Soils, sludges and treated bio-wastes — Detection and enumeration of *Escherichia coli* in sludges, soils and organic fertilisers of similar consistency to the matrices validated – Part 1: Membrane filtration method for quantification

Boden, Schlamm und behandelte Bio-abfälle — Quantitativer Nachweis von Escherichia coli aus Schlämmen, Böden, Dügemittels und Bodenverbesserern Kultursubstraten sowie Bioabfällen – Teil 1: Membranfiltrationsverfahren

S*ols, boues et bio-déchets traités — Détection et dénombrement de Escherichia coli dans les boues, les sols et les fertilisants organiques de consistance similaire aux matrices validées – Partie 1 : Méthode par filtration sur membrane*

ICS:

Descriptors: *E.coli*, sludges, soils, organic fertilisers

Document type: European Standard
Document subtype:
Document stage: Working Document
Document language: E

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Foreword

This document has been prepared in the framework of the project Horizontal.

This document is a working document.

The following TC's have been involved in the preparation of the standard: TC 308.

The standard is divided into three parts:

- part 1 describes a membrane filtration method for quantification,
- part 2 describes a miniaturised semi-quantitative MPN method,
- part 3 describes a semi-quantitative macromethod.

This standard is applicable and validated for several types of matrices. The table below indicates which ones.

Material	Validated	Document
Mesophilic anaerobic digested sewage sludge		
Anaerobic treated biowaste		
Pelletised air dried sludge		
Digested sewage sludge presscake		
Composted sewage sludge		
Composted green waste		
Composted biowaste		

Introduction

This document is developed in the framework of the project 'Horizontal'. It is the result of a desk study "Hygienic Parameters Feasibility of Horizontal Standards for *Escherichia coli* and *Salmonella* spp. in sludges, soils, soil improvers, growing media and biowastes" and aims at evaluation of the latest developments in assessing *E. coli* in sludge, soil and organic fertilisers. After discussion with all parties concerned in CEN and selection of a number of test methods described in this study the standard has been developed further as a horizontal method and has been validated within the project ”Horizontal”.

Sludges, soils, soil and organic fertilizers can contain pathogenic micro-organisms such as *Salmonella* spp. which occur mainly in the intestinal tract of humans and animals and are transmitted through faecal contamination. The use of such contaminated materials in agriculture may cause outbreaks of infection due to the production of contaminated food and animal foodstocks. They may also be transmitted to wild animals. There is a need to monitor the efficacy of storage and treatment processes to control pathogens such as *Salmonella* spp., and application rates to land.

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.

Suitable quality control procedures, at least those described in ISO 8199:2005, have to be applied.

WARNING — "Waste and sludge samples can contain hazardous and inflammable substances. They can contain pathogens and be liable to biological action. Consequently, it is recommended that these samples should 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".

The texts of the chapters 1 to 12 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

1 Scope

This part of the European standard describes a membrane filtration procedure for the quantitative detection, by culture of individual colonies on chromogenic agar media, of *Escherichia coli*. in sludges, soils and organic fertilisers of similar consistency to the matrices validated. This part of the standard 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. A liquid enrichment and most probable number estimation method may be suited to such a purpose.

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.

The method has a limit of detection of approximately 27 *E. coli* cfu.g^{-1} wet weight [ENV ISO 13843], dependent on the solids content which at high concentrations (> 10 % (w/v)) may restrict filtration of the sample volume through the membrane if not first diluted.

2 Normative references

These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, only the edition cited applies. For undated references the latest edition of the publication referred to applies (including any amendments).

EN ISO 5667-13:1997, *Water quality — Sampling — Part 13: Guidance on sampling of sludges from sewage and water treatment works*.

EN 12880:2000, *Characterisation of sludges — Determination of dry residue and water content*.

ISO 8199:2005, *Water quality — General guidance on the enumeration of micro-organisms by culture*.

ENV ISO 13843:2001, *Water Quality — Guidance on validation of microbiological methods.*

ISO 5725:1994, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*.

