Soils, Sludges and treated bio-wastes — Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowastes — Part 3: Macromethod (Most Probable Number) in liquid medium

Boden, Schlamm und behandelte Bio-abfälle — Quantitativer Nachweis von Escherichia coli aus Schlämmen, Böden, Düngemitteln und Bodenverbesserern, Kultursubstraten sowie Bioabfällen — Teil 3: Makromethode (MPN) in Flüssigmedium

Sols, boues et bio-déchets traités — Détection et dénombrement de Escherichia coli dans les boues, les sols, et les biodéchets — Partie 3 : Macrométhode (NPP) par ensemencement en milieu liquide

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Contents

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Foreword

This document has been prepared in the frameworkof the project Horizontal.

This document is a working document.

The standard is divided into three parts:

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

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

Material	Validated	Document
Soil	Not validated yet	
Sludge	Not validated yet	
Biowaste	Not validated yet	
Soil improvers	Not validated yet	

Introduction

This document is developed in the framework of the project 'Horizontal'. It is the result of a desk study "Hygienic Parameters Feasibility of Horizontal Standards for *Escherichia coli* and *Salmonella* spp. In sludges, soils, and biowastes" and aims at evaluation of the latest developments in assessing *Salmonella* spp. 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 in the project 'Horizontal'.

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

Escherichia coli is a non-pathogenic, Gram negative bacterium with faecal origin. Consequently, it can be used as an indicator of faecal contamination. It can also be used to monitor the effectiveness of pasteurization or disinfection treatments but it is comparatively sensitive (to heat, high pH) and cannot therefore reflect the behaviour of all pathogens in these materials. Suitable quality control procedures, at least those described in ISO 8199:2005, have to be applied.

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

The texts of the chapters 1 to 13 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

1 Scope

This part of the European standard describes a most probable number (MPN) method for the quantitative detection of *Escherichia coli* in sludges, soils, and biowastes. It allows further differentiation within the test than part 2 of this standard. It is suitable to evaluate the log reduction of *E.coli* through treatment as well as the quality of the end product.

This method can be used irrespective of the dry residue content of the test material.

The method has a limit of detection of approximately 10 MPN/g wet weight sludge.

2 Normative references

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

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*.

3 Terms and definitions

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

3.1
Escherichia coli
Escherichia coli which belong to the family of Enterobacteriaceae, are Gram-negative, non-sporulating, rod-shaped, lactose positive bacteria 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
for the purpose of the present method, the following *E. coli* definition shall apply: - β-glucuronidase-positive and able to hydrolyse 4-methylumbelliferyl-B-D-glucoronide (MUG) when at an incubation temperature of 44 °C in the specified liquid medium. In addition, indole shall be produced from tryptophan and gas produced from lactose.

3.3
dry residue
the dry mass portion of the sample obtained after the specified drying process. It is expressed as percent or in grams per kilogram.

[EN 12880:2000, 3.1].

3.4
Most probable number (MPN)
The MPN is statistically determined number which represents the number of bacteria most likely present in a sample. In this method the MPN is calculated from the number of positive gas-fluorescence and indole tubes and expressed as the number *E. coli* per 1 g wet weight of sample.

4 Symbols and abbreviations

4.1
MPN: Most Probable Number

4.2
MUG: 4-methyl-umbelliferyl-β-D-glucuronide

4.3
E. coli: Escherichia coli

5 Principle

This method is based on that described by Schindler 1991 (Fluorocult™ or equivalent).

a) Suspension of the sample in 0.9 % m/V sodium chloride;

b) Serial dilutions of this suspension in the same diluent (from 10^{-1} up to 10^{-7})

c) Transfer of 3 x 1mL out of each dilutions step into 3 tubes containing 9 mL MUG fluorocult lauryl sulfate broth;

d) Incubation at (44 ± 1) °C for (40 ± 4) h;

e) Detection of gas production, fluorescence and indole formation;

f) Quantification by the MPN technique.

6 Verification of interferences

7 Reagents, diluents and culture media

7.1 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. 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.

7.2 *"FluorocultÔ Lauryl sulfate broth"* (MUG, 4-methyl-umbelliferyl-β-D-glucuronide) prepared according the manufacturer or:

Mix the following substances in 1 000 mL water in a 2 000 mL flat bottom flask, while heating in a boiling water bath (5.16):

— 20,0 g trypsin digested peptone from meat

— 5,0 g lactose

— 5,0 g sodium chloride

— 0,1 g sodium lauryl sulfate

— 2,75 g di-potassium hydrogen phosphate

— 2,75 g potassium di-hydrogen phosphate

— 1,0 g L- tryptophan

— 0,1 g 4-methyl-umbelliferyl- ß-D-glucuronide

mix carefully and distribute into culture tubes in 9 mL portions and add a Durham tube (5.11) to each portion. Adjust the pH to (6,8 ± 0,1) using (1 mol/L) sodium hydroxide solution. Sterilize in an autoclave (steam sterilizer) (5.3) for (15 ± 1) min at (121 ± 3) °C.

