Soils, sludges and treated bio-wastes — Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowastes — Part 2: Miniaturised method (Most Probable Number) by inoculation in liquid medium

Boden, Schlamm und behandelle Bio-abfälle — Quantitativer Nachweis von Escherichia coli aus Schlämmen, Böden, Dügemitels und Bodenverbesserern, Kultursubstraten sowie Bioabfällen — Teil 2: Miniaturisiertes Verfahren durch Animpfen in Flüssigmedium (MPN-Verfahren)

Sols, boues et bio-déchets traités — Détection et dénombrement de Escherichia coli dans les boues, les sols, les engrais, les amendements organiques et les biodéchets — Partie 2 : Méthode miniaturisée : (Nombre le Plus Probable) par ensemencement en milieu liquide

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Contents

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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

The standard is divided into three parts :

- Part 1 gives a membrane filtration method,
- Part 2 gives a miniaturised semi-quantitative MPN method,
- Part 3 gives a semi-quantitative macro method.

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

Material	Validated	Document
Soil	Note yet validated	
Sludge	Note yet validated	
Biowaste	Note yet validated	
Soil improvers	Note yet validated	

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* sp. in sludge, soils, and biowastes” and aims at evaluation of the latest developments in assessing *E. coli* in sludge, soil, treated biowaste and neighbouring fields. 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 modular horizontal method and has been validated within the project “Horizontal”.

Sludge, soils, and biowastes can contain pathogenic micro-organisms such as *Escherichia coli* which occur mainly in the intestinal tract of humans and animals and are transmitted through faecal contamination. The use of such pathogen-contaminated materials in agriculture may cause outbreaks of infection due to the production of contaminated food and animal feedstocks and may also be transmitted to wild animals. Consequently, there is a need to monitor rates to land.

Escherichia coli is a non-pathogenic, Gram negative bacterium with an exclusive faecal origin. Consequently, it can be used as an indicator of faecal contamination. It can also be used to monitor the effectiveness of 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 8199:2001, have to be applied.

WARNING — "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may 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 13 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

1 Scope

This part of this European standard describes a miniaturized most probable number (MPN) method for the quantitative detection of *Escherichia coli* in sludge, soils, and biowastes. It is suitable to evaluate the Log reduction of *E. coli* through treatment as well as the quality of the end product.

This method has a detection limit (5 %) of 67,4 *E. coli* per g of wet weight with an upper detection limit of 7,07 x 10^7, and a quantification range of 6,0 Log.

2 Normative references

This European standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

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

EN 12880:2000, *Characterisation of Sludge - Determination of Dry Residue and Water Content.*

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

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

NF EN 12176 : 1998, Characterization of sludge – Determination of pH value

3 Definitions

For the purposes of this European Standard, 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, lactose positive, and able to grow at 44 °C. Most *E. coli* strains are able to produce indole from tryptophane and are *β*-glucuronidase-positive.

3.2
method definition
b-glucuronidase-positive micro-organism able to hydrolyse 4-methylumbelliferyl-β-D-glucuronide (MUG) when growing at an incubation temperature of 44 °C in the specified liquid medium.

3.3
dry matter
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, 3.1].

4 Symbols and abbreviations

MUG: 4-methylumbelliferyl-β-D-glucuronide

SMD: Special Microplate Diluent

cfu: colony forming unit

MPN: Most Probable Number

CN: characteristic number

5 Principle

This method is based on EN ISO 9308-3:1998: "Water quality - Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium". The following text describes sample preparation in order to prepare a liquid suspension, after which the analysis is performed following EN ISO 9308-3, reaching Most Probable Number results in 100 mL. The number of *E. coli* is then calculated to express the number of *E*. *coli* per g of sludge. In order to perform the analysis of *E*. *coli* in sludge, soils, and biowastes, this document describes the whole procedure corresponding to the one of the EN ISO 9308-3.

