of hydrochloric acid.

The reagent will be ready for use after 6 h to 7 h (indicated by a yellow colour). Store in the refrigerator and protect from light.

WARNING — Kovacs reagent is harmful if swallowed, irritating to the respiratory system and to the skin. It is recommended to use it in a flow cabinet.

7.4 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:

7.4.1 Apparatus for sterilisation by dry heat (oven) or steam (autoclave).

7.4.2 Thermostatic incubator adjustable to (44 ± 1) °C.

7.4.3 Tunnel drier or vertical laminar airflow cabinet (preferably class II).

7.4.4 Homogeniser

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

7.4.6 Ultraviolet observation chamber (Wood's Lamp, 366 nm wavelength).

7.4.7 Portable refractometer (optional).

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

7.4.9 Sterile test tubes, 40 ml volume, or flasks with similar capacity.

7.4.10 Sterile flasks, of nominal capacities e.g. 250 ml.

7.4.11 Sterile graduated pipettes, glass or disposable plastic ware, capable of dispersing 2 ml and 18 ml volumes.

7.4.12 Adjustable or pre-set 8-channel multi-pipette or any other suitable system used for measuring and distributing 200 µl per well.

7.4.13 Sterile tips for multi-pipette.

7.4.14 Equipment for membrane filtration, according to ISO 8199, including membrane filters with a nominal pore size of 0,2 µm, for sterilisation of liquid media.

7.4.15 Sterile microtitre plates – 96 wells, 350 µl, flat-bottomed, non-fluorescent.

7.4.16 Sterile adhesive covering-strips for sealing microtitre plates.

7.4.17 Sterile Petri dishes, 90 mm in diameter.

7.4.18 Analytical balance

7.4.19 Laboratory spatula

7.4.20 Vortex mixer

7.4.21 Stirrer and magnetic bars

7.5 Sampling

7.5.1 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.

Bursting glass bottles containing sludge can produce micro-organism contaminated shrapnel. Plastic bottles can also burst and produce a hazardous spray and aerosol.

See also the Warning note in the introduction.

7.5.2 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.

7.5.3 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.

7.6 Procedure

7.6.1 Sample preparation

7.6.1.1 General

Weigh a representative sub-sample of 25 g (wet weight) into a homogeniser bag (7.4.5) with an integrated mesh if large debris is to be excluded.

Add an appropriate volume of peptone saline suspension (6.1) so that the final weight is 100 g. Place the homogeniser bag (7.4.5) in a homogeniser (7.4.4) and homogenise for 1 min.

7.6.1.2 For lime-treated materials

Adjust the pH to (7,0 ± 0,5) with 1 mol/l hydrochloric acid. 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 (7.4.10).

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 neutralisation procedure shall be used (see e.g. EN 1040).

7.6.2 Analysis

7.6.2.1 Preparation of dilutions

Prepare the 1/2 dilution in SMD (6.2) and then serial ten fold dilutions from 1/20 to 1/2 000 000 also in SMD as follows:

Vigorously stir the primary suspension (9.1.2) in order to obtain a homogeneous suspension and, using a sterile pipette, immediately transfer 18 ml of this homogenised suspension to a first tube (7.4.9) containing 18 ml of SMD (6.2) to obtain the 1/2 dilution.

Using a fresh pipette (7.4.11), transfer 2 ml of this first dilution (homogenised by handle shaking) to a second 18 ml SMD (6.2) tube to obtain the 1/20 dilution.

From this second tube (1/20 dilution carefully homogenised by handle shaking) proceed to another 1/10 dilution giving the following dilution 1/200, while adding 2 ml of 1/20 dilution in 18 ml of SMD (6.2).

Continue as above until all the dilutions to 1/2 000 000 have been prepared.

Appropriate precautions should be taken as aerosols may be created by the diluting and pipetting.

7.6.2.2 Inoculation and incubation of microtitre plates

Inoculate a microtitre plate (7.4.15) containing the MUG/EC medium in each well (6.3) while distributing each dilution from 1/20 to 1/2 000 000, each in 16 consecutive wells (the 1/2 dilution is only used for the preparation of the serial dilutions):

Transfer the contents of the last dilution tube (1/2 000 000) to an empty sterile Petri dish of 90 mm diameter (7.4.17).

Using a multi-channel pipette (7.4.12) with 8 sterile tips (7.4.13), distribute 200 µl per well into 16 wells of the microtitre plate (7.4.15) corresponding to this last dilution (use the two last columns on the left side of the microtitre plate corresponding to the 11A to 11H and 12A to 12H columns).

For subsequent dilutions (1/200, 1/2 000, etc…), operate in an identical manner using for each successive dilution the two following 8 wells columns of the microtitre plate. The two last 8 wells columns on the left of the microtitre plate should correspond to the 1/20 dilution.

Alternatively, any other suitable system (7.4.12) may be used to distribute 200 µl of each dilution per well.

WARNING — Beware of contamination via an overflow from one well to another.

Once the microtitre plate is inoculated, cover with the disposable adhesive tape (7.4.16) provided for this purpose. Incubate the microtitre plate in an incubator (7.4.2) at (44 ± 1) °C during (48 ± 4) h.

The microtitre plates should be handled with care, without tilting.

7.6.2.3 Reading

Place each microtitre plate with the adhesive on, in the UV observation chamber (7.4.6). Note the number of positive blue fluorescent wells for each dilution.

Then, using an adjustable pipette with a sterile tip, distribute 15 µl of Kovac's reagent in each blue fluorescent well. Wait 1 min to 2 min for the colour change. Note the number of positive red-top wells for each dilution.

Consider all wells that are blue fluorescent and red topped positive.

7.6.3 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 described in EN 15934. This shall be performed in parallel with the microbiological analysis.

7.7 Expression of results

7.7.1 Determination of the characteristic number

For each of the six inoculated dilutions, note the number of positive wells and identify the corresponding characteristic number (CN) according to the instructions given in ISO 8199 for MPN calculation:

The CN corresponds to the number of positive wells of the three last dilutions giving a number of positive wells > 0 (see Table 5).

When it is possible, choose three serial dilutions for which results are neither totally positive nor totally negative. If it is not possible, it is better to choose the three serial dilutions with positive results than negative results (see Table 5, examples A and B).

If less than three serial dilutions give positive results, start from the dilution containing the higher concentration in sample and follow with the two next dilutions (see Table 5, example C).

If there are only positive wells for one of the serial dilutions, start from this dilution and select the previous and the next dilutions (see Table 5, example D).

Table 5 — Examples for the determination of the characteristic number

Example	Dilution						CN
	1/20	1/200	1/2 000	1/20 000	1/200 000	1/2 000 000	
A	16	16	9	3	0	0	**16/9/3**
B	16	16	9	7	1	0	**9/7/1**
C	12	5	0	0	0	0	**12/5/0**
D	0	1	0	0	0	0	**0/1/0**
E	**16**	**16**	**12**	**5**	**0**	**0**	**16/12/5**

In example E (Table 5), the CN will be 16/12/5 for dilution 1/200, 1/2 000, 1/20 000.

7.7.2 Calculation of the MPN and its confidence interval

The MPN is a statistical estimation of the density of micro-organisms, assumed to correspond to a Poisson distribution in the volumes inoculated. Confidence intervals are attached to this MPN.

The MPN corresponding to the identified CN can be obtained from the MPN_i Table 5. The confidence limits at 95 % are attached to this MPN_i.

Proceed then as follows:

Identify the intermediary MPN (MPN_i) from the CN directly in Table 5. The final MPN per ml of suspension, taking into account the dilution steps corresponding to the CN, is calculated from MPN_i as follows:

1) If CN correspond to dilutions 1/20, 1/200 and 1/2 000, MPN.ml^{-1} suspension = MPN_i

2) If CN correspond to dilutions 1/200, 1/2 000 and 1/20 000, MPN.ml^{-1} suspension = $10 \times MPN_i$

3) If CN correspond to dilutions 1/2 000, 1/20 000 and 1/200 000, MPN.ml^{-1} suspension = $100 \times MPN_i$

4) If CN correspond to dilutions 1/20 000, 1/200 000 and 1/2 000 000, MPN.ml^{-1} suspension = $1\,000 \times MPN_i$

5) The result per g of wet weight is then calculated as follows:

   E. coli MPN g^{-1} wet weight = MPN_{ww} = MPN $\times$ 100 ml of total suspension / 25 g

6) The result may be converted in MPN g^{-1} of dry matter as follows:

   E. coli MPN g^{-1} dry matter = MPN_{dw} = $MPN_{ww} \times 100$ / % of dry matter

In the example E of Table 5:

If CN = 16/12/5 for dilutions 1/200, 1/2 000 and 1/20 000, the MPN_i statistical Table 5 gives 1 758,2 ml^{-1}, with a lower limit of 1 018,2 ml^{-1} and an upper limit of 3 036,2 ml^{-1}.

The MPN is then 17 582 ml^{-1} suspension (lower limit = 10 182 ml^{-1}; upper limit = 30 362 ml^{-1}).

The result per g wet weight is then:

70 328 *E. coli* MPN g^{-1} wet weight (lower limit = 40 728 MPN g^{-1} wet weight; upper limit = 121 448 MPN g^{-1} wet weight).

If none of the wells is positive, express the result in the following form: < n ml^{-1},

n being the MPN for 1 positive well under the dilution conditions employed.

If all the wells are positive, express the result in the following form: > n ml^{-1},

n being the MPN lower limit for all positive wells under the dilution conditions employed.

Low and upper limits shall always be given.