7.3 NaCl solution (0.9 % m/V)

Mix the following substance in 1 000 mL water in a 2 000 mL flat bottom flask.

— 9,0 g sodium chloride

sterilize in an autoclave (steam sterilizer) (5.3) for (15 ± 2) min at (121 ± 3) °C and fill into 500 mL flasks (5.1) in 180 mL portions or into culture tubes (5.9) in 9 mL portions under sterile conditions.

7.4 NaOH solution (1 mol/L)

7.5 Kovacs reagent

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

— 5,0 g 4-di-methylamino benzaldehyde

— 75,0 mL n-butanol

— 25,0 mL hydrochloric acid. (p = 1,18 g/mL)

Dissolve 4-dimethylamino benzaldehyde in n-butanol and add slowly the hydrochloric acid. Store in the refrigerator and protect from light.

8 Apparatus

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

Usual microbiological laboratory equipment, and in particular:

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

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

8.3 Autoclave (Steam sterilizer).

8.4 Refrigerator.

8.5 Sterile plastics culture dishes, with lid of about 90 mm in diameter.

8.6 Graduated pipettes, of nominal capacities 1 and 10 mL.

8.7 Apparatus for shaking the culture tubes.

8.8 Culture tubes, 25 mL capacity, or equivalent containers.

8.9 Vortex mixer suitable for of 25 mL capacity culture tubes or equivalent containers.

8.10 Durham – tubes.

8.11 pH meter, with temperature compensation and pH measuring cell.

8.12 Boiling water bath.

8.13 UV-lamp (366 nm).

8.14 Laboratory spatula

9 Sampling and hazards

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible, preferably chilled at (5 ± 3) °C.

9.1 General

Samples are liable to ferment, particularly if untreated, and may contain pathogenic micro-organisms. It is essential to keep them away from any food or drink, and to protect any cuts. 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 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.

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 Introduction.

10 Procedure

10.1 Sample preparation

Place 20 g (wet weight) of sample into 180 mL 0.9 % sterile NaCl solution. Shake at a minimum of 150 rpm for 30 min. up to 20 h at (5 ± 3) °C.

NOTE 1 A 20 hour shaking is recommended for all samples. In the case of homogeneous samples (e.g. sewage sludge) the shaking time may be reduced, but not less than 30 min. For inhomogeneous samples (e.g. compost) the shaking time of 20h shall be used.

NOTE 2 For disinfectant (e.g. lime, peracetic acid) treated sludges, a suitable pre-treatment for neutralising the disinfecting agent is required. For lime treated materials adjust the pH to 7,0 ± 0,5 with 1 mol/L hydrochloric acid. For other relevant chemicals (e.g. peracetic acid), a suitable neutralisation procedure must be used (see for example EN 1040 for neutralizers).

10.2 Analysis

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

— Prepare a serial tenfold dilution (1 mL of prepared suspension (8.1) + 9 mL 0.9 % sterile NaCl solution) up to 10^{-7}.

— From each dilution step, transfer 1 mL per tube (5.1) into 3 tubes containing 9 mL MUG lauryl sulfate broth and a Durham tube (7.2) each.

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

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

— Add 0.5 mL NaOH 1N (7.4) to each “gas positive” tube and examine for fluorescence with 366 nm UV-light (5.17). Consider all fluorescent tubes as positive culture.

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

NOTE 1- The number of dilution steps depends on type of substrate being tested. For untreated substrate a dilution up to 10^{-7} should be carried out. However, for treated substrate, dilution up to 10^{-4} should be sufficient.

NOTE 2-The primary solution (described in 8.1) is already diluted as 10^{-1}. From this primary dilution, transfer 1 mL into three tubes each containing 9 mL MUG lauryl sulfate broth (7.2), as a first dilution step.

NOTE 3– if all tubes in the last three dilution steps (10^{-5}, 10^{-6} and 10^{-7} or 10^{-2}, 10^{-3} and 10^{-4}) are positive (gas+/fluorescence+/indole+), prepare 10^{-8} and 10^{-9} or 10^{-5} and 10^{-6} dilutions steps and transfer 1 mL per tube (5.1) into 3 tubes containing 9 mL MUG lauryl sulfate broth (7.2) and a Durham tube (5.10) each. Store all tubes from the dilution steps of the sample at 4 °C until the final result is obtained.