3 Definitions

For the purposes of this standard**Erreur ! Source du renvoi introuvable.**, the following terms and definitions apply.

3.1
Escherichia coli
Escherichia coli, belongs to the family of Enterobacteriaceae, is Gram-negative, non-sporulating, rod-shaped bacteria, able to ferment lactose and to grow at 44℃. Most *E. coli* strains are able to produce indole from tryptophan and are *β*-glucuronidase-positive

3.2
method definition
β-glucuronidase-positive able to hydrolyse 5-bromo-4-chloro-3-indolyl-β-glucuronide (BCIG) when growing at the incubation temperature of 44℃ on an Membrane Lactose Glucuronide Agar (MLGA) medium

3.3
cfu, colony forming unit
growth of individual bacterial cells into visible colonies on agar media, including on membrane filters overlaying the agar media

3.4
vegetative bacteria
bacteria which are capable of normal growth in broth or on agar media without pre-culture resuscitation

3.5
sub-lethally damaged bacteria
bacteria which have been stressed but not killed by storage or subsequent treatment by, for example, mesophilic anaerobic digestion, lime stabilisation or composting and therefore may not be recovered

3.6
resuscitation
recovery to vegetative growth of sub-lethally damaged bacteria previously incapable of growth on agar media

3.7
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

3.8
dry residue
the dry mass portion of the material obtained after the specified drying process. It is expressed as percent or in grams per kilogram [EN 12880:2000]

4 Symbols and abbreviations

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

DS: Dry Solid

MLGA: Membrane Lactose Glucuronide Agar

E. coli: *Escherichia coli*

5 Principle

The homogenised diluted sample is filtered, the membrane filter recovered aseptically and incubated on membrane lactose glucuronide agar (MLGA), initially at (30 ± 1)℃ for (4 ± 0.5) h. Subsequently, the temperature is increased to (44 ± 1)℃ for (16 ± 2) h. The presence of *E. coli* is indicated by green colonies resulting from the hydrolysis of BCIG.

6 Reagents, diluents and culture media

6.1 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 [ISO 8199:2005].

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

6.2 Peptone saline solution

Bacteriological peptone	1.0 g
Sodium chloride	8.5 g
Distilled water	1 000 mL

Dissolve the Bacteriological peptone and sodium chloride into distilled water. 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℃.

Sterilise in the autoclave (7.1) at (121 ± 3)℃ for (15 ± 1) min. Store at (5 ± 3)℃ for a maximum of 3 months.

6.3 Membrane Lactose Glucuronide Agar (MLGA)

6.3.1 5-bromo-4-chloro-3-indolyl-β-glucuronide (BCIG) suspension

BCIG, monohexylammonium salt	0.2 g
95 % aqueous ethanol	2.5 mL
1 M sodium hydroxide	0.5 mL

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

6.3.2 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 (7.8) 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.

6.4 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	1000 mL

Suspend the ingredients in 1000 ml of distilled water. Bring to the boil whilst stirring continuously to dissolve all ingredients completely. Adjust the pH (7.8) to (7.0 ± 0.5).

Sterilise in the autoclave (7.1) 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.

7 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199:2005.

Usual microbiological laboratory equipment and in particular:

7.1 Apparatus for sterilisation - autoclave

7.2 Thermostatic incubator(s) regulated at (30 ± 1)°C and/or (44 ± 1)°C

7.3 Homogeniser

7.4 Centrifuge capable of centrifuging 50 mL at 200 *g* to 300 *g*.

7.5 Membrane filters, 0.45 µm gridded, cellulose nitrate

7.6 Glass fibre pre-filter discs, 47 mm diameter, pore size 2.7 µm

7.7 Vacuum pump

7.8 Vacuum manifold – magnetic filter bases and cups

7.9 Sterile homogeniser bags, 250 mL volume, with or without integrated mesh to exclude large particulate matter

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

7.11 Sterile universals of 20 mL volume, or containers with similar capacity

7.12 Sterile pipettes, glass or disposable plastic ware, capable of dispensing 1 and 10 mL volumes

7.13 Sterile conical centrifuge tubes, 50 mL volume, disposable plastic

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

7.15 Analytical balance

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

7.17 Vortex mixer

7.18 pH meter with an accuracy of ± 0.1

7.19 Beakers or containers 100 mL, 250 mL and 1000 mL

7.20 Laboratory spatula

7.21 Boiling bath

7.22 Bunsen burner

7.23 Sterile forceps

7.24 Filter funnels

8 Sampling

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.