7.8 Performance data

7.8.1 MPN Statistical table

Table 6 — MPN statistical table for microtitre plate 96 wells six dilutions (1/20 to 1/2 000 000) / 16 wells seeded per dilution *(1 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
0/16	0/16	1/16	5,60	0,80	41,00
0/16	0/16	2/16	11,30	2,70	46,70
0/16	1/16	0/16	5,60	0,80	41,00
0/16	1/16	1/16	11,30	2,70	46,70
0/16	1/16	2/16	16,90	5,20	54,90
0/16	2/16	0/16	11,30	2,70	46,80
0/16	2/16	1/16	17,00	5,30	54,90
0/16	3/16	0/16	17,00	5,30	55,00
0/16	3/16	1/16	22,70	8,10	63,50
0/16	4/16	0/16	22,80	8,20	63,60
1/16	0/16	0/16	5,80	0,80	41,00
1/16	0/16	1/16	11,60	2,90	47,10
1/16	0/16	2/16	17,40	5,50	55,50
1/16	1/16	0/16	11,70	2,90	47,20
1/16	1/16	1/16	17,50	5,50	55,60
1/16	1/16	2/16	23,30	8,40	64,40
1/16	2/16	0/16	17,50	5,50	55,70
1/16	2/16	1/16	23,30	8,50	64,50
1/16	2/16	2/16	29,20	11,60	73,30
1/16	3/16	0/16	23,40	8,50	64,60
1/16	3/16	1/16	29,30	11,70	73,40
1/16	4/16	0/16	29,40	11,70	73,60
1/16	4/16	1/16	35,20	15,10	82,50
1/16	5/16	0/16	35,30	15,10	82,60
2/16	0/16	0/16	12,00	3,00	47,60
2/16	0/16	1/16	17,90	5,70	56,30
2/16	0/16	2/16	23,90	8,80	65,40

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
2/16	1/16	0/16	18,00	5,70	56,40
2/16	1/16	1/16	24,00	8,80	65,50
2/16	1/16	2/16	30,00	12,10	74,60
2/16	2/16	0/16	24,10	8,80	65,60
2/16	2/16	1/16	30,10	12,10	74,70
2/16	2/16	2/16	36,20	15,60	83,90
2/16	3/16	0/16	30,20	12,20	74,80
2/16	3/16	1/16	36,30	15,70	84,00
2/16	4/16	0/16	36,40	15,70	84,20
2/16	4/16	1/16	42,50	19,30	93,40
2/16	5/16	0/16	42,60	19,40	93,60
3/16	0/16	0/16	18,5	6	57,2
3/16	0/16	1/16	24,7	9,2	66,5
3/16	0/16	2/16	30,9	12,6	75,9
3/16	0/16	3/16	37,1	16,2	85,4
3/16	1/16	0/16	24,8	9,2	66,6
3/16	1/16	1/16	31	12,6	76,1
3/16	1/16	1/16	37,3	16,2	85,5
3/16	2/16	0/16	31,1	12,7	76,2
3/16	2/16	1/16	37,4	16,3	85,7
3/16	2/16	2/16	43,6	20	95,2
3/16	3/16	0/16	37,5	16,4	85,8
3/16	3/16	1/16	43,8	20,1	95,3
3/16	3/16	2/16	50,1	23,9	104,9
3/16	4/16	0/16	43,9	20,2	95,5
3/16	4/16	1/16	50,2	24	105,1
3/16	5/16	0/16	50,4	24,1	105,3
3/16	5/16	1/16	56,7	28	114,9
3/16	6/16	0/16	56,9	28,1	115,1

Table 6 *(2 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
4/16	0/16	0/16	25,5	9,6	67,8
4/16	0/16	1/16	32	13,2	77,5
4/16	0/16	2/16	38,4	16,9	87,3
4/16	0/16	3/16	44,9	20,8	97
4/16	1/16	0/16	32,1	13,2	77,6
4/16	1/16	1/16	38,5	17	87,4
4/16	1/16	2/16	45	20,8	97,2
4/16	1/16	3/16	51,5	24,8	107,1
4/16	2/16	0/16	38,6	17	87,6
4/16	2/16	1/16	45,1	20,9	97,4
4/16	2/16	2/16	51,7	24,9	107,3
4/16	3/16	0/16	45,3	21	97,6
4/16	3/16	1/16	51,8	25	107,5
4/16	3/16	2/16	58,4	29,1	117,4
4/16	4/16	0/16	52	25,1	107,7
4/16	4/16	1/16	58,6	29,2	117,7
4/16	4/16	2/16	65,2	33,3	127,7
4/16	5/16	0/16	58,8	29,3	118
4/16	5/16	1/16	65,4	33,4	128
4/16	6/16	0/16	65,6	33,6	128,3
5/16	0/16	0/16	33,1	13,8	79,2
5/16	0/16	1/16	39,8	17,7	89,3
5/16	0/16	2/16	46,5	21,7	99,4
5/16	0/16	3/16	53,2	25,9	109,6
5/16	1/16	0/16	39,9	17,8	89,5
5/16	1/16	1/16	46,6	21,8	99,7
5/16	1/16	2/16	53,4	26	109,9
5/16	1/16	3/16	60,2	30,2	120,1
5/16	2/16	0/16	46,8	21,9	99,9
5/16	2/16	1/16	53,6	26,1	110,1
5/16	2/16	2/16	60,4	30,3	120,4
5/16	2/16	3/16	67,3	34,6	130,8
5/16	3/16	0/16	53,7	26,2	110,4
5/16	3/16	1/16	60,6	30,4	120,7
5/16	3/16	2/16	67,5	34,7	131,1

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
5/16	4/16	0/16	60,8	30,5	121
5/16	4/16	1/16	67,7	34,9	131,4
5/16	4/16	2/16	74,6	39,2	141,9
5/16	5/16	0/16	67,9	35	131,7
5/16	5/16	1/16	74,9	39,4	142,3
5/16	6/16	0/16	75,1	39,6	142,6
5/16	6/16	1/16	82,1	44	153,3
5/16	7/16	0/16	82,4	44,2	153,7
6/16	0/16	0/16	41,2	18,6	91,5
6/16	0/16	1/16	48,2	22,8	102,1
6/16	0/16	2/16	55,3	27,1	112,7
6/16	0/16	3/16	62,2	31,5	123,3
6/16	1/16	0/16	48,4	22,9	102,3
6/16	1/16	1/16	55,4	27,2	112,9
6/16	1/16	2/16	62,2	31,6	123,6
6/16	1/16	3/16	69,7	36,1	134,4
6/16	2/16	0/16	55,6	27,3	113,2
6/16	2/16	1/16	62,8	31,8	124
6/16	2/16	2/16	69,9	36,3	134,8
6/16	2/16	3/16	77,1	40,8	145,7
6/16	3/16	0/16	63	31,9	124,3
6/16	3/16	1/16	70,1	36,4	135,1
6/16	3/16	2/16	77,4	41	146,1
6/16	4/16	0/16	70,4	36,6	135,5
6/16	4/16	1/16	77,6	41,2	146,5
6/16	4/16	2/16	84,9	45,8	157,6
6/16	5/16	0/16	77,9	41,3	146,9
6/16	5/16	1/16	85,2	46	158
6/16	5/16	2/16	92,6	50,7	169,2
6/16	6/16	0/16	85,5	46,2	158,4
6/16	6/16	1/16	92,9	50,9	169,7
6/16	7/16	0/16	93,2	51,1	170,2

Table 6 *(3 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
7/16	0/16	0/16	50,1	23,9	104,9
7/16	0/16	1/16	57,5	28,5	116
7/16	0/16	2/16	64,9	33,1	127,1
7/16	0/16	3/16	72,3	37,8	138,4
7/16	1/16	0/16	57,6	28,6	116,3
7/16	1/16	1/16	65,1	33,2	127,5
7/16	1/16	2/16	72,6	37,9	138,8
7/16	1/16	3/16	80,1	42,7	150,2
7/16	2/16	0/16	65,3	33,4	127,8
7/16	2/16	1/16	72,8	38,1	139,2
7/16	2/16	2/16	80,4	42,9	150,6
7/16	2/16	3/16	88	47,7	162,2
7/16	3/16	0/16	73,1	38,3	139,5
7/16	3/16	1/16	80,7	43,1	151
7/16	3/16	2/16	88,3	47,9	162,7
7/16	3/16	3/16	96	52,8	174,5
7/16	4/16	0/16	80,9	43,3	151,5
7/16	4/16	1/16	88,6	48,1	163,2
7/16	4/16	2/16	96,3	53	175
7/16	5/16	0/16	88,9	48,3	163,6
7/16	5/16	1/16	96,7	53,3	175,5
7/16	5/16	2/16	104,5	58,2	187,6
7/16	6/16	0/16	97	53,5	176
7/16	6/16	1/16	104,9	58,5	188,1
7/16	7/16	0/16	105,3	58,7	188,7
8/16	0/16	0/16	59,8	30	119,6
8/16	0/16	1/16	67,8	34,8	131,3
8/16	0/16	2/16	75,5	39,8	143,2
8/16	0/16	3/16	83,4	44,8	155,2
8/16	1/16	0/16	67,9	35	131,7
8/16	1/16	1/16	75,9	39,9	143,6
8/16	1/16	2/16	83,7	45	155,6
8/16	1/16	3/16	91,7	50,1	167,8
8/16	2/16	0/16	76	40,1	144
8/16	2/16	1/16	84	45,2	156,1
8/16	2/16	2/16	92	50,3	168,3
8/16	2/16	3/16	100,1	55,4	180,8
8/16	3/16	0/16	84,3	45,4	156,5
8/16	3/16	1/16	92,3	50,5	168,9
8/16	3/16	2/16	100,5	55,7	181,3
8/16	3/16	3/16	108,7	60,9	194
8/16	4/16	0/16	92,7	50,7	169,4
8/16	4/16	1/16	100,9	55,9	181,9
8/16	4/16	2/16	109,1	61,1	194,7
8/16	4/16	3/16	117,4	66,4	207,6

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
8/16	5/16	0/16	101,3	56,1	182,5
8/16	5/16	1/16	109,5	61,4	195,3
8/16	5/16	2/16	117,9	66,7	208,3
8/16	6/16	0/16	109,9	61,7	195,9
8/16	6/16	1/16	118,3	67	209
8/16	6/16	2/16	126,8	72,3	222,3
8/16	7/16	0/16	118,8	67,3	209,7
8/16	7/16	1/16	127,3	72,6	223,1
8/16	8/16	0/16	127,8	72,9	223,9
9/16	0/16	0/16	70,7	36,8	135,9
9/16	0/16	1/16	78,9	42	148,4
9/16	0/16	2/16	87,3	47,3	161,1
9/16	0/16	3/16	95,7	52,6	174
9/16	1/16	0/16	79,7	42,2	148,9
9/16	1/16	1/16	87,6	47,5	161,6
9/16	1/16	2/16	96,1	52,9	174,6
9/16	1/16	3/16	104,6	58,3	187,8
9/16	2/16	0/16	87,9	47,7	162,1
9/16	2/16	1/16	96,5	53,1	175,2
9/16	2/16	2/16	105,1	58,6	188,4
9/16	2/16	3/16	113,7	64,1	201,9
9/16	3/16	0/16	96,8	53,4	175,7
9/16	3/16	1/16	105,5	58,8	189,1
9/16	3/16	2/16	114,2	64,4	202,6
9/16	3/16	3/16	123	69,9	216,4
9/16	4/16	0/16	105,9	59,1	189,7
9/16	4/16	1/16	114,7	64,7	203,3
9/16	4/16	2/16	123,5	70,3	217,2
9/16	4/16	3/16	132,5	75,9	231,4
9/16	5/16	0/16	115,1	65	204
9/16	5/16	1/16	124	70,6	218
9/16	5/16	2/16	133,1	76,2	232,3
9/16	5/16	3/16	142,2	81,9	246,8
9/16	6/16	0/16	124,6	70,9	218,8
9/16	6/16	1/16	133,6	76,6	233,1
9/16	6/16	2/16	142,8	82,3	247,8
9/16	7/16	0/16	134,5	76,9	234
9/16	7/16	1/16	143,4	82,6	248,7
9/16	7/16	2/16	152,7	88,4	263,8
9/16	8/16	0/16	144	83	249,7
9/16	8/16	1/16	153,4	88,8	264,9
9/16	9/16	0/16	154	89,2	265,9

