Soils, sludges and treated bio-wastes — Detection and enumeration of intestinal enterococci in sludges, soils and treated bio-wastes – Part 2: Miniaturised method (Most Probable Number) by inoculation in liquid medium

Boden, Schlamm und behandelte Bio-abfälle — Quantitativer Nachweis von Enterococci aus Schlämmen, Böden und Behandelte Bioabfällen – Teil 2: Miniaturisiertes Verfahren durch Animpfen in Flüssigmedium (MPN Verfahren)

S*ols, boues et bio-déchets traités — Détection et dénombrement des enterococci intestinaux dans les boues, les sols et les biodéchets traités – Partie 2 : Méthode miniaturisée (Nombre le Plus Probable) par ensemencement en milieu liquide*

ICS:

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Foreword

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

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

This document does not replace any existing CEN method.

The standard is divided into two parts:

- part 1 describes a membrane filtration method for quantification,
- part 2 describes a miniaturised most probable number method.

This document is currently undergoing ruggedness trials.

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

Material	Validated	Document
Raw sewage sludge		
Mesophilic anaerobic digested sewage sludge		
Anaerobic treated biowaste		
Pelletised air dried sludge		
Digested sewage sludge presscake		
Lime treated sewage sludge		
Composted sewage sludge		
Composted green waste		
Sludge amended nutrient weak soil		

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 intestinal enterococci and *Clostridium perfringens* in sludges, soils, soil improvers, growing media and biowastes" and aims at evaluation of the latest developments in assessing intestinal enterococci in sludge, soil and treated biowastes. 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 will be validated within the project ”Horizontal”.

Sludges, soils and treated biowastes can contain pathogens 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.

Enterococci are round (cocci) Gram positive bacteria often forming in pairs or chains, difficult to distinguish from Streptococci on physical characteristics alone. They are normally non pathogenic commensal bacteria present in the intestines of humans and animals (*E. faecium* and *E. faecalis*). However, they can cause infection as opportunistic pathogens such as urinary tract infections, bacteremia, bacterial endocarditis, diverticulitis, and meningitis. Most important feature of this genus is their high level of endemic antibiotic resistance. Enterococci are now believed to provide a higher correlation than faecal coliform with many of the human pathogens often found in sewage so could be a suitable candidate for analysis in sludges, soils and biowastes.

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 draft European standard describes a miniaturized most probable number (MPN) method for the quantitative detection of intestinal enterococci in sludge, soils and biowastes. It is suitable to evaluate the log reduction of enterococci through treatment as well as the quality of the end product.

The user should, prior to analysis, validate the method for the particular type of sample they wish to analyse: sludges, soils, soil improvers, growing media (e.g. compost) and biowastes.

The method has a limit of detection of approximately 169 MPN/g wet weight [ENV ISO 13843:2001].

2 Normative references

The following normative references are cited at appropriate places in the text. They were referred to extensively and offer indispensable advice for the application of this method. 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.*

3 Terms and definitions

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

3.1
enterococci
enterococci are gram-positive, catalase-negative, facultative anaerobes that grow as diplococci in short chains. They can be differentiated from other catalase-negative gram-positive cocci by their production of beta-D-glucosidase, their ability to hydrolyse aesculin to produce dextrose and aesculetin in the presence of 40% bile salts, growth in 6.5% sodium chloride at 45°C, reduce 2,3,5-triphenyltetrazolium chloride (TTC) to formazan and produce pyrrolidonylarylamidase (*i.e.* PYR reaction)

3.2
method definition
the medium contains a defined substrate consisting of 4-methyl-umbelliferyl β-D-glucoside. When this substrate is hydrolyzed by the β-D-glucosidase produced by the enterococci, it produces 4-methyl umbelliferone as a blue fluorescent hydrolysis product, as well as the D-glucose product that can be used as a substrate for bacterial growth

3.3
MPN, most probable number
every tube (or plate, etc.) whose inoculum contains even one viable organism will produce detectable growth or change. The individual tubes of the sample are independent

The essence of the MPN method is to dilute the sample to such a degree that 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