8.1 General

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.

See also the Warning note in the introduction.

8.2 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.

8.3 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.

See also the Warning note in Introduction.

9 Procedure

9.1 Sample preparation

Weigh a representative 10 g (wet weight) of the sample as received into a 250 mL container (7.19).

Add an appropriate volume of peptone saline solution (6.2) so that the final weight is 100 g and mix thoroughly using a vortex mixer (7.17).

Place in homogeniser bag (7.9) and place in the homogeniser (7.3) 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.

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 (7.18).

NOTE 1 **If the pH drops below 4.5 during the neutralisation process, start a new analysis with a fresh test portion.**

NOTE 2 **For other relevant treatment chemicals (e.g. peracetic acid), a suitable oxidant neutralisation procedure must be used (see for example EN 1040:1997)[(1)].**

NOTE: Centrifugation and Pre-filtration (Annex B - informative)

The variation in the level of solid material contained within the matrices applicable to this standard 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 it necessary then centrifugation and pre-filtration should be applied.

Centrifugation

Transfer the homogeniser bag contents to two disposable centrifuge tubes (7.13) and centrifuge the two 50 mL aliquots at 200*g* to 300*g* for 3 min.

Pre-filtration

Decant the supernatant from the tubes in a beaker and filter through a glass-fibre pre-filter (7.6) using a filter funnel with receiver (7.24) to remove fine debris.

The filter funnels should have been sterilised in a boiling bath (7.21) prior to analysis, the filter funnels are removed from the boiling bath using sterile forceps (7.23) and attached to the vacuum pump (7.7).

The glass fibre filter (7.6) is placed on the filter funnel using sterile tweezers (7.14) 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 minutes before being used again.

9.2 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}-10^{-3} with peptone saline solution (7.2). This will permit the enumeration of up to 10^4 *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} (for example, untreated sludge may contain 10^8 -10^9 *E. coli* per g wet weight).

Prepare the relevant number of sterile universals (7.11) according to the number of selected dilutions; add 9 mL of sterile peptone saline solution (6.2) to each.

Using a sterile pipette (7.12); transfer 1 mL of the filtrate to the first universal containing 9 mL of peptone saline solution and mix thoroughly using a vortex mixer (7.17).

Using a fresh pipette (7.12), transfer 1 mL of the diluted sample to the second universal containing 9 mL of peptone saline solution and mix thoroughly using a vortex mixer (7.17).

Continue as above until all the dilutions have been prepared.

9.3 Membrane Filtration

Transfer the magnetic filter base (7.8) from the boiling water bath (7.21) to the manifold using sterilised forceps (7.23).

NOTE Aseptic technique should be followed at all times.

Membranes (7.5) are removed from their packets with sterilised tweezers (7.14) and placed grid side up on to the magnetic filter base. Membranes that have torn are dropped or which touch any object must be discarded. Membranes should only be handled by the edge with tweezers designed for that purpose. The tweezers must be sterilised first by immersing them in ethanol which is subsequently flamed off using a Bunsen burner (7.22), 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 must 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 must 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 (6.3). 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 must be discarded. The magnetic filter base must 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 must be totally immersed in boiling water for at least two minutes before being removed to continue filtration.

A positive control suspension containing 10^2 -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.

9.4 Resuscitation and enumeration of colonies on chromogenic agar

Remove the filter from the housing using sterile tweezers (7.13) and transfer to the surface of a 55 mm diameter MLGA plate (6.3). Incubate plates initially at (30 ± 1)°C for (4 ± 0.5) h. Subsequently, increase the temperature to (44 ± 1)°C for (16 ± 2) h.