Table 6 *(4 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
10/16	0/16	0/16	82,8	44,4	154,3
10/16	0/16	1/16	91,7	50,1	167,8
10/16	0/16	2/16	100,6	55,8	181,6
10/16	0/16	3/16	109,7	61,5	195,6
10/16	1/16	0/16	92	50,3	168,4
10/16	1/16	1/16	101	56	182,2
10/16	1/16	2/16	110,2	61,8	196,3
10/16	1/16	3/16	119,4	67,7	210,8
10/16	2/16	0/16	101,5	56,3	182,9
10/16	2/16	1/16	110,6	62,1	197,1
10/16	2/16	2/16	119,9	68	211,6
10/16	2/16	3/16	129,4	73,9	226,4
10/16	3/16	0/16	111,1	62,4	197,8
10/16	3/16	1/16	120,5	68,3	212,4
10/16	3/16	2/16	129,9	74,3	227,3
10/16	3/16	3/16	139,6	80,3	242,6
10/16	4/16	0/16	121	68,7	213,2
10/16	4/16	1/16	130,5	74,6	228,2
10/16	4/16	2/16	140,2	80,7	243,6
10/16	4/16	3/16	150	86,7	259,4
10/16	5/16	0/16	131,1	75	229,1
10/16	5/16	1/16	140,8	81,1	244,6
10/16	5/16	2/16	150,7	87,1	260,5
10/16	5/16	3/16	160,7	93,3	276,8
10/16	6/16	0/16	141,4	81,4	245,6
10/16	6/16	1/16	151,4	87,6	261,6
10/16	6/16	2/16	161,4	93,7	278
10/16	6/16	3/16	171,6	99,9	294,8
10/16	7/16	0/16	152,1	88	262,7
10/16	7/16	1/16	162,2	94,2	279,2
10/16	7/16	2/16	172,4	100,4	296,2
10/16	8/16	0/16	162,9	94,6	280,5
10/16	8/16	1/16	173,2	100,9	297,6
10/16	9/16	0/16	174,1	101,4	298,9
11/16	0/16	0/16	96,6	53,2	175,4
11/16	0/16	1/16	106,2	59,3	190,2
11/16	0/16	2/16	116	65,5	205,4
11/16	0/16	3/16	125,9	71,7	220,9
11/16	1/16	0/16	106,7	59,6	190,9
11/16	1/16	1/16	116,5	65,8	206,2
11/16	1/16	2/16	126,5	72,1	221,8
11/16	1/16	3/16	136,6	78,5	237,9
11/16	1/16	4/16	147	84,9	254,5
11/16	2/16	0/16	117	66,2	207
11/16	2/16	1/16	127,1	72,5	222,8
11/16	2/16	2/16	137,3	78,9	239
11/16	2/16	3/16	147,7	85,3	255,6
11/16	2/16	4/16	158,2	91,8	272,8
11/16	3/16	0/16	127,7	72,8	223,7
11/16	3/16	1/16	137,9	79,3	240
11/16	3/16	2/16	148,4	85,7	256,8
11/16	3/16	3/16	159	92,3	274,1
11/16	3/16	4/16	169,8	98,8	291,9
11/16	4/16	0/16	138,6	79,7	241,1
11/16	4/16	1/16	149,1	86,2	257,9
11/16	4/16	2/16	159,8	92,7	275,4
11/16	4/16	3/16	170,7	99,3	293,3
11/16	5/16	0/16	149,8	86,6	259,1
11/16	5/16	1/16	160,6	93,2	276,7
11/16	5/16	2/16	171,6	99,9	294,7
11/16	5/16	3/16	182,7	106,5	313,4
11/16	6/16	0/16	161,4	93,7	278
11/16	6/16	1/16	172,4	100,4	296,2
11/16	6/16	2/16	183,7	107,1	315
11/16	6/16	3/16	195,1	113,8	334,4
11/16	7/16	0/16	173,3	100,9	297,7
11/16	7/16	1/16	184,6	107,7	316,6
11/16	7/16	2/16	196,2	114,4	336,2
11/16	8/16	0/16	185,6	108,2	318,3
11/16	8/16	1/16	197,2	115	338,1
11/16	8/16	2/16	209	121,9	358,5
11/16	9/16	0/16	198,3	115,7	339,9
11/16	9/16	1/16	210,2	122,5	360,5
11/16	10/16	0/16	211,4	123,2	362,6

Table 6 *(5 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
12/16	0/16	0/16	112,6	63,4	200,1
12/16	0/16	1/16	123,2	70	216,7
12/16	0/16	2/16	134	76,8	233,7
12/16	0/16	3/16	145	83,7	251,4
12/16	1/16	0/16	123,8	70,4	217,6
12/16	1/16	1/16	134,7	77,2	234,8
12/16	1/16	2/16	145,8	84,1	252,6
12/16	1/16	3/16	157,1	91,1	271
12/16	1/16	4/16	168,7	98,1	290
12/16	2/16	0/16	135,3	77,7	235,9
12/16	2/16	1/16	146,5	84,6	253,8
12/16	2/16	2/16	157,9	91,6	272,3
12/16	2/16	3/16	169,6	98,7	291,5
12/16	2/16	4/16	181,6	105,8	311,5
12/16	3/16	0/16	147,3	85,1	255
12/16	3/16	1/16	158,8	92,1	273,7
12/16	3/16	2/16	170,6	99,3	293,1
12/16	3/16	3/16	182,6	106,4	313,2
12/16	3/16	4/16	194,9	113,7	334,1
12/16	4/16	0/16	159,6	92,6	275,1
12/16	4/16	1/16	171,5	99,8	294,6
12/16	4/16	2/16	183,6	107,1	314,9
12/16	4/16	3/16	196	114,3	336
12/16	4/16	4/16	208,7	121,7	357,9
12/16	5/16	0/16	172,4	100,4	296,2
12/16	5/16	1/16	184,7	107,7	316,7
12/16	5/16	2/16	197,2	115	338
12/16	5/16	3/16	209,9	122,4	360,1
12/16	5/16	4/16	223	129,8	383,1
12/16	6/16	0/16	185,7	108,3	318,5
12/16	6/16	1/16	198,3	115,7	339,9
12/16	6/16	2/16	211,2	123,1	362,3
12/16	6/16	3/16	224,4	130,6	385,6
12/16	7/16	0/16	199,5	116,4	342
12/16	7/16	1/16	212,5	123,9	364,5
12/16	7/16	2/16	225,8	131,4	388,1
12/16	7/16	3/16	239,4	139	412,6
12/16	8/16	0/16	213,8	124,6	366,8
12/16	8/16	1/16	227,2	132,2	390,6
12/16	8/16	2/16	241	139,8	415,4
12/16	9/16	0/16	228,7	133	393,2
12/16	9/16	1/16	242,6	140,7	418,2
12/16	9/16	2/16	256,8	148,4	444,4
12/16	10/16	0/16	244,2	141,5	421,1
12/16	10/16	1/16	258,5	149,3	447,6
13/16	0/16	0/16	131,7	75,4	230,1
13/16	0/16	1/16	143,6	82,8	249,1
13/16	0/16	2/16	155,8	90,3	268,9
13/16	0/16	3/16	168,4	97,9	289,5
13/16	0/16	4/16	181,3	105,7	311,1
13/16	1/16	0/16	144,4	83,3	250,4
13/16	1/16	1/16	156,7	90,9	270,3
13/16	1/16	2/16	169,4	98,5	291,2
13/16	1/16	3/16	182,4	106,4	312,9
13/16	1/16	4/16	195,8	114,3	335,7
13/16	2/16	0/16	157,6	91,4	271,8
13/16	2/16	1/16	170,4	99,2	292,8
13/16	2/16	2/16	183,5	107	314,8
13/16	2/16	3/16	197,1	115	337,8
13/16	2/16	4/16	211	123	362
13/16	3/16	0/16	171,4	99,8	294,5
13/16	3/16	1/16	184,7	107,7	316,7
13/16	3/16	2/16	198,3	115,7	340
13/16	3/16	3/16	212,4	123,8	364,4
13/16	3/16	4/16	226,9	132	390
13/16	4/16	0/16	185,8	108,4	318,7
13/16	4/16	1/16	199,6	116,5	342,2
13/16	4/16	2/16	213,8	124,6	366,9
13/16	4/16	3/16	228,5	132,9	392,8
13/16	4/16	4/16	243,6	141,2	420,1
13/16	5/16	0/16	200,9	117,2	344,4
13/16	5/16	1/16	215,3	125,4	369,4
13/16	5/16	2/16	230,1	133,8	395,6
13/16	5/16	3/16	245,3	142,2	423,3
13/16	5/16	4/16	261	150,7	452,3
13/16	6/16	0/16	216,7	126,3	372
13/16	6/16	1/16	231,7	134,7	398,5
13/16	6/16	2/16	247,1	143,2	426,5
13/16	6/16	3/16	263	151,7	455,9
13/16	6/16	4/16	279,4	160,3	486,9
13/16	7/16	0/16	233,3	135,6	401,5
13/16	7/16	1/16	248,9	144,1	429,8
13/16	7/16	2/16	265	152,8	459,7
13/16	7/16	3/16	281,6	161,5	491,1
13/16	7/16	4/16	298,7	170,3	524,1