The detection and enumeration of *E*. *coli* from biological wastes and soils requires the following stages:

a) sample preparation: suspension of sludge in peptone saline solution;

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

c) examination of the microtiter plates under ultraviolet light at 366 nm in the dark after an incubation period of 36 h minimum and 72 h maximum at (44 ± 1) °C. The presence of *E*. *coli* is indicated by a blue fluorescence resulting from hydrolysis of MUG;

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

6 Verification of interferences

7 Reagents, diluents and culture media

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). If the media are not used immediately, preserve them in the dark at (5 ± 3) °C for up to one month in conditions avoiding any alterations in their composition.

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

7.1 Peptone saline solution

Per litre:

a) 1 g of casein peptone;

b) 8,5 g of sodium chloride.

Sterilise the solution in a steam steriliser (7.1) at (121 ± 3) °C for (15 ± 1) min.

7.2 Special Microplate Diluent (SMD)

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

Sterilize in the autoclave (7.1) at (121 ± 3) °C for (15 ± 1) min.

The bromophenol blue solution is prepared by adding 0,04 g in 100 mL of 50 % ethanol. It is only used to colour the SMD blue and avoid confusing with demineralised or distilled water. Pour 18 mL fraction into sterile tubes.

NOTE 1 A typical analysis of a commercially available and suitable synthetic sea salt is given in informative Annex B.

NOTE 2 SMD ready to use is commercially available by several suppliers.

7.3 Demineralized or distilled water

Demineralized or distilled water free from substances inhibiting growth under test conditions.

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

7.4 Culture medium: MUG/EC medium

Composition:

Tryptone	40 g
Salicin	1 g
Triton x 100	1 g
MUG* (4-methyl-umbelliferyl β-D-glucuronide)	100 mg
Demineralized or distilled water	1 000 mL

* the fluorogenic constitutent MUG is dissolved in 2 mL of N-N-dimethylformamide.

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

Successively add Tryptone, Salicin and Triton to one litre of water, while maintaining a gentle heat and magnetic stirring, then bring to the boil until completely dissolved. Allow to cool and add the MUG solution.

Adjust the pH to (6,9 ± 0,2).

Sterilize by filtration with membranes of average pore size 0,2 µm (7.14).

Distribute in 96-well microtitre plates (7.15) with a volume of 100 µL of media in each well (minimum capacity 350 µL) and dehydrate immediately in a tunnel drier or laminar airflow cabinet (7.3).

The manufacturing of the medium shall meet the quality criteria given in Annex D.

NOTE Some microtiter plates containing the MUG/EC medium already distributed are commercially available.

7.5 Kovac's reagent

Use a commercial product according to the manufacturer's instruction or prepare as follows:

- 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-dimethylaminobenzaldehyde in 75 mL of isoamyl alcohol and heat in a water bath at 60°C for 5 min. Then add slowly 25 mL 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.

8 Apparatus

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

Usual microbiological laboratory equipment and in particular:

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

8.2 Thermostatic incubator regulated at (44 ± 1) °C.

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

8.4 Homogeniser (e.g. Stomacher^R, Seward Laboratories or equivalent).

8.5 Sterile homogeniser bags, 250 mL volume, with or without integrated mesh to exclude large particulate matter (e.g. Stomacher^R, Seward Laboratories 6041, 6041/STR or equivalent).

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

WARNING — UV light causes irritation of eye and skin. Use protective glasses and gloves.

8.7 Portable refractometer (optional).

8.8 pH meter with an accuracy of ± 0.1.