3.4
vegetative bacteria
bacteria 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
presumptive positives
isolates which are believed to be enterococci, but are not yet confirmed

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

MPN Most Probable Number

QSR Quarter Strength Ringers

UV Ultra violet

CN Characteristic number

5 Principle

This method is based on EN 7899-1:2000: “Water quality – Detection and enumeration of intestinal enterococci Part 1 – Miniaturised method (Most Probable Number) for surface and wastewater”. The following text describes sample preparation in order to prepare a liquid suspension, after which the analysis is performed following EN 7899-1, reaching Most Probable Number results in 100 mL. The number of enterococci is then calculated to express the number of enterococci per g of sludge.

In order to perform the analysis of enterococci in sludge, soils and biowastes, this document describes the whole procedure corresponding to EN 7899-1. The detection and enumeration of enterococci from biological wastes and soils requires the following stages:

a) sample preparation: suspension of sludge in special microplate diluent (or other suitable diluent);

b) inoculation of the diluted sample in two rows of microtitre plate wells (16 wells) containing dehydrated culture medium;

c) examination of the microtitre plates under ultraviolet light at 366 nm in the dark after an incubation period of 36 h minimum and 48 h maximum at (43 ± 1)°C. The presence of enterococci is indicated by a blue fluorescence resulting from hydrolysis of 4-methyl-umbelliferyl β-D-glucoside;

d) results are given as most probable number per gram of sludge (wet weight).

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 Special Microplate Diluent (SMD)

6.2.1 Bromophenol blue solution (optional)

Bromophenol blue	0.04 g
50% ethanol	100 mL

Dissolve bromophenol blue in 100mL 50% ethanol.

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

6.2.2 SMD

Synthetic sea salt	0.04 g
Bromophenol blue solution (optional)	10 mL
Distilled water	1 000 mL

Dissolved the reagents in the water. Pour 18 mL fraction into sterile tubes.

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

6.3 Quarter Strength Ringers solution (QSR solution)

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride $6H_2O$	0.12 g
Sodium bicarbonate	0.05 g

Dissolve by heating in 1 L deionised or distilled water.

Ringer's is commercially available in tablet form where one tablet is added to 500 mL water for quarter strength ringers.

Solution is steam sterilised (autoclaved) at (121 ± 3)ºC for (15 ± 1) minutes. Check the pH (7.0 ± 0.2).

6.4 Culture medium: MUD/SF medium

6.4.1 Solution A

Tryptose	40.0 g
KH_2PO_4	10 g
D(+)-galactose	2 g
Polyoxyethylenesorbitan monooleate (or Tween 80)	1.5 mL
Deionised water	900 mL

Add Tryptose, KH_2PO_4, galactose and Tween 80 to 900 mL water, whilst gently heating with stirring. Then bring to the boil until completely dissolved. Allow to cool.

6.4.2 Solution B

$NaCO_3$	4.0 g
Nalidixic acid	250 mg
Deionised water	50 mL

Add reagents to the water, whilst gently heating with stirring. Allow to cool.

6.4.3 Solution C

Thallium (I) acetate	2 g
2,3,5-triphenyltetrazolium chloride	0.1 g

Deionised water	50 mL

WARNING **Thallium (I) acetate is toxic. Harmful by inhalation, contact with skin. Use a chemical fume hood, wear gloves and eye protection.**

Add reagents to 50 mL water, whilst gently heating with stirring. Allow to cool.

6.4.4 Solution D

MUD (4-methylumbelliferyl-β-D-glucoside)	150 mg
N,N-dimethylformamide	2 mL

WARNING – *N,N*-dimethylformamide is toxic. Harmful by inhalation, in contact with skin and swallowing. May cause cancer. Use a chemical fume hood, wear eye protection and gloves.

6.4.5 Complete medium

Mix together solutions A, B, C and D. Adjust pH to (7.5 ± 0.2). Filter sterilise. Distribute 100 µL to each well of microtitre plates and immediately dehydrate in tunnel drier or laminar air cabinet.