Table 6 *(6 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
13/16	8/16	0/16	250,8	145,1	433,2
13/16	8/16	1/16	267	153,9	463,5
13/16	8/16	2/16	283,8	162,6	495,4
13/16	8/16	3/16	301,2	171,5	528,9
13/16	9/16	0/16	269,1	154,9	467,4
13/16	9/16	1/16	286,1	163,8	499,8
13/16	9/16	2/16	303,7	172,8	533,8
13/16	9/16	3/16	321,8	181,8	569,7
13/16	10/16	0/16	288,5	165	504,2
13/16	10/16	1/16	306,3	174,1	538,9
13/16	10/16	2/16	324,6	183,2	575,3
13/16	11/16	0/16	308,9	175,4	544
13/16	11/16	1/16	327,5	184,6	581
14/16	0/16	0/16	155,3	90	267,9
14/16	0/16	1/16	169,1	98,4	290,7
14/16	0/16	2/16	183,5	107	314,7
14/16	0/16	3/16	198,4	115,7	340,1
14/16	0/16	4/16	213,9	124,6	366,9
14/16	1/16	0/16	170,2	99	292,5
14/16	1/16	1/16	184,7	107,7	316,8
14/16	1/16	2/16	199,8	116,5	342,5
14/16	1/16	3/16	215,4	125,5	369,7
14/16	1/16	4/16	231,7	134,7	398,7
14/16	2/16	0/16	186	108,5	318,9
14/16	2/16	1/16	201,2	117,4	344,9
14/16	2/16	2/16	217,1	126,5	372,5
14/16	2/16	3/16	233,5	135,7	401,9
14/16	2/16	4/16	250,7	145,1	433,1
14/16	3/16	0/16	202,7	118,2	347,5
14/16	3/16	1/16	218,7	127,4	375,4
14/16	3/16	2/16	235,4	136,7	405,2
14/16	3/16	3/16	252,8	146,2	436,9
14/16	3/16	4/16	270,9	155,9	470,7
14/16	3/16	5/16	289,8	165,7	506,8
14/16	4/16	0/16	220,4	128,3	378,4
14/16	4/16	1/16	237,3	137,8	408,6
14/16	4/16	2/16	254,9	147,4	440,8
14/16	4/16	3/16	273,3	157,1	475,2
14/16	4/16	4/16	292,4	167,1	511,9
14/16	4/16	5/16	312,4	177,2	551

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
14/16	5/16	0/16	239,2	138,8	412,1
14/16	5/16	1/16	257	148,5	444,8
14/16	5/16	2/16	275,7	158,4	479,8
14/16	5/16	3/16	295,1	168,4	517,2
14/16	5/16	4/16	315,5	178,7	557
14/16	5/16	5/16	336,7	189,1	599,4
14/16	6/16	0/16	259,2	149,7	448,9
14/16	6/16	1/16	278,2	159,7	484,5
14/16	6/16	2/16	297,9	169,9	522,6
14/16	6/16	3/16	318,6	180,2	563,2
14/16	6/16	4/16	340,1	190,8	606,4
14/16	7/16	0/16	280,7	161	489,4
14/16	7/16	1/16	300,8	171,3	528,1
14/16	7/16	2/16	321,8	181,8	569,5
14/16	7/16	3/16	343,7	192,5	613,7
14/16	7/16	4/16	366,6	203,4	660,5
14/16	8/16	0/16	303,7	172,8	533,8
14/16	8/16	1/16	325	183,4	576,1
14/16	8/16	2/16	347,4	194,2	621,2
14/16	8/16	3/16	370,7	205,4	669
14/16	8/16	4/16	394,9	216,8	719,5
14/16	9/16	0/16	328,4	185,1	582,9
14/16	9/16	1/16	351,1	196,1	628,9
14/16	9/16	2/16	374,9	207,4	677,8
14/16	9/16	3/16	399,6	219	729,4
14/16	9/16	4/16	425,4	231	783,4
14/16	10/16	0/16	355	197,9	636,9
14/16	10/16	1/16	379,2	209,4	686,8
14/16	10/16	2/16	404,5	221,2	739,5
14/16	10/16	3/16	430,8	233,5	794,7
14/16	11/16	0/16	383,7	211,5	696,2
14/16	11/16	1/16	409,5	223,6	750,1
14/16	11/16	2/16	436,3	236,1	806,5
14/16	11/16	3/16	464,2	249,1	864,9
14/16	12/16	0/16	414,7	226	761
14/16	12/16	1/16	442,1	238,8	818,6
14/16	12/16	2/16	470,6	252,1	878,3

Table 6 *(7 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
15/16	0/16	0/16	186,2	108,6	319,2
15/16	0/16	1/16	203,2	118,5	348,4
15/16	0/16	2/16	221,1	128,8	379,8
15/16	0/16	3/16	240,1	139,3	413,7
15/16	0/16	4/16	260,1	150,2	450,5
15/16	1/16	0/16	204,9	119,5	351,2
15/16	1/16	1/16	223	129,8	383,1
15/16	1/16	2/16	242,3	140,5	417,7
15/16	1/16	3/16	262,6	151,5	455,3
15/16	1/16	4/16	284,2	162,8	496,1
15/16	2/16	0/16	225	130,9	386,6
15/16	2/16	1/16	244,5	141,8	421,8
15/16	2/16	2/16	265,2	152,9	460,1
15/16	2/16	3/16	287,2	164,4	501,9
15/16	2/16	4/16	310,6	176,2	547,4
15/16	2/16	5/16	335,5	188,5	597,1
15/16	3/16	0/16	246,8	143	426
15/16	3/16	1/16	267,9	154,3	465,1
15/16	3/16	2/16	290,3	166	507,8
15/16	3/16	3/16	314,2	178	554,5
15/16	3/16	4/16	339,6	190,5	605,4
15/16	3/16	5/16	366,6	203,4	660,7
15/16	4/16	0/16	270,7	155,8	470,3
15/16	4/16	1/16	293,5	167,6	514
15/16	4/16	2/16	317,9	179,9	561,8
15/16	4/16	3/16	343,8	192,6	614
15/16	4/16	4/16	371,5	205,8	670,8
15/16	4/16	5/16	401	219,6	732,2
15/16	5/16	0/16	296,8	169,3	520,4
15/16	5/16	1/16	321,7	181,7	569,4
15/16	5/16	2/16	348,3	194,7	623
15/16	5/16	3/16	376,6	208,2	681,4
15/16	5/16	4/16	406,9	222,3	744,5
15/16	5/16	5/16	439	237,3	812
15/16	6/16	0/16	325,6	183,7	577,3
15/16	6/16	1/16	353,3	196,9	632,4
15/16	6/16	2/16	382	210,7	692,5
15/16	6/16	3/16	413	225,2	757,4
15/16	6/16	4/16	446	240,6	826,8
15/16	6/16	5/16	480,9	257	899,8
15/16	7/16	0/16	359,4	199,2	642,1
15/16	7/16	1/16	387,5	213,3	704
15/16	7/16	2/16	419,4	228,2	770,9
15/16	7/16	3/16	453,4	244	842,3
15/16	7/16	4/16	489,3	261	917,3
15/16	7/16	5/16	527,1	279,3	994,8

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
15/16	8/16	0/16	393,3	216	716,1
15/16	8/16	1/16	426,1	231,3	785
15/16	8/16	2/16	461,1	247,7	858,5
15/16	8/16	3/16	498,2	265,2	935,6
15/16	8/16	4/16	531,3	284,2	1 015
15/16	8/16	5/16	576,2	304,8	1 095,5
15/16	9/16	0/16	433,2	234,6	799,8
15/16	9/16	1/16	469,2	251,5	875,5
15/16	9/16	2/16	507,5	269,7	954,8
15/16	9/16	3/16	547,7	289,5	1 036,2
15/16	9/16	4/16	589,7	310,9	1 118,6
15/16	9/16	5/16	633,2	334	1 200,7
15/16	10/16	0/16	477,8	255,5	893,3
15/16	10/16	1/16	516,6	274,5	974,8
15/16	10/16	2/16	558,9	295,1	1 058,4
15/16	10/16	3/16	602,3	317,5	1 142,6
15/16	10/16	4/16	647,3	341,6	1 226,5
15/16	10/16	5/16	693,6	367,3	1 309,5
15/16	11/16	0/16	527,6	279,5	995,9
15/16	11/16	1/16	570,6	301,1	1 081,6
15/16	11/16	2/16	615,6	324,5	1 167,7
15/16	11/16	3/16	662,7	349,7	1 253,4
15/16	11/16	4/16	710	376,7	1 338,1
15/16	11/16	5/16	758,9	405,1	1 421,8
15/16	12/16	0/16	583,1	307,5	1 105,9
15/16	12/16	1/16	629,6	332	1 194
15/16	12/16	2/16	677,8	358,5	1 281,6
15/16	12/16	3/16	727,4	386,7	1 368,2
15/16	12/16	4/16	778,1	416,4	1 453,8
15/16	13/16	0/16	644,6	340,1	1 221,6
15/16	13/16	1/16	694,5	367,9	1 311,1
15/16	13/16	2/16	745,8	397,4	1 399,7
15/16	13/16	3/16	798,4	428,5	1 487,5
15/16	13/16	4/16	851,9	460,9	1 574,8
15/16	14/16	0/16	712,3	378	1 342,2
15/16	14/16	1/16	765,6	409	1 432,9
15/16	14/16	2/16	829,6	441,5	1 523
15/16	14/16	3/16	875,6	475,3	1 612,9
16/16	0/16	0/16	231,2	134,4	397,6
16/16	0/16	1/16	254,5	147,2	440,2
16/16	0/16	2/16	280	160,7	488,1
16/16	0/16	3/16	308	174,9	542,3
16/16	0/16	4/16	338,8	190,1	603,8