Enumerate typical green colonies by eye, only plates within the range 10-100 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.

NOTE **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.**

9.5 Confirmation of colony identity

The typical colonies are sub-cultured onto selective MacConkey agar (7.4). The MacConkey subculture plates are incubated at (36 ±2)℃ 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.

9.6 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 described in EN 12880:2000. This shall be performed in parallel with the microbiological analysis.

10 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 must be taken into account to estimate the total number of typical colonies to calculate the final result [ISO 8199:2005].

$$c = \frac{n}{v}$$

where:

c = *E. coli* concentration per g wet weight of original sample

n = total number of typical *E. coli* colonies on the selected filter membranes: $n = n1 + n2 + \ldots$

v = total volume filtered through the selected filter membranes (from 1 ml): $v = v1 + v2 + \ldots$

NOTE **The dilution factor of the dilution A taken for filtration step should not be forget 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 x 1 x 0.01) + (0.1 x 1 x 0.001)

$c = 96 / 0.0011$ = 8.7 10^4 cfu/g ww

Numbers present per g dry weight of sample are calculated according to:

$$c = \frac{n}{ve} \times 100$$

e = % dry residue of the original wet sample.

11 Performance data

Information concerning the repeatability and reproducibility of the procedure, obtained by interlaboratory tests of the validation study (European scale Interlaboratory trial) performed during the FP6 EU Horizontal-Hyg project is given in Annex A (informative).

12 Test report

The test report shall contain the following information:

a) reference to this part of this European Standard;

b) all information necessary for complete identification of the sample;

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

d) results of the determination according to Clause 9; and

e) any detail not specified in this part of this European Standard and any other factor which may have affected the results.

Annex A
(informative)

Performance data of the interlaboratory comparison

A.1 Objective of the interlaboratory comparison

In a European wide laboratory comparison study according to ISO 5725-2, the performance characteristics of the standard “Detection and enumeration of *Escherichia coli* in sludges, soils and treated biowaste. Part1: Membrane filtration method for quantification” were established.

A.2 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 broad as possible, because the standard 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 [5]).

Table A.2-1 provides a list of the type of matrices chosen for *E. coli* detection.

Table A.2-1 Matrices types tested in the interlaboratory comparison trial

Matrice type	Short name
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 designed by project Horizontal-Hygiene on the basis of each laboratory being given 3 laboratory samples of each of the 2 batches of the seven matrices to be tested.

A.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 (Table A.3-1).

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

Table A.3-1 — 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 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).

A.4 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 A.4-1).

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 standard 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: $r_{test} = f.\sqrt{2}.s_{r.test}$ with the critical range factor f = 2.

NOTE The above relationship refers to the difference that may be found between two measurement results performed each on two laboratory samples obtained under the same conditions. The value f = 2 used in the factor $f.\sqrt{2}$ 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 recommended in the Guide to the expression of Uncertainty in Measurement (GUM). 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 standard 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: $R = f.\sqrt{2}.s_R$ with the critical range factor f = 2.

NOTE The above relationship refers to the difference that may be found between two measurement results performed each on two laboratory samples obtained under the same conditions. The value f = 2 used in the factor $f.\sqrt{2}$ 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 recommended in the Guide to the expression of Uncertainty in Measurement (GUM). 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 $k.s_R$ 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 $f.\sqrt{2}$ (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 A.4-1 (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 standard 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 $r = 2\sqrt{2}.s_r$ and $R = 2\sqrt{2}.s_R$.