Note **Ready prepared microtitre plates are available commercially**

7 Apparatus

With the exception of equipment supplied sterilised, all should be sterilised before use in accordance with ISO 8199:2005.

Usual microbiological laboratory equipment and in particular:

7.1 Apparatus for sterilisation – either dry heat (oven) or steam (autoclave).

7.2 Thermostatic incubator(s) at (43 ± 1)ºC

7.3 Tunnel drier or vertical laminar flow cabinet (Class II preferably)

7.4 Homogeniser

7.5 Sterile homogeniser bags – 250 mL volume with or without integrated mesh to remove large particles

7.6 pH meter with an accuracy of ± 0.1

7.7 Ultraviolet observation chamber (Wood's Lamp, 366 nm)

WARNING – UV light can damage eyes and skin. Use protective goggles and gloves

7.8 Bunsen burner

7.9 Pipettors – capable of dispensing 100 µL and 1 mL

7.10 Sterile graduated pipettes – capable of dispensing 2-10 mL

7.11 Adjustable or pre-set 8 channel multi-pipette for measuring and distributing 200 µL per well

7.12 Sterile tips

7.13 Sterile microtitre plates – 96 well, 350 µL, flat bottomed, non-fluorescent

7.14 Sterile adhesive cover strips for sealing microtitre plates

7.15 Sterile containers – plastic or glassware capable of holding up to 250 mL

7.16 Sterile universals – plastic or glassware, capable of holding up to 250 mL

7.17 Sterile Petri dishes – 90 mm diameter

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

7.19 Vortex mixer

7.20 Laboratory spatula

8 Sampling

Take samples of at least 500 g wet weight and deliver them to the laboratory as quickly as possible (within 24 h). In order to prevent propagation or inactivation of intestinal enterococci 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 of paramount importance to adhere to national and international regulations relating to bio hazardous samples when handling and transporting samples.

It is also essential to keep them away from any food or drink, and to protect any cuts.

See also the Warning note in introduction.

8.2 Storage

Do not store these samples on an open bench in the laboratory. If samples are to be stored, store them in well labelled containers, preferable plastic, at (5 ± 3)°C for no more than 72 h after receipt.

8.3 Handling

Good laboratory practice and cleanliness 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 when opening sludge samples is usually flammable and so should be carried out away from naked flames and all equipment should be flame proof.

See also the Warning note in introduction.

9 Procedure

9.1 Sample preparation

Weigh a representative 25 g (wet weight) of the homogenised sample.

Add an appropriate volume of QSR solution so that the final volume is 250 mL and mix thoroughly using a vortex mixer.

For lime treated sludges adjust the pH to (7.0 ± 0.5) with 1 mol/L hydrochloric acid.

NOTE 1 If the pH drops below 4.5 whilst neutralising the sample, a new sample should be prepared.

NOTE 2 If other chemical treatment is used on sludge samples to be tested a suitable neutralisation procedure should be adopted.

If sludge sample is expected to have a high dry weight content (*e.g.* >10%, press-cake, pelletised sludge) a homogeniser bag with an integrated mesh to remove large particles should be used. Place sample in a homogeniser and mix for 2 min.

NOTE 3 If sludge contains sharp objects, for example compost, sample should not be stomached but an alternative method of homogenisation should be used e.g. Pulsifier™. If none available sample should be stomached in 30 s bursts for 1 min.

This first preparation of sample is deemed dilution A.

9.2 Preparation of dilutions

Prepare serial dilutions from dilution A in SMD:

Mix the primary suspension dilution A, and using a sterile pipette aseptically transfer 18 mL into a sterile tube containing 18 mL of SMD (1:2 dilution). Mix this dilution and using a new sterile pipetted transfer 2 mL of this first dilution (1:2) into a sterile tube containing 18 mL of SMD.

Continue this procedure until the 1:2 000 000 dilution has been prepared.

9.3 Analysis

Transfer the content of the tube of 1:20 dilution to an empty sterile Petri dish (90 mm). Distribute 200 µL to 16 wells of a microtitre plate using multi-channel pipettor. For subsequent dilutions operate in the same way, changing the Petri dish and pipette tips.