8.9 Sterile test tubes of 40 mL volume, or flasks with similar capacity**.**

8.10 Sterile flasks, of nominal capacities e.g. 250 mL.

8.11 Sterile graduated pipettes, glass or disposable plastic ware, capable of dispersing 2 mL and 18 mL.

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

8.13 Sterile tips for multi-pipette**.**

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

8.15 Sterile microtiter plates – 96 wells, 350 µL, flat-bottomed, non-fluorescent**.**

8.16 Sterile adhesive covering strips for sealing microtitre plates.

8.17 Sterile Petri dishes, 90 mm in diameter.

8.18 Scale

8.19 Spatula laboratory

8.20 Vortex mixer

8.21 Stirrer and magnetic bars

9 Sampling and hazards

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 hours). In order to prevent propagation or inactivation of *E. coli* during transport to the laboratory and subsequent storage, the necessary precautions depending upon the matrix shall be taken.

NOTE Generally chilling the sample to (5±3)°C is recommended.

9.1 General

Samples are liable to ferment, particularly if untreated, 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 biohazardous 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.

9.2 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 a maximum period of 36 hours.

9.3 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 the introduction.

10 Procedure

10.1 Sample preparation

10.1.1 Determination of the dry matter content

The dry matter of the sample is measured using the method described in EN 12880:2000.

10.1.2 Suspension preparation

Weigh a representative sub-sample of 25 g (wet weight) into a homogeniser bag (8.5) with an integrated mesh if large debris is to be excluded.

Add an appropriate volume of peptone saline suspension (7.1) so that the final weight is 100 g. Place the homogeniser bag (8.5) in a homogeniser (8.4) and homogenize for 1 min.

Process without delay.

NOTE For sanitised (e.g. lime, peracetic acid) and treated sludge, a suitable neutralisation procedure must be used (see for example EN 1040:1997 or NF EN 12176 (1998)).

10.2 Analysis

10.2.1 Preparation of dilutions

Prepare 1/2 dilution in SMD (7.2) and then serial ten fold dilutions from 1/20 to 1/2 000 000.

Vigorously stir the primary suspension (10.1.2) in order to obtain a homogeneous suspension and, using a sterile pipette, immediately transfer 18 mL of this homogenised suspension to a first tube (8.9) containing 18 mL of SMD (1/2 dilution).

Using a fresh pipette (8.11), transfer 2 mL of this first dilution (homogenised by handle shaking) to a second 18 mL SMD tube (1/20 dilution).

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

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

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

10.2.2 Inoculation and incubation of microtitre plates

Inoculate a microtitre plate (8.15) containing the MUG/EC medium in each well (7.4) while distributing each dilution from 1/20 to 1/2 000 000 in 16 wells each (dilution ½ 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 (8.17).

Using a multi-channel pipette (8.12) with 8 sterile tips (8.13), distribute 200 µL per wells into 16 wells of the microtitre plate (8.15) corresponding to this last dilution (use the 2 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 2 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 (8.12) 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 (8.16) provided for this purpose.
Incubate the microtitre plate in an incubator (8.2) at (44 ± 1) °C for a minimum of 36 hours, and a maximum of 72 hours.

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

10.2.3 Reading

Place each microtitre plate with the adhesive on, in the UV observation chamber (8.6).
Note the number of positive blue fluorescent wells for each dilution.

Then, using a multi-channel pipette with 8 sterile tips, distribute 15 µL of Kovac's reagent in each well. Wait 1-2 min for the colour change.
Note the number of positive red-top wells for each dilution.

Consider all blue fluorescent and red-top wells as being positive.

11 Expression of results

11.1 Determination of the characteristic number

For each of the 6 inoculated dilutions, note the number of positive wells and identify the corresponding characteristic number (CN) according to the instructions given in ISO 8199 for NPP calculation:

The CN corresponds to the number of positive wells of the 3 last dilutions giving a number of positive wells > 0 (table 1)

When it is possible, choose 3 serial dilutions for which results are neither totally positive, neither totally negative. If it is not possible, it is better to choose the 3 serial dilutions with positive results than negative results (table 1, examples a and b).

If less than 3 serial dilutions give positive results use the dilution containing the higher concentration in sample and the 2 next dilutions (table 1, example c).

If only one wells serial gives positive results, use this dilution and the previous and the next dilution (table 1 example d).