Table 6 *(8 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
16/16	1/16	0/16	257,5	148,8	445,6
16/16	1/16	1/16	283,6	162,5	494,9
16/16	1/16	2/16	312,3	177,1	550,8
16/16	1/16	3/16	344,1	192,7	614,5
16/16	1/16	4/16	379,3	209,5	687
16/16	1/16	5/16	418,4	227,7	768,8
16/16	2/16	0/16	287,3	164,4	501,9
16/16	2/16	1/16	316,8	179,3	559,7
16/16	2/16	2/16	349,6	195,3	625,8
16/16	2/16	3/16	386,2	212,7	701,3
16/16	2/16	4/16	426,9	231,7	786,6
16/16	2/16	5/16	472	252,8	881,3
16/16	3/16	0/16	321,6	181,7	569,1
16/16	3/16	1/16	355,5	198,1	637,8
16/16	3/16	2/16	393,4	216,1	716,4
16/16	3/16	3/16	435,9	235,8	805,5
16/16	3/16	4/16	483,1	258	904,3
16/16	3/16	5/16	535,1	283,2	1 010,9
16/16	3/16	6/16	591,5	311,8	1 121,9
16/16	4/16	0/16	361,6	201,1	650,4
16/16	4/16	1/16	406,3	219,7	732,5
16/16	4/16	2/16	445,5	240,3	825,7
16/16	4/16	3/16	495	263,7	929
16/16	4/16	4/16	549,6	290,4	1 040
16/16	4/16	5/16	608,9	320,9	1 155,1
16/16	4/16	6/16	672	355,2	1 271,2
16/16	5/16	0/16	409,3	223,5	749,7
16/16	5/16	1/16	455,8	245,2	847,3
16/16	5/16	2/16	503,1	269,9	955,5
16/16	5/16	3/16	565,3	298,4	1 071,1
16/16	5/16	4/16	627,7	331	1 190,5
16/16	5/16	5/16	694,2	367,7	1 310,6
16/16	5/16	6/16	763,7	407,9	1 429,8
16/16	5/16	7/16	835,5	450,9	1 548,2
16/16	6/16	0/16	462,8	250,4	870,6
16/16	6/16	1/16	521,7	276,6	983,9
16/16	6/16	2/16	582,5	307,1	1 104,5
16/16	6/16	3/16	648,3	342,2	1 228,5
16/16	6/16	4/16	713,2	381,5	1 352,7
16/16	6/16	5/16	794,9	424,4	1 476,3
16/16	6/16	6/16	867,1	470,1	1 599,2
16/16	6/16	7/16	944,3	517,7	1 722,4

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
16/16	7/16	0/16	536,8	284,1	1 014,5
16/16	7/16	1/16	601,2	316,9	1 140,5
16/16	7/16	2/16	670,9	354,6	1 269,3
16/16	7/16	3/16	744,9	396,9	1 398,1
16/16	7/16	4/16	822,1	442,8	1 526,5
16/16	7/16	5/16	901,8	491,4	1 654,8
16/16	7/16	6/16	983,2	541,9	1 783,9
16/16	7/16	7/16	1 065,4	593,8	1 914,7
16/16	7/16	8/16	1 150,9	646,7	2 048
16/16	8/16	0/16	621,7	327,8	1 179,2
16/16	8/16	1/16	695,7	368,5	1 313,2
16/16	8/16	2/16	774,1	414,1	1 447,2
16/16	8/16	3/16	855,8	463,2	1 581,1
16/16	8/16	4/16	940	515,1	1 715,6
16/16	8/16	5/16	1 026,2	568,8	1 851,7
16/16	8/16	6/16	1 114,3	623,9	1 990,3
16/16	8/16	7/16	1 204	680	2 132
16/16	8/16	8/16	1 295,5	736,9	2 277,5
16/16	9/16	0/16	723,1	384,2	1 360,9
16/16	9/16	1/16	806,4	433,3	1 500,7
16/16	9/16	2/16	893,1	486,1	1 641
16/16	9/16	3/16	982,5	541,5	1 782,8
16/16	9/16	4/16	1 074,2	598,7	1 927,1
16/16	9/16	5/16	1 167,9	657,4	2 074,9
16/16	9/16	6/16	1 263,8	717,2	2 226,8
16/16	9/16	7/16	1 361,6	777,9	2 383,3
16/16	9/16	8/16	1 461,6	839,5	2 545
16/16	9/16	9/16	1 563,8	901,7	2 712,1
16/16	10/16	0/16	842,4	455,1	1 559,4
16/16	10/16	1/16	934,8	511,8	1 707,3
16/16	10/16	2/16	1 030,1	571,2	1 857,8
16/16	10/16	3/16	1 128	632,5	2 012
16/16	10/16	4/16	1 228,5	695,2	2 170,8
16/16	10/16	5/16	1 331,4	759,2	2 334,9
16/16	10/16	6/16	1 437	824,3	2 505
16/16	10/16	7/16	1 545,2	890,4	2 681,5
16/16	10/16	8/16	1 656,3	957,4	2 865,2
16/16	10/16	9/16	1 770,2	1 025,3	3 056,4

Table 6 *(9 of 9)*

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
16/16	11/16	0/16	981,7	541	1 781,4
16/16	11/16	1/16	1 083,9	604,8	1 942,4
16/16	11/16	2/16	1 189,2	670,7	2 108,6
16/16	11/16	3/16	1 297,7	738,3	2 280,9
16/16	11/16	4/16	1 409,3	807,3	2 460,2
16/16	11/16	5/16	1 524,1	877,6	2 647
16/16	11/16	6/16	1 642,6	949,2	2 842,4
16/16	11/16	7/16	1 764,7	1 022	3 047,1
16/16	11/16	8/16	1 890,7	1 095,9	3 261,7
16/16	11/16	9/16	2 020,7	1 170,9	3 487,3
16/16	11/16	10/16	2 155,1	1 246,9	3 724,8
16/16	12/16	0/16	1 145,4	643,3	2 039,3
16/16	12/16	1/16	1 259,6	714,6	2 220,3
16/16	12/16	2/16	1 377,8	787,9	2 409,3
16/16	12/16	3/16	1 500,1	863	2 607,7
16/16	12/16	4/16	1 626,8	939,7	2 816,2
16/16	12/16	5/16	1 758,2	1 018,2	3 036,2
16/16	12/16	6/16	1 894,7	1 098,3	3 268,9
16/16	12/16	7/16	2 036,5	1 179,9	3 515
16/16	12/16	8/16	2 184	1 263,1	3 776,5
16/16	12/16	9/16	2 337,8	1 347,8	4 055,1
16/16	12/16	10/16	2 497,8	1 433,8	4 351,6
16/16	12/16	11/16	2 664,9	1 521,2	4 668,4
16/16	13/16	0/16	1 341,9	765,7	2 351,8
16/16	13/16	1/16	1 472,4	846	2 562,5
16/16	13/16	2/16	1 608,6	928,8	2 786
16/16	13/16	3/16	1 750,7	1 013,7	3 023,4
16/16	13/16	4/16	1 899,5	1 101,1	3 276,9
16/16	13/16	5/16	2 055,5	1 190,7	3 548,3
16/16	13/16	6/16	2 219,3	1 282,6	3 839,8
16/16	13/16	7/16	2 391,6	1 376,9	4 154
16/16	13/16	8/16	2 573,3	1 473,6	4 493,8
16/16	13/16	9/16	2 764,9	1 572,5	4 861,5
16/16	13/16	10/16	2 967,2	1 673,7	5 260,3
16/16	13/16	11/16	3 181,5	1 777,7	5 693,9
16/16	13/16	12/16	3 408,3	1 884,4	6 164,7

Characteristic Number			MPN_i in 1 ml	Lower limit	Upper limit
16/16	14/16	0/16	1 587	915,7	2 750,3
16/16	14/16	1/16	1 741,7	1 008,4	3 008,2
16/16	14/16	2/16	1 905,3	1 104,4	3 286,8
16/16	14/16	3/16	2 078,8	1 203,9	3 589,3
16/16	14/16	4/16	2 263,4	1 307	3 919,5
16/16	14/16	5/16	2 460,3	1 413,8	4 281,5
16/16	14/16	6/16	2 671,1	1 524,5	4 680,4
16/16	14/16	7/16	2 897,6	1 639,2	5 121,9
16/16	14/16	8/16	3 141,5	1 758,5	5 612,1
16/16	14/16	9/16	3 405	1 882,8	6 157,7
16/16	14/16	10/16	3 689,7	2 012,9	6 763,3
16/16	14/16	11/16	3 997,9	2 150	7 434
16/16	14/16	12/16	4 331,7	2 296,2	8 171,4
16/16	14/16	13/16	4 693,3	2 454,4	8 974,6
16/16	15/16	0/16	1 912,4	1 108,6	3 299,2
16/16	15/16	1/16	2 108,2	1 220,6	3 641,5
16/16	15/16	2/16	2 320,4	1 338,3	4 023,2
16/16	15/16	3/16	2 551,8	1 462,3	4 453,1
16/16	15/16	4/16	2 806	1 593,3	4 941,6
16/16	15/16	5/16	3 087	1 732,2	5 501,3
16/16	15/16	6/16	3 399,7	1 880,4	6 146,7
16/16	15/16	7/16	3 749,8	2 039,9	6 893
16/16	15/16	8/16	4 143,8	2 214,1	7 755,4
16/16	15/16	9/16	4 588,4	2 408,4	8 741,7
16/16	15/16	10/16	5 090,1	2 630,4	9 849,9
16/16	15/16	11/16	5 654,3	2 889,9	11 063,3
16/16	15/16	12/16	6 284,4	3 196,4	12 355,9
16/16	15/16	13/16	6 985,8	3 559,1	13 712
16/16	15/16	14/16	7 762,6	3 982,9	15 129
16/16	15/16	15/16	8 616,7	4 467,7	16 618,6
16/16	16/16	0/16	2 398,1	1 380,4	4 165,9
16/16	16/16	1/16	2 682,4	1 530,3	4 702,1
16/16	16/16	2/16	3 009,4	1 694,5	5 344,8
16/16	16/16	3/16	3 392,1	1 876,9	6 130,6
16/16	16/16	4/16	3 849,5	2 084,4	7 109,5
16/16	16/16	5/16	4 407,8	2 329,5	8 340,4
16/16	16/16	6/16	5 100,2	2 635	9 872,1
16/16	16/16	7/16	5 963,9	3 038,1	11 707,4
16/16	16/16	8/16	7 022,5	3 578,6	13 780,7
16/16	16/16	9/16	8 299,46	4 285,9	16 071,4

7.8.2 Performance data of the interlaboratory comparison

7.8.2.1 Matrices — Method B

The interlaboratory comparison of the miniaturised method for semi-quantification of *E. coli* in soil, sludge and treated biowaste took place from May to July 2007. It was carried out with 14 European laboratories on 7 different matrices. The matrices selected for the interlaboratory comparison were chosen to represent soil, sludge and biowaste as broadly as possible, because Method B will find general application across different

types of soil and soil related materials (detailed information can be found in the final report on the interlaboratory comparison study).

Table 7 provides a list of the matrices chosen for *E. coli* detection.