Cover the microtitre plate and seal with tape, supplied with ready made plates or Parafilm.

NOTE Care should be taken to avoid contamination of wells by tipping of microtitre plate.

Incubate at (43 ± 1)ºC for a minimum of 36 h and maximum of 48 h.

9.4 Resuscitation and enumeration of enterococci in microtitre plates

Observe each covered microtitre plate in the UV observation chamber. Consider all wells in which a blue fluorescence is observed as being positive.

For each dilution, note the number of positive wells,

Example:

Dilution	Positive wells
1/20	16 of 16
1/200	12 of 16
1/2 000	1 of 16

9.5 Confirmation

The media used in the described method does not require confirmation but biochemical tests can be applied such as test strips or equivalent (See Annex B).

9.6 Determination of the dry residue content

The numbers of enterococci 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

10.1 Calculation of MPN

Refer to the MPN table in Annex A. Alternatively use software in EN ISO 7899-1:1998 to calculate the MPN or refer to appropriate MPN tables.

For each chosen dilution note the number of positive wells. A Characteristic Number (CN) of 3 figures will be retained. In all cases, the lowest characteristic number has to be chosen.

To calculate the MPN result for dilutions analysed:

If CN correspond to dilutions 1/20, 1/200 and 1/2 000 MPN mL^{-1} suspension use CN number

If CN correspond to dilutions 1/200, 1/2 000 and 1/20 000 MPN mL^{-1} suspension use 10 x CN number

If CN correspond to dilutions 1/2 000, 1/20 000 and 1/200 000 MPN mL^{-1} suspension use 100 x CN number

If CN correspond to dilutions 1/20 000, 1/200 000 and 1/2 000 000 MPN mL^{-1} suspension use 1 000 x CN number

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

MPN g^{-1} wet weight = MPN mL^{-1} x 250 mL of total original suspension / 25 g

If it is necessary the result may be converted in MPN g^{-1} of dry matter as follows:

Enterococci MPN g^{-1} dry matter = MPN g^{-1} wet weight x 100 / % of dry matter

If none of the well are positive record the result as < n/g where n is the MPN for 1 positive well according the dilution.

Example

1/20	16 positive out of 16
1/200	12 positive out of 16
1/2 000	5 positive out of 16
1/20 000	0 positive out of 16

1/200 000 0 positive out of 16

Retain 16/12/5 = CN.

Using the current table 6 dilutions, CN = 16/12/5 corresponds to MPN = 1758.2 per mL of the dilution 1/20.

Therefore, MPN mL^{-1} suspension = 1758.2 x 10 = 17582

Result per g wet weight, MPN g^{-1} = 172.4 x 250 / 25 = 175 820

11 Performance data

First performance data of the procedure, following the ruggedness study (European scale intralaboratory trial) [3] performed during the FP6 EU Horizontal-Hyg project is given in Annex C (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)

MPN Statistical Table for Microtiter plate 96 wells

6 dilutions (1/20 to 1/2000000) / 16 wells seeded per dilution

Table A.1

Characteristic Number			MPNi 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
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

Characteristic Number			MPNi 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			MPNi 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

Characteristic Number			MPN/ 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/ 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

Characteristic Number			MPNi 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

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
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

Characteristic Number			MPN 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

Characteristic Number			MPN in 1 ml	Lower limit	Upper limit
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

Characteristic Number			MPNi 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			MPNi 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