Table 1 : Examples for determination of the characteristic number

	1/20	1/200	1/2 000	1/20 000	1/200 000	1/2 000 000	CN
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

EXAMPLE

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

In this example, the CN will be 16/12/5 for dilution 1/200, 1/2 000, 1/20 000.

11.2 Calculation of the MPN and its confidence interval

The Most Probable Number (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 given in Annex A (informative). 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 the table in Annex A;

— the final MPN per 1 mL of suspension (10.1.2), taking into account the dilution steps corresponding to the CN, is calculated from MPN_i as follows:

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

* If CN correspond to dilutions 1/200, 1/2 000 and 1/20 000, MPN.mL $^{-1}$ suspension = 10 x MPN_i

* If CN correspond to dilutions 1/2 000, 1/20 000 and 1/200 000, MPN.mL $^{-1}$ suspension = 100 x MPN_i

* If CN correspond to dilutions 1/20 000, 1/200 000 and 1/2 000 000, $MPN_A.mL^{-1}$ suspension = 1 000 x MPN_i

Expression of results: MPN g^{-1} of wet weight or MPN g^{-1} dry matter

The result per g of wet weight is then calculated as follows:
E. coli MPN g^{-1} wet weight = MPN_B = MPN_A x 100 ml of total suspension / 25 g

If it is necessary the result may be converted in MPN g^{-1} of dry matter as follows:
E. coli MPN g^{-1} dry matter = $MPN_C = MPN_B \times 100$ / % of dry matter

In the example of § 11.1:

If CN = 16/12/5 for dilutions 1/200, 1/2 000 and 1/20 000, the MPN_i statistical table in Annex A 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:
E. coli = 70 328 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.

Note Low and upper limits should always be given.

12 Performance data

Performance data in terms of repeatability and reproducibility obtained using intra and inter-laboratory test is given in Annex C (informative).

This Annex C will be completed by the results of the validation study (European scale Interlaboratory trial) that will be performed during the FP6 EU HORIZONTAL-HYG project

13 Test report

The test report shall contain the following information:

a) A reference to this European Standard including its date of publication;

b) Sampling report including precise identification of the sample

c) specific reporting on the considered quantification standard

d) expression of results according to clause 10

e) any deviation from this standard, and any facts which may have influenced the result.

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			MPNi 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			MPNi 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

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			MPNi 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			MPNi 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	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)

SYNTHETIC SEA SALT

B.1 Major ion composition of a convenient ocean 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	($SO4^=$)	6.602	2 631
Magnesium	(Mg^{++})	3,230	1 256
Calcium	(Ca^{++})	1.013	400
Potassium	(K^+)	1.015	401
Bicarbonate	(HCO_3^-)	0.491	194
Borate	(B)	0.015	6,0
Strontium	(Sr^{++})	0.001	7,5
	SOLIDS TOTAL	86.11%	34 089,50
Water	(H_2O)	13,88	
	TOTAL	99,99%	

B. 2 Example for preparation from defined substances:

Three basal solutions are to be made as follows:

Solution A

$CaCl_2$, $2H_2O$	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

$MgSO_4,7H_2O$	190,0 g
$MgCl_2.6H_2O$	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.

Annex C
(informative)

Performance data of the method

Intralaboratory trials carried out in 2001 allowed a first characterisation of the method in comparison with another enumeration on agar plates: « Chromocult ».

The quantitative technique described (Microtiter plate 96 wells: 6 dilutions- 16 replicates per dilution) does not generate any significant error as opposed to the « Chromocult (Merck) » method which shows a recovery yield slightly (but significantly) lower than 100 % (80 % – 85 %) for composted sludge samples.

In intralaboratory trials, the dispersion can be totally attributed to sampling (dispersion linked to the constitution of aliquot fractions, described by the Poisson distribution).

An interlaboratory trial (French scale) has subsequently been carried out, with 13 participants and 5 different materials.