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

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

$\log(r_1) - \log(r_2) \leq 0.9$ (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 A.4-1 — Summary of *E. coli* Membrane filtration method 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 labs
Mesophilic anaerobic digested sewage sludge	< 26.96[(1)]	-	-	-	-	0	13
	< 26.96[(1)]	-	-	-	-	3	13
Anaerobic treated biowaste	1784260	2.8	59.4	2	-	33	13
	2898637	3.5	72	-	-	36	12
Pelletised air dried sludge	< 26.96[(1)]	-	-	-	-	0	13
	385222	7.1	29465.7	-	1	36	13
Digested sewage sludge presscake	6632	3.2	7499.5	-	-	35	12
	3651	4.1	655.8	-	-	36	12
Composted sewage sludge	94944250	11.9	34.8	1	-	33	12
	968981	5.9	88.1	1	-	36	13
Composted green waste	824211	3.6	51.7	1	-	36	13
	433007	2.5	32.3	-	-	39	13
Composted biowaste	7883[(2)]	262.7 [(2)]	262.7 [(2)]	-	-	12	13
	16967925	4.3	33.6	-	-	39	13

[(1)] Theoretical limit of detection with a probability of 95% calculated for the method

[(2)] 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.

Annex B
(informative)

Prefiltration and centrifugation : comparison tests

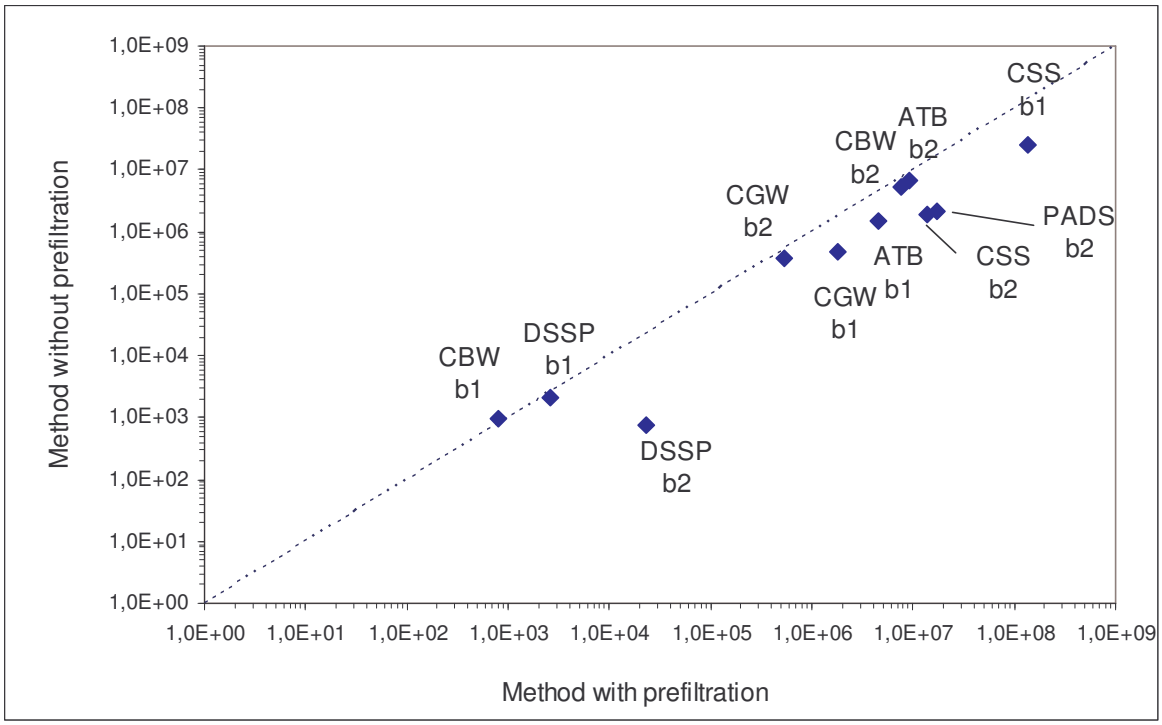
In parallel with the validation study, a short comparative study was performed by two labs to evaluate the interest of the prefiltration and centrifugation step according to various matrices to be analysed (see Table A.2-1: Matrices tested during the validation study).

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 prefiltration 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 6 analyses of the same matrix batch with and without the prefiltration 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 prefiltration and centrifugation step of each set of matrix are shown on Graph B.1.



Graph B.1: Biplot representations of *E. coli* filtration method results, with and without prefiltration; b1= first batch analysed; b2 = second batch analysed.

Wilcoxon test p-value:

p-value = 0.0044

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

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