Characteristic Number			MPN/ 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/ 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	1015
15/16	8/16	5/16	576,2	304,8	1095,5
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	1036,2
15/16	9/16	4/16	589,7	310,9	1118,6
15/16	9/16	5/16	633,2	334	1200,7
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	1058,4
15/16	10/16	3/16	602,3	317,5	1142,6
15/16	10/16	4/16	647,3	341,6	1226,5
15/16	10/16	5/16	693,6	367,3	1309,5
15/16	11/16	0/16	527,6	279,5	995,9
15/16	11/16	1/16	570,6	301,1	1081,6
15/16	11/16	2/16	615,6	324,5	1167,7
15/16	11/16	3/16	662,7	349,7	1253,4
15/16	11/16	4/16	710	376,7	1338,1
15/16	11/16	5/16	758,9	405,1	1421,8
15/16	12/16	0/16	583,1	307,5	1105,9
15/16	12/16	1/16	629,6	332	1194
15/16	12/16	2/16	677,8	358,5	1281,6
15/16	12/16	3/16	727,4	386,7	1368,2
15/16	12/16	4/16	778,1	416,4	1453,8
15/16	13/16	0/16	644,6	340,1	1221,6
15/16	13/16	1/16	694,5	367,9	1311,1
15/16	13/16	2/16	745,8	397,4	1399,7
15/16	13/16	3/16	798,4	428,5	1487,5
15/16	13/16	4/16	851,9	460,9	1574,8
15/16	14/16	0/16	712,3	378	1342,2
15/16	14/16	1/16	765,6	409	1432,9
15/16	14/16	2/16	829,6	441,5	1523
15/16	14/16	3/16	875,6	475,3	1612,9
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

Characteristic Number			MPNi 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	1010,9
16/16	3/16	6/16	591,5	311,8	1121,9
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	1040
16/16	4/16	5/16	608,9	320,9	1155,1
16/16	4/16	6/16	672	355,2	1271,2
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	1071,1
16/16	5/16	4/16	627,7	331	1190,5
16/16	5/16	5/16	694,2	367,7	1310,6
16/16	5/16	6/16	763,7	407,9	1429,8
16/16	5/16	7/16	835,5	450,9	1548,2
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	1104,5
16/16	6/16	3/16	648,3	342,2	1228,5
16/16	6/16	4/16	713,2	381,5	1352,7
16/16	6/16	5/16	794,9	424,4	1476,3
16/16	6/16	6/16	867,1	470,1	1599,2
16/16	6/16	7/16	944,3	517,7	1722,4

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
16/16	7/16	0/16	536,8	284,1	1014,5
16/16	7/16	1/16	601,2	316,9	1140,5
16/16	7/16	2/16	670,9	354,6	1269,3
16/16	7/16	3/16	744,9	396,9	1398,1
16/16	7/16	4/16	822,1	442,8	1526,5
16/16	7/16	5/16	901,8	491,4	1654,8
16/16	7/16	6/16	983,2	541,9	1783,9
16/16	7/16	7/16	1065,4	593,8	1914,7
16/16	7/16	8/16	1150,9	646,7	2048
16/16	8/16	0/16	621,7	327,8	1179,2
16/16	8/16	1/16	695,7	368,5	1313,2
16/16	8/16	2/16	774,1	414,1	1447,2
16/16	8/16	3/16	855,8	463,2	1581,1
16/16	8/16	4/16	940	515,1	1715,6
16/16	8/16	5/16	1026,2	568,8	1851,7
16/16	8/16	6/16	1114,3	623,9	1990,3
16/16	8/16	7/16	1204	680	2132
16/16	8/16	8/16	1295,5	736,9	2277,5
16/16	9/16	0/16	723,1	384,2	1360,9
16/16	9/16	1/16	806,4	433,3	1500,7
16/16	9/16	2/16	893,1	486,1	1641
16/16	9/16	3/16	982,5	541,5	1782,8
16/16	9/16	4/16	1074,2	598,7	1927,1
16/16	9/16	5/16	1167,9	657,4	2074,9
16/16	9/16	6/16	1263,8	717,2	2226,8
16/16	9/16	7/16	1361,6	777,9	2383,3
16/16	9/16	8/16	1461,6	839,5	2545
16/16	9/16	9/16	1563,8	901,7	2712,1
16/16	10/16	0/16	842,4	455,1	1559,4
16/16	10/16	1/16	934,8	511,8	1707,3
16/16	10/16	2/16	1030,1	571,2	1857,8
16/16	10/16	3/16	1128	632,5	2012
16/16	10/16	4/16	1228,5	695,2	2170,8
16/16	10/16	5/16	1331,4	759,2	2334,9
16/16	10/16	6/16	1437	824,3	2505
16/16	10/16	7/16	1545,2	890,4	2681,5
16/16	10/16	8/16	1656,3	957,4	2865,2
16/16	10/16	9/16	1770,2	1025,3	3056,4