Detection limit

— quantification limit = 410 *E. coli* per g dry matter ($\alpha = 5$ %) , 762 *E. coli* per g dry matter ($\alpha = 1$ %) (these values are slightly higher than that of Chromocult method, which are 369 and 530 for $\alpha = 5$ % and 1 %, respectively);

— dispersion of results described by the Poisson distribution ;

— uncertainty of result described in the graph next page.

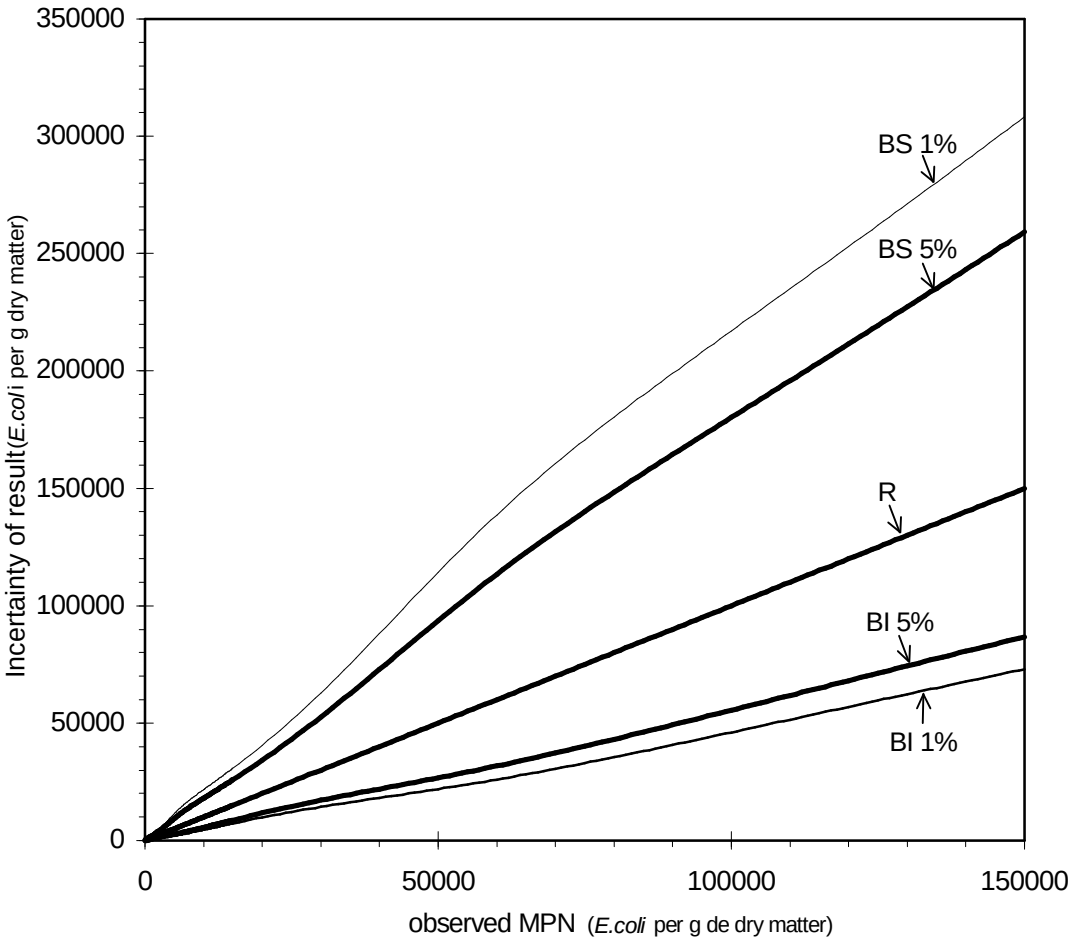
350000
300000
250000
200000
150000
100000
50000
0
Incertainty of result(E.coli per g dry matter)
0
50000
100000
150000
observed MPN (E.coli per g de dry matter)
BS 1%
BS 5%
R
BI 5%
BI 1%