Table 7 — Matrices tested in the interlaboratory comparison trial

Matrix type
Mesophilic anaerobic digested sewage sludge
Anaerobic treated biowaste
Pelletised air-dried sludge
Digested sewage sludge presscake
Composted sewage sludge
Composted green waste
Composted biowaste

In the interlaboratory comparison study, the following starting points were used:

The laboratory samples were all taken from a large batch of the different matrices according to the normal practice. The choice was made to analyse only spiked samples to obtain positive results. The spiking, the mixing and the sub-sampling were carried out as needed to prepare representative laboratory samples of approximately 150 g from the large batch sample. These were sent out by courier to each of the participating laboratories.

The experimental plan requested information on the basis of each laboratory being given 3 laboratory samples of each of the 2 batches of the seven matrices to be tested.

7.8.3 First assessment of the precision of the method

The statistical evaluation was conducted according to ENV ISO 13843. The limit of detection, the upper limit of quantification, the range of quantification and the results of dispersion U^2 were obtained (Table 8).

The limit of detection corresponds to the number of particles (germs per test portion) when the probability of a negative result is 5 % (superior limit of the confidence interval of the null result).

Poisson distribution corresponds to the random distribution of the number of particles at the moment of sampling a perfectly homogenised suspension.

The relative variance U^2 corresponds to the relative standard deviation squared ratio of the standard deviation squared and the mean squared as $U^2 = s^2 / m^2$.

NOTE 1 This statistic is commonly used to express dispersion or uncertainty of microbiological test results.

Table 8 — Summary of components of the *E. coli* Miniaturised method (MPN) precision

Limit of detection 5 % *E. coli*/g wet weight	Upper limit of quantification 5 % *E. coli*/g wet weight	Range of quantification Log10 unit	Results of dispersion U^2
67,40	$7{,}07\ 10^7$	6,0	0,1

NOTE 2 In judging the results it is important to consider that they do not depend on the experimental data but only on the design of the measurement protocol (random variation).

7.8.4 Interlaboratory comparison results

The statistical evaluation was conducted according to ISO 5725-2. The average values, the repeatability and the reproducibility were obtained (Table 9).

The repeatability corresponds to the maximum difference that can be expected (with a 95 % statistical confidence) between one test result and another, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present method by the same laboratory using its own facilities and testing laboratories samples obtained from the same primary field sample and prepared under identical procedures.

The repeatability limit was calculated using the relationship: $r_{\text{test}} = f \cdot \sqrt{2} \cdot s_{r.\text{test}}$ with the critical range factor $f = 2$.

NOTE 1 The above relationship refers to the difference that may be found between two measurements results performed each on two laboratory samples obtained under the same conditions. The value $f = 2$ used in the factor $f \cdot \sqrt{2}$ corresponds to the theoretical factor of 1,96 for a pure normal distribution at 95 % statistical confidence. Also, this value $f = 2$ corresponds to the usual value $k = 2$ of the coverage factor. However, it may be necessary to a use a larger value for f in situations as described Clause 12.

The reproducibility, like repeatability, corresponds to the maximum difference that can be expected (with a 95 % statistical confidence) between one test result and another test result obtained by another laboratory, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present method by two different laboratories using their own facilities and testing laboratory samples obtained from the same primary field sample and prepared under identical procedures.

This reproducibility limit was calculated using the relationship: $R = 2\ f \cdot \sqrt{2} \cdot s_R$ with the critical range factor $f = 2$.

NOTE 2 The above relationship refers to the difference that may be found between two measurements results performed each on two laboratory samples obtained under the same conditions. The value $f = 2$ used in the factor $f \cdot \sqrt{2}$ corresponds to the theoretical factor of 1,96 for a pure normal distribution at 95 % statistical confidence. Also, this value $f = 2$ corresponds to the usual value $k = 2$ of the coverage factor. In the case when reference is made to the dispersion of the values that could reasonably be attributed to the parameter being measured, the dispersion limit is equal to $k \cdot s_R$ with the usual value $k = 2$, resulting in a dispersion limit lower than the reproducibility limit (i.e. a ration of $\sqrt{2}$). However, it may be necessary to use a larger value $f \cdot \sqrt{2}$ (or k) in situations as described in Clause 9.

In case of relatively heterogeneous materials, the repeatability and the reproducibility limits may be larger than the values given in Table 9 (this means that the value chosen for the critical range factor f is larger than 2 as well as for the coverage factor k for dispersion). This is because the extreme results may have been obtained in accordance with the present method and/or be caused by the variability within, or in between, the laboratory samples.

For the calculations, as the test results were expressed on a log scale, the standard deviations in repeatability and Reproducibility conditions, respectively s_r and s_R were also expressed on a log scale. The expression of

repeatability and reproducibility in terms of maximum difference that can be expected between one test and another is given then by the limit of repeatability and Reproducibility respectively $r = 2\sqrt{2} \cdot s_r$ and $R = 2\sqrt{2} \cdot s_R$.

In order to make easier the interpretation, the values of repeatability and reproducibility are expressed in terms of maximal difference between two independent measurements on log scale, with a confidence level of 95 %:

EXAMPLE 1 Assuming r_1 and r_2 two independent measurements observed for a given method in repeatability conditions with $r_1 > r_2$:

$\log(r_1) - \log(r_2) \leq 0,9$ (95 % of the cases), corresponding to almost 1 log of difference between results.

The deviations between test results obtained under repeatability and reproducibility conditions can also be expressed by the maximal ratio between two independent measurements on natural scale (number of germs), with a confidence level of 95 %.

EXAMPLE 2 $\log(r_1) - \log(r_2) \leq 0,9$ (95 % of the cases)

then $r_1 / r_2 \leq 10^{0,9}$

then $r_1 / r_2 \leq 7,9$

thus r_1 is significantly higher than r_2 if $r_1 / r_2 > 7,9$.

Table 9 — Summary of *E. coli* MPN results of inter-laboratory comparison

Matrix	Overall mean (*E. coli*/g wet weight)	Repeat-ability (Ratio)	Reproduc-ibility (Ratio)	Discarded outliers (Statistical fitness)	Removed data (Other reasons)	Total number of data	Total number of labo-ratoties
Mesophilic anaerobic digested sewage sludge	< 67,40[a]	–	–	–	–	0	14
	< 67,40[a]	–	–	–	–	3	14
Anaerobic treated biowaste	1 378 339	2,4	10,5	1	–	30	13
	2 470 799	5,3	38,1	–	–	36	13
Pelletised air dried sludge	97	25,0	74,9	–	–	24	13
	724 659	10,5	56 115,6	–	–	33	14
Digested sewage sludge presscake	6 205	23,8	37 364,2	–	1	30	14
	2 099	4,4	4 718,9	–	–	39	11
Composted sewage sludge	30 307 023	11,9	11,9	1	–	18	13
	2 036 471	7,2	21,5	–	–	36	13
Composted green waste	1 809 954	4,5	4,5	1	–	36	13
	396 173	4,2	11,0	1	–	36	13
Composted biowaste	16 868[b]	1 863,6[b]	1 863,6[b]	–	–	21	13
	14 915 052	18,1	–	1	–	24	13

[a] Theoretical limit of detection with a probability of 95 % calculated for the method.

[b] Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.

NOTE In judging the results, it is important to consider the concentrations levels, at which measurements have been carried out.

7.9 Preparation of synthetic sea salt

7.9.1 Major ion composition of a convenient ocean synthetic sea salt

Table 10 — Composition of synthetic sea salt

Major ion	Total weight %	Ionic concentrations at 34 $^{0}/_{00}$ salinity mg/l
Chloride (Cl^-)	47,470	18 740
Sodium (Na^+)	26,280	10 454
Sulfate (SO_4^{2-})	6,602	2 631
Magnesium (Mg^{2+})	3,230	1 256
Calcium (Ca^{2+})	1,013	400
Potassium (K^+)	1,015	401
Bicarbonate (HCO_3^-)	0,491	194
Borate (B)	0,015	6,0
Strontium (Sr^{2+})	0,001	7,5
Solids total	86,11	34 089,50
Water (H_2O)	13,88	
Total	99,99	

7.9.2 Example for preparation from defined substances

Three basal solutions are to be made as follows:

Solution A

$CaCl_2 \cdot 2H_2O$	86,6 g
KCl	43,5 g
$SrCl_2.6H_2O$	0,07 g
Distilled Water	Make up to 1 000 ml

Solution B

$NaHCO_3$	5,15 g
$Na_2B_4O_7$	3,0 g
Distilled Water	Make up to 1 000 ml

Solution C

$MgSO_4 \cdot 7H_2O$	190,0 g
$MgCl_2 \cdot 6H_2O$	147,0 g
Distilled Water	Make up to 1 000 ml

The diluent is made by adding to 960 distilled water 10 ml of solution A, 10 ml of solution B, 20 ml of solution C, and then 14,9 g sodium chloride, mixing until completely dissolved and setting the pH to (7,5 ± 0,2).

The diluent is distributed into containers of desired volumes, and sterilised by autoclaving at (121 ± 3) °C for (15 ± 1) min.

7.10 Quality criteria for the manufacturing of the medium in microtitre plates (*E. coli*)

For each of the criteria which follow, a quality control has to be made on each batch of manufactured microtitre plates. The microtitre plates to be tested are taken at random or in a systematic way to constitute a sample in accordance with ISO 3951, respecting the general control level n° II of the normal control.

The threshold of positivity of a microtitre plate is defined as being the fluorescence level leading to a positive reading without ambiguity, to the eye, under a Wood's lamp (366 nm) (7.4.6).

The quality criteria to be respected are the following:

a) The background noise: absence of positive well in each microtitre plate of the sample, after inoculation with sterile special diluent and incubation of 48 h at 44 °C. The medium background noise of the sample has to be inferior to 25 % of the positivity threshold defined above and the variation coefficient has to be inferior to 10 %.

b) Average level of fluorescence: this is the geometric mean of the fluorescence signal obtained from the 96 wells of a microtitre plate inoculated uniformly with 200 µl per well of a suspension of *E. coli* WR1[2)] containing 500 germs per ml of Special Microplate Diluent (7.3.3), and incubation for 48 h at 44 °C. The average signal so obtained has to be at least twice the threshold of positivity and variation coefficient has to be inferior to 10 %.

c) Fertility: is calculated as the ratio of the number of germs observed with the batch of microtitre plates under test to the number of germs expected with a stable reference material (target value). The level of concentration should be brought up to around the maximum of the precision of the method, e.g. about one germ per well (500/100 ml). The stability and the homogeneity (target value and confidence intervals) of the reference material should have been determined with one (or several) batch(es) of microtitre plates already accepted. The threshold of acceptance of the microtitre plates tested is 0,66 to 1,5 of the target value. The variation coefficient should be inferior to 10 % (in logarithmic units).