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
16/16	11/16	0/16	981,7	541	1781,4
16/16	11/16	1/16	1083,9	604,8	1942,4
16/16	11/16	2/16	1189,2	670,7	2108,6
16/16	11/16	3/16	1297,7	738,3	2280,9
16/16	11/16	4/16	1409,3	807,3	2460,2
16/16	11/16	5/16	1524,1	877,6	2647
16/16	11/16	6/16	1642,6	949,2	2842,4
16/16	11/16	7/16	1764,7	1022	3047,1
16/16	11/16	8/16	1890,7	1095,9	3261,7
16/16	11/16	9/16	2020,7	1170,9	3487,3
16/16	11/16	10/16	2155,1	1246,9	3724,8
16/16	12/16	0/16	1145,4	643,3	2039,3
16/16	12/16	1/16	1259,6	714,6	2220,3
16/16	12/16	2/16	1377,8	787,9	2409,3
16/16	12/16	3/16	1500,1	863	2607,7
16/16	12/16	4/16	1626,8	939,7	2816,2
16/16	12/16	5/16	1758,2	1018,2	3036,2
16/16	12/16	6/16	1894,7	1098,3	3268,9
16/16	12/16	7/16	2036,5	1179,9	3515
16/16	12/16	8/16	2184	1263,1	3776,5
16/16	12/16	9/16	2337,8	1347,8	4055,1
16/16	12/16	10/16	2497,8	1433,8	4351,6
16/16	12/16	11/16	2664,9	1521,2	4668,4
16/16	13/16	0/16	1341,9	765,7	2351,8
16/16	13/16	1/16	1472,4	846	2562,5
16/16	13/16	2/16	1608,6	928,8	2786
16/16	13/16	3/16	1750,7	1013,7	3023,4
16/16	13/16	4/16	1899,5	1101,1	3276,9
16/16	13/16	5/16	2055,5	1190,7	3548,3
16/16	13/16	6/16	2219,3	1282,6	3839,8
16/16	13/16	7/16	2391,6	1376,9	4154
16/16	13/16	8/16	2573,3	1473,6	4493,8
16/16	13/16	9/16	2764,9	1572,5	4861,5
16/16	13/16	10/16	2967,2	1673,7	5260,3
16/16	13/16	11/16	3181,5	1777,7	5693,9
16/16	13/16	12/16	3408,3	1884,4	6164,7

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
16/16	14/16	0/16	1587	915,7	2750,3
16/16	14/16	1/16	1741,7	1008,4	3008,2
16/16	14/16	2/16	1905,3	1104,4	3286,8
16/16	14/16	3/16	2078,8	1203,9	3589,3
16/16	14/16	4/16	2263,4	1307	3919,5
16/16	14/16	5/16	2460,3	1413,8	4281,5
16/16	14/16	6/16	2671,1	1524,5	4680,4
16/16	14/16	7/16	2897,6	1639,2	5121,9
16/16	14/16	8/16	3141,5	1758,5	5612,1
16/16	14/16	9/16	3405	1882,8	6157,7
16/16	14/16	10/16	3689,7	2012,9	6763,3
16/16	14/16	11/16	3997,9	2150	7434
16/16	14/16	12/16	4331,7	2296,2	8171,4
16/16	14/16	13/16	4693,3	2454,4	8974,6
16/16	15/16	0/16	1912,4	1108,6	3299,2
16/16	15/16	1/16	2108,2	1220,6	3641,5
16/16	15/16	2/16	2320,4	1338,3	4023,2
16/16	15/16	3/16	2551,8	1462,3	4453,1
16/16	15/16	4/16	2806	1593,3	4941,6
16/16	15/16	5/16	3087	1732,2	5501,3
16/16	15/16	6/16	3399,7	1880,4	6146,7
16/16	15/16	7/16	3749,8	2039,9	6893
16/16	15/16	8/16	4143,8	2214,1	7755,4
16/16	15/16	9/16	4588,4	2408,4	8741,7
16/16	15/16	10/16	5090,1	2630,4	9849,9
16/16	15/16	11/16	5654,3	2889,9	11063,3
16/16	15/16	12/16	6284,4	3196,4	12355,9
16/16	15/16	13/16	6985,8	3559,1	13712
16/16	15/16	14/16	7762,6	3982,9	15129
16/16	15/16	15/16	8616,7	4467,7	16618,6
16/16	16/16	0/16	2398,1	1380,4	4165,9
16/16	16/16	1/16	2682,4	1530,3	4702,1
16/16	16/16	2/16	3009,4	1694,5	5344,8
16/16	16/16	3/16	3392,1	1876,9	6130,6
16/16	16/16	4/16	3849,5	2084,4	7109,5
16/16	16/16	5/16	4407,8	2329,5	8340,4
16/16	16/16	6/16	5100,2	2635	9872,1
16/16	16/16	7/16	5963,9	3038,1	11707,4
16/16	16/16	8/16	7022,5	3578,6	13780,7
16/16	16/16	9/16	8299,46	4285,9	16071,4