Figure C.1

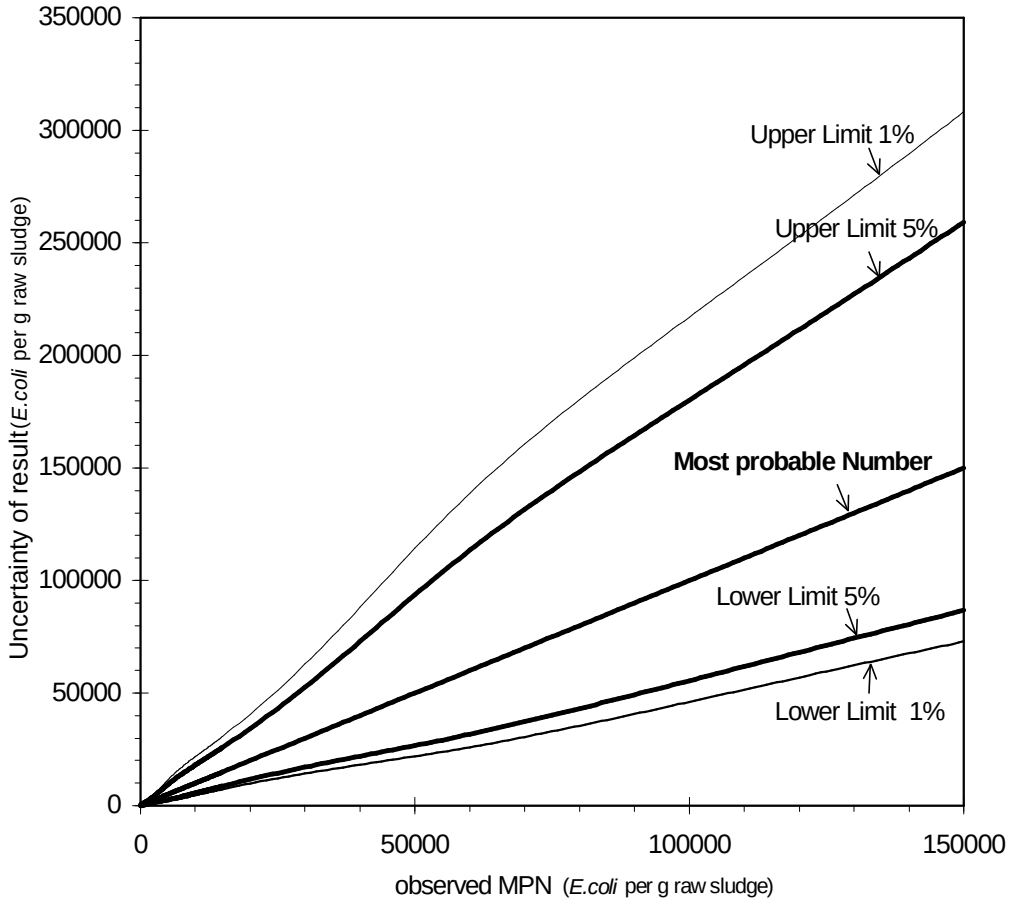

Figure C.2

Enumeration of *E. coli* in sludge samples: uncertainty of results.

Annex D
(normative)

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 light (366 nm).

The quality criteria to be respected are the following:

1) 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 %;

2) 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* containing 500 germs per mL of Special Microplate Diluent (7.2), 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 %;

3) 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, that is to say 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 are: 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 48h at 44°C.

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

* *E. coli* WR1 can be obtained NCTC, UK (NCTC number 13167).

Bibliography

ISO 9308-3:1998, *Water quality: - Detection and enumeration of Escherichia coli and coliform bacteria in surface and waste water - Part 3: Miniaturized method (most Probable Number) by inoculation in liquid medium.*

CEN/TC 308 – doc525:2001, *Revision of Directive 86/278/EEC (3rd Draft).*