The strain to be tested is *E. coli* WR1 and the incubation is 48 h at 44 °C.

The batch is rejected if any of the criteria is not respected.

2) *E. coli* WR1 is the trade name of a product supplied by NCTC, UK (NCTC number 13167). This information is given for the convenience of users of this Technical Report and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

8 Method C — Macromethod (Most Probable Number) in liquid medium

8.1 Scope

Method C specifies a most probable number (MPN) method for the semi-quantitative detection of *Escherichia coli* in sludges, treated biowaste and soils of similar consistency to the matrices validated. It is suitable to evaluate the log reduction of *E.coli* through treatment as well as the quality of the end product. Method C can be used irrespective of the dry residue content of the test material. Method C has a limit of detection of approximately 10 *E. coli* MPN/g wet weight (see ENV ISO 13843).

8.2 Principle

The essence of the MPN method is to dilute the sample to such a degree that the inocula in the tubes will sometimes but not always contain viable organisms. The "outcome", i.e., the number of tubes and the number of tubes with growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions incubating tubes at several dilutions. The MPN is the number which makes the observed outcome most probable.

This method is based on (see also [1]):

— preparation of the homogenised sample suspension of the sample in 0,9 % (mass/volume, e.g. 9 g/l) sodium chloride solution;

— serial dilutions of this suspension in the same diluents (from 10^{-1} up to 10^{-7});

— transfer of 3 x 1 ml out of each dilution step into three tubes containing 9 ml Fluorocult™ lauryl sulfate broth[3)];

— incubation at (44 ± 1) °C for (40 ± 4) h;

— detection of gas production, fluorescence and indole formation;

— quantification by the MPN technique.

8.3 Reagents, diluents and culture media

8.3.1 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 providing that they are shown to be of equivalent performance in the test.

8.3.2 **Sodium chloride solution,** NaCl, 9 g/l

Mix the sodium chloride in water in a 2 000 ml flat bottom flask (8.4.1). Fill in 180 ml portions into 500 ml flasks (8.4.1) or in 9 ml portions into culture tubes (8.4.9). Adjust the pH to (7,0 ± 0,2) at 25 °C.

3) Fluorocult™ lauryl sulfate broth is an example of a suitable product available commercially. This information is given for the convenience of the users of this Technical Report and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

Sterilise by autoclaving (8.4.3) at (121 ± 3) °C for (15 ± 2) min.

8.3.3 Fluorocult™ lauryl sulfate broth with MUG[1)]

Trypsin digested peptone from meat	20,0 g
Lactose	5,0 g
Sodium chloride	5,0 g
Sodium lauryl sulphate	0,1 g
Di-potassium hydrogen phosphate	2,75 g
Potassium di-hydrogen phosphate	2,75 g
L- tryptophan	1,0 g
4-methyl-umbelliferyl- ß-D-glucuronide (MUG)	0,1 g
Demineralised water	1 000 ml

Mix carefully the following substances in 1 000 ml of water in a 2 000 ml (8.4.1) flat bottom flask, while heating in a boiling water bath (8.4.16). Adjust the pH (8.4.11) to (6,8 ± 0,1) using 1 mol/l sodium hydroxide solution. Distribute the solution in 9 ml portions into culture tubes and add a Durham tube (8.4.11) to each portion.

Sterilise in an autoclave (steam steriliser) (8.4.3) for (15 ± 1) min at (121 ± 3) °C.

8.3.4 Kovac's reagent

4-di-methylamino benzaldehyde ($C_9H_{11}NO$)	5,0 g
Isoamyl alcohol ($C_5H_{12}O$)	75,0 ml
Hydrochloric acid (ρ = 1,18 g/ml)	25,0 ml

Dissolve the 4-dimethylamino benzaldehyde in the isoamyl alcohol and heat in a water bath at 60 °C for 5 min. Then, add slowly the hydrochloric acid.

The reagent will be ready for use after 6 to 7 h (indicated by a yellow colour). Store in the refrigerator and protect from light.

WARNING — Kovacs reagent is harmful if swallowed, irritating to the respiratory system and to the skin. It is recommended to use it in a flow cabinet.

8.3.5 Sodium hydroxide, NaOH, 1mol/l.

8.4 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:

8.4.1 Wide-mouth glass flasks or beakers for example 125 ml, 200 ml, 500 ml and 2 000 ml.

8.4.2 Thermostatic incubators regulated at (44 ± 1) °C (static).

8.4.3 Autoclave (Steam steriliser).

8.4.4 Refrigerator

8.4.5 Sterile plastics Petri dishes, with lid of about 90 mm in diameter.

8.4.6 Graduated pipettes, of nominal capacities 1 ml and 10 ml.

8.4.7 Shaking device

8.4.8 Culture tubes, 25 ml capacity, or equivalent containers.

8.4.9 Vortex mixer suitable for 25 ml capacity culture tubes or equivalent containers.

8.4.10 Durham-tubes

8.4.11 pH-meter, with temperature compensation and pH measuring cell.

8.4.12 Boiling water bath

8.4.13 UV-lamp (366 nm wavelength)

8.4.14 Laboratory spatula

8.4.15 Analytical balance

8.5 Sampling

8.5.1 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, cool the sample at (5 ± 3) °C.

Samples are liable to ferment and can 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.

8.5.2 Sample storage

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

8.5.3 Sample 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.

8.6 Procedure

8.6.1 Sample preparation

Place 20 g (wet weight) of sample into 180 ml sterile sodium chloride solution (8.3.2). Shake at a minimum of 150 rpm for 20 h at (5 ± 3) °C.

20 h shaking is recommended for all samples. In the case of liquid homogeneous samples (e.g. anaerobic digested sewage sludge) the shaking time may be reduced, but not less than 30 min.

For disinfectant (e.g. lime, peracetic acid) treated sludges, a suitable pre-treatment for neutralising the disinfecting agent is required. For lime treated materials adjust the pH to (7,0 ± 0,5) with 1 mol/l hydrochloric acid. For other relevant chemicals (e.g. peracetic acid), a suitable neutralisation procedure shall be used (see for example EN 1040 for neutralisers).

8.6.2 Analysis

Take an aliquot of 1 ml out of the primary prepared suspension (see 8.1) from sample preparation.

Prepare a serial tenfold dilution up to 10^{-7}: 1 ml of prepared suspension (see 8.1) + 9 ml of sterile sodium chloride solution (8.3.2).

From each dilution step, transfer 1 ml per tube into 3 tubes containing 9 ml of Fluorocult™ lauryl sulfate broth[1)] with MUG (8.3.3) and a Durham tube (8.4.10) each.

Incubate at (44 ± 1) °C for (40 ± 4) h.

Observe the Durham tube for gas formation. Consider all tubes with gas as positive culture.

Add 0.5 ml of sodium hydroxide (8.4.5) to each “gas positive” tube and examine for fluorescence with 366 nm UV-light (8.4.13). Consider all fluorescent tubes as positive culture.

Add a 0,5 cm layer of Kovács reagent to each “gas and fluorescence positive” tube and watch for the colour change (cherry red after 1 min to 2 min). Consider all tubes with a red circle as positive culture.

The number of dilution steps depends on type of matrix being tested. For untreated matrix a dilution up to 10^{-7} should be carried out. However, for treated matrix, dilution up to 10-4 should be sufficient.

The primary solution (described in 8.1) is already diluted as 10^{-1}. From this primary dilution, transfer 1 ml into three tubes each containing 9 ml Fluorocult™ lauryl sulfate broth with MUG (8.3.3), as a first dilution step.

If all tubes in the last three dilution steps (10^{-5}, 10^{-6} and 10^{-7} or 10^{-2}, 10^{-3} and 10^{-4}) are positive (gas+/fluorescence+/indole+), prepare 10^{-8} and 10^{-9} or 10^{-5} and 10^{-6} dilutions steps and transfer 1 ml per tube into three tubes containing 9 ml Fluorocult™ lauryl sulfate broth with MUG (8.3.3) and a Durham tube (8.4.10) each. Store all tubes from the dilution steps of the sample at 4 °C until the final result is obtained.

8.6.3 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 described in EN 15934. This shall be performed in parallel with the microbiological analysis.

8.7 Expression of the results

For each of the seven dilutions (from 10^{-1} to 10^{-7}), note the number of positive tubes (gas+/fluorescence+/indol+) (between 0 and 3). Identify the characteristic number composed of three digits, corresponding to the number of positive tubes of the three last dilutions giving a number of positive tubes >0.

Calculate the MPN corresponding to the identified characteristic number using the De Man table (see Table 12), by multiplying the MPN index by the dilution factor. The result corresponds to a MPN per ml of primary prepared suspension.

EXAMPLE

Table 11 — Example for the determination of the MPN of *E. coli*

	Gas+/Fluorescence+/Indol+						
Dilution step	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}+	**10^{-7}+**
Tube 1	+	+	+	+	-	-	**-**
Tube 2	+	+	+	-	+	-	**-**
Tube 3	+	+	+	-	-	-	**-**
Characteristic number	3	3	**3**	**1**	**1**	0	**0**
MPN index	**7,5**						
Dilution factor	**10^{-3}**						
Result: MPN *E.coli*/g (wet weight) sample	**7,5 x 10^{3}**						

The result per gram of sample material is MPN *E. coli*/g (wet weight) of original sample.

Numbers present per dry weight of sludge are calculated according to Formula (3):

$$N_{\mathrm{d}} = N_{\mathrm{w}} \cdot 100 / e \qquad (3)$$

where

N_{d} is the bacterial count in MPN/g dry weight;

N_{w} is the bacterial count in MPN *E. coli*/g wet weight;

e is the dry mass of the original wet (as received) sample.