Annex B
(informative)

Performance data

Samples of raw and pelleted sludge alongside positive controls (*Enterococci faecalis* NCTC 775) were analysed using the methods described herein. To compare the results samples were also analysed by spread plating onto OAA (oxolinic aesculin azide) agar.

Although MUD/SF is designed as a confirmed media and as such no further confirmation should be necessary, in this research wells were confirmed on bile aesculin agar and analysed for catalase activity. Also some wells were analysed using the API system. Confirmation of presumptive positive colonies was conducted on all wells if less than ten were present and at least ten colonies if more were present.

The results found that the numbers of enterococci counted on OAA agar were significantly higher than the MPN results obtained using MUD/SF for raw sludges ($t = 5.69$, 4 d.f., $p = 0.005$) (Table 2). Numbers for pelleted sludge samples and positive control samples were not significantly different between the two methods ($p = 0.277$ (4 d.f.) and $p = 0.679$ (4 d.f.) respectively) (Table 2). This method was found to be particularly useful if the dry solids are high but the number of target bacteria present is low as the presence of high solids masked colonies on membrane filters.

Confirmation of the positive wells gave 100% agreement with both BAA / catalase test and API confirmations.

Table 1 Numbers of enterococci in sludge samples using MUD/SF (MPN/g) or OAA (cfu/g).

Sample	Media	Mean cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 4)	Min cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 4)	Max cfu/g (OAA) or MPN/g (MUD/SF) ww (n = 4)	St. dev. cfu/g (OAA) or MPN/g (MUD/SF) ww	p-value (t-test) *(Mann Whitney Rank sum)
Raw	**MUD/SF**	26,325	24,048	28,090	2,069	
	OAA	84,000	64,000	96,000	17,436	0.005
Pelleted	**MUD/SF**	20,142	3,342	49,520	25,530	
	OAA	1,600	800	2,800	1,058	0.277
Positive control	**MUD/SF**	37,100	27,828	53,676	14,389	
	OAA	32,667	22,000	40,000	9,452	0.679
Negative control	**MUD/SF**	0	0	0		
	OAA	0	0	0		
Blank	**MUD/SF**	0	0	0		
	OAA	0	0	0		

Key: cfu	colony forming unit
MPN	most probable number
ww	wet weight
n	number
OAA	oxolinic aesculin azide agar
Min	minimum
Max	maximum
St. dev.	standard deviation

Annex C
(informative)

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 CA.1 — Summary of components of the precision of the intestinal enterococci microtiter plate method

Limit of detection (5%) *Enterococci* /g wet weight	**Upper limit of quantification (5%)** *Enterococci* /g wet weight	**Range of quantification** Log10 unit	**Results of dispersion U^2**
168.51	$1.77\ 10^8$	6	0.1

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).**

Bibliography

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