8.8 Performance data

8.8.1 MPN Statistical table for 3-tubes MPN procedure

Table 12 — MPN statistical table for 3-tubes MPN procedure according to de Man (see also [2])

Characteristic number			MPN index	Confidence limits			
1st digit	2nd digit	3rd digit		≥ 95 %	≥ 95 %	≥ 99 %	≥ 99 %
0	0	0	< 0,30	0,00	0,94	0,00	1,40
0	0	1	0,30	0,01	0,95	0,00	1,40
0	1	0	0,30	0,01	1,00	0,00	1,60
0	1	1	0,61	0,12	1,70	0,05	2,50
0	2	0	0,62	0,12	1,70	0,05	2,50
0	3	0	0,94	0,35	3,50	0,18	4,60
1	0	0	0,36	0,02	1,70	0,01	2,50
1	0	1	0,72	0,12	1,70	0,05	2,50
1	0	2	1,1	0,4	3,5	0,2	4,6
1	1	0	0,71	0,13	2,00	0,06	2,70
1	1	1	1,1	0,4	3,5	0,2	4,6
1	2	0	1,1	0,4	3,5	0,2	4,6
1	2	1	1,5	0,5	3,8	0,2	5,2
1	3	0	1,6	0,5	3,8	0,2	5,2
2	0	0	0,93	0,15	3,50	0,07	4,60
2	0	1	1,4	0,4	3,5	0,2	4,6
2	0	2	2,0	0,5	3,8	0,3	5,2
2	1	0	1,5	0,4	3,8	0,2	5,2
2	1	1	2,0	0,5	3,8	0,2	5,2
2	1	2	2,7	0,9	9,4	0,5	14,2
2	2	0	2,1	0,5	4,0	0,2	5,6
2	2	1	2,8	0,9	9,4	0,5	14,2
2	2	2	3,5	0,9	9,4	0,5	14,2
2	3	0	2,9	0,9	9,4	0,5	14,2
2	3	1	3,6	0,9	9,4	0,5	14,2
3	0	0	2,3	0,5	9,4	0,3	14,2
3	0	1	3,8	0,9	10,4	0,5	15,7
3	0	2	6,4	1,6	18,1	1,0	25,0
3	1	0	4,3	0,9	18,1	0,5	25,0
3	1	1	7,5	1,7	19,9	1,1	27,0
3	1	2	12	3	36	2	44
3	1	3	16	3	38	2	52
3	2	0	9,3	1,8	36,0	1,2	43,0
3	2	1	15	3	38	2	52
3	2	2	21	3	40	2	56
3	2	3	29	9	99	5	152
3	3	0	24	4	99	3	152
3	3	1	46	9	198	5	283
3	3	2	110	20	400	10	570
3	3	3	> 110				

8.8.2 Repeatability and reproducibility

8.8.2.1 Matrices — Method C

The interlaboratory comparison of the macro method for quantification of *E. coli* in soil, sludge and treated biowaste took place from May to July 2007. It was carried out with 14 European laboratories on 7 different matrices. The matrices selected for the interlaboratory comparison were chosen to represent soil, sludge and biowaste as broadly as possible, because the method will find general application across different types of soil and soil related materials (detailed information can be found in the final report on the interlaboratory comparison study [3]).

Matrices types tested in the interlaboratory comparison trial:

— Mesophilic anaerobic digested sewage sludge;

— Anaerobic treated biowaste;

— Pelletised air-dried sludge;

— Digested sewage sludge presscake;

— Composted sewage sludge;

— Composted green waste;

— Composted biowaste.

In the interlaboratory comparison study, the following starting points were used:

The laboratory samples were all taken from a large batch of the different matrices according to the normal practice. The choice was made to analysed only spiked samples so as to obtain positive results. The spiking, the mixing and the sub-sampling were carried out as needed to prepare representative laboratory samples of approximately 150 g from the large batch sample. These were sent out by courier to each of the participating laboratories.

The experimental plan requested information on the basis of each laboratory being given 3 laboratory samples of each of the 2 batches of the seven matrices to be tested.

8.8.3 First assessment of the precision of the method

The statistical evaluation was conducted according to ISO 13843. The limit of detection, the upper limit of quantification, the range of quantification and the results of dispersion U^2 were obtained (see Table 13).

The limit of detection corresponds to the number of particles (germs per test portion) when the probability of a negative result is 5 % (superior limit of the confidence interval of the null result).

Poisson distribution corresponds to the random distribution of the number of particles at the moment of sampling a perfectly homogenised suspension.

The relative variance U^2 corresponds to the relative standard deviation squared ratio of the standard deviation squared and the mean squared as:

$$U^2 = s^2 / m^2 \quad (4)$$

where

U^2 is the relative variance;

s^2 is the standard deviation squared;

m^2 is the mean squared.

NOTE 1 This statistic is commonly used to express dispersion or uncertainty of microbiological test results.

Table 13 — Summary of components of the *E. coli* Macro method precision

Limit of detection 5% *E. coli* /g wet weight	Upper limit of quantification 5% *E. coli* /g wet weight	Range of quantification Log10 unit	Results of dispersion U^2
8,99	$4{,}65 \cdot 10^4$	5,7	0,8

NOTE 2 In judging the results it is important to consider that they do not depend on the experimental data but only on the design of the measurement protocol (random variation).

8.8.4 Interlaboratory comparison results

The statistical evaluation was conducted according to ISO 5725-2. The average values, the repeatability and the reproducibility were obtained (Table 14).

The repeatability corresponds to the maximum difference that can be expected (with a 95 % statistical confidence) between one test result and another, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present method by the same laboratory using its own facilities and testing laboratories samples obtained from the same primary field sample and prepared under identical procedures.

The repeatability limit was calculated using the relationship: $r_{\text{test}} = f \cdot \sqrt{2} \cdot s_{r.\text{test}}$ with the critical range factor $f = 2$.

NOTE 1 The above relationship refers to the difference that may be found between two measurements results performed each on two laboratory samples obtained under the same conditions. The value $f = 2$ used in the factor $f \cdot \sqrt{2}$ corresponds to the theoretical factor of 1,96 for a pure normal distribution at 95 % statistical confidence. Also, this value $f = 2$ corresponds to the usual value $k = 2$ of the coverage factor. However, it may be necessary to a use a larger value for f in situations as described Clause 12.

The reproducibility, like repeatability corresponds to the maximum difference that can be expected (with a 95 % statistical confidence) between one test result and another test result obtained by another laboratory, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present method by two different laboratories using their own facilities and testing laboratory samples obtained from the same primary field sample and prepared under identical procedures.

This reproducibility limit was calculated using the relationship: $R = 2 f \cdot \sqrt{2} \cdot s_R$ with the critical range factor $f = 2$.

NOTE 2 The above relationship refers to the difference that may be found between two measurements results performed each on two laboratory samples obtained under the same conditions. The value $f = 2$ used in the factor $f \cdot \sqrt{2}$ corresponds to the theoretical factor of 1,96 for a pure normal distribution at 95 % statistical confidence. Also, this value $f = 2$ corresponds to the usual value $k = 2$ of the coverage factor. In the case when reference is made to the dispersion of the values that could reasonably be attributed to the parameter being measured, the dispersion limit is equal to $k \cdot s_R$ with the usual value $k = 2$, resulting in a dispersion limit lower than the reproducibility limit (i.e. a ration of $\sqrt{2}$). However, it may be necessary to use a larger value $f \cdot \sqrt{2}$ (or k) in situation as described in Clause 9.

In case of relatively heterogeneous materials, the repeatability and the reproducibility limits may be larger than the values given in Table 9 (this means that the value chosen for the critical range factor f is larger than 2 as well as for the coverage factor k for dispersion). This is because the extreme results may have been obtained

in accordance with the present method and/or be caused by the variability within, or in between, the laboratory samples.

For the calculations, as the test results were expressed on a log scale, the standard deviations in repeatability and Reproducibility conditions, respectively s_r and s_R were also expressed on a log scale. The expression of repeatability and reproducibility in terms of maximum difference that can be expected between one test and another is given then by the limit of repeatability and Reproducibility respectively $r = 2\sqrt{2} \cdot s_r$ and $R = 2\sqrt{2} \cdot s_R$.

In order to make easier the interpretation, the values of repeatability and reproducibility are expressed in terms of maximal difference between two independent measurements on log scale, with a confidence level of 95 %:

EXAMPLE 1 Assuming r_1 and r_2 two independent measurements observed for a given method in repeatability conditions with $r_1 > r_2$:

$\log(r_1) - \log(r_2) \leq 0{,}9$ (95 % of the cases), corresponding to almost 1 log of difference between results.

The deviations between test results obtained under repeatability and reproducibility conditions can also be expressed by the maximal ratio between two independent measurements on natural scale (number of germs), with a confidence level of 95 %:

EXAMPLE 2 $\log(r_1) - \log(r_2) \leq 0{,}9$ (95 % of the cases)

then $r_1 / r_2 \leq 10^{0,9}$

then $r_1 / r_2 \leq 7{,}9$

thus r_1 is significantly higher than r_2 if $r_1 / r_2 > 7{,}9$.

Table 14 — Summary of *E. coli* Macromethod results of inter-laboratory comparison

Matrix	Overall mean (*E, coli* /g wet weight)	Repeatability (Ratio)	Reproducibility (Ratio)	Discarded outliers (Statistical fitness)	Removed data (Other reasons)	Total number of data	Total number of labo-ratories
Mesophilic anaerobic digested sewage sludge	< 8,99[a]	-	-	-	-	3	14
	< 8,99[a]	-	-	-	-	3	14
Anaerobic treated biowaste	832837	7,0	25,7	2	-	27	13
	1547828	10,8	61,9	-	-	33	13
Pelletised air dried sludge	< 8,99[a]	-	-	-	-	0	13
	394368	9,2	2632,7	-	-	27	14
Digested sewage sludge presscake	3898	9,7	73661,6	-	-	39	13
	1288	20,1	3505,1	-	-	39	13
Composted sewage sludge	> 4,65 10^6	-	-	-	-	0	13
	833659	18,8	85,3	-	-	30	13
Composted green waste	818474	13,4	90,5	-	-	33	13
	193497	8,5	49,6	-	-	36	13
Composted biowaste	1531[c]	3255,0[c]	3255,0[c]	-	-	30	13
	> 4,65 10^{6} [b]	-	-	-	-	6	13

[a] Theoretical limit of detection with a probability of 95 % calculated for the method.

[b] Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.

[c] The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully.

9 Test report

The test report shall contain the following information:

a) a reference to this Technical Report and the applied Method;

b) complete identification of the sample;

c) details of sample pre-treatment, if carried out;

d) expression of results according to the specifications of the methods (6.7, 7.7, 8.7 as appropriate);

e) any details not specified in this Technical Report or which are optional, as well as any factor which may have affected the results.

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[12] ENV ISO 13843:2001, *Water quality — Guidance on validation of microbiological methods (ISO 13843)*

[13] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

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