10.3 Determination of the dry residue content

The numbers of *E. coli* may be calculated per wet weight or dry weight. For the latter, it is necessary to determine the dry residue of the sample using the method described in EN 12880. This shall be performed in parallel with the microbiological analysis.

11 Expression of the results

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

Calculate the MPN corresponding to the identified characteristic number using DE MAN table (1983 – European Journal Applied Microbiol. Biotechnol. 17, 301-305. Annex A). The result corresponds to a MPN per mL of primary prepared suspension.

For example:

Dilution step	Gas+/Fluorescence+/Indol+		
10^{-1}	+	+	+
10^{-2}	+	+	+
10^{-3}	+	+	+
10^{-4}	+	-	-
10^{-5}	-	+	-
10^{-6}	-	-	-
10^{-7}	-	-	-
Characteristic number	3	1	1
MPN table	7.5		
Dilution factor	10^{-3}		
Result: MPN *E.coli*/g (wet weight) sample	$7,5 \times 10^{3}$		

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

Numbers present per dry weight of sludge are calculated according to:

$$N_d = N_w \times 100/e$$

Where

Nd = bacterial count in MPN g dry weight

e = % dry mass of the original wet (as received) sample

and

N_w = bacterial count in MPN *E.coli*/g wet weight

12 Test report

The test report shall contain the following information:

a) reference to this European standard;

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

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

d) results of the determination according to clause 9.

Any detail not specified in this European Standard and any other factor which may have affected the results.

13 Performance data

Information concerning the repeatability and reproducibility of the procedure, obtained by interlaboratory tests, is given in Annex B (informative).

Annex A
(informative)

MPN Statistical Table for 3-Tubes MPN procedure (de Man *et al*., 1983)

Characteristic number			MPN index	Confidence limits			
1st digit	2nd digit	3rd digit		³ 95 %	³ 95 %	³ 99 %	³ 99 %
0	0	0	< 0.30	0.00	0.94	0.00	1.40
0	0	1	0.30	0.01	0.95	0.00	1.40
0	1	0	0.30	0.01	1.00	0.00	1.60
0	1	1	0.61	0.12	1.70	0.05	2.50
0	2	0	0.62	0.12	1.70	0.05	2.50
0	3	0	0.94	0.35	3.50	0.18	4.60
1	0	0	0.36	0.02	1.70	0.01	2.50
1	0	1	0.72	0.12	1.70	0.05	2.50
1	0	2	1.1	0.4	3.5	0.2	4.6
1	1	0	0.71	0.13	2.00	0.06	2.70
1	1	1	1.1	0.4	3.5	0.2	4.6
1	2	0	1.1	0.4	3.5	0.2	4.6
1	2	1	1.5	0.5	3.8	0.2	5.2
1	3	0	1.6	0.5	3.8	0.2	5.2
2	0	0	0.93	0.15	3.50	0.07	4.60
2	0	1	1.4	0.4	3.5	0.2	4.6
2	0	2	2.0	0.5	3.8	0.3	5.2
2	1	0	1.5	0.4	3.8	0.2	5.2
2	1	1	2.0	0.5	3.8	0.2	5.2
2	1	2	2.7	0.9	9.4	0.5	14.2
2	2	0	2.1	0.5	4.0	0.2	5.6
2	2	1	2.8	0.9	9.4	0.5	14.2
2	2	2	3.5	0.9	9.4	0.5	14.2
2	3	0	2.9	0.9	9.4	0.5	14.2
2	3	1	3.6	0.9	9.4	0.5	14.2
3	3	0	2.3	0.5	9.4	0.3	14.2
3	0	1	3.8	0.9	10.4	0.5	15.7
3	0	2	6.4	1.6	18.1	1.0	25.0
3	1	0	4.3	0.9	18.1	0.5	25.0
3	1	1	7.5	1.7	19.9	1.1	27.0
3	1	2	12	3	36	2	44
3	1	3	16	3	38	2	52
3	2	0	9.3	1.8	36.0	1.2	43.0
3	2	1	15	3	38	2	52
3	2	2	21	3	40	2	56
3	2	3	29	9	99	5	152
3	3	0	24	4	99	3	152
3	3	1	46	9	198	5	283

Characteristic number			MPN index	Confidence limits			
1st digit	2nd digit	3rd digit		³ 95 %	³ 95 %	³ 99 %	³ 99 %
3	3	2	110	20	400	10	570
3	3	3	> 110				

Annex B
(informative)

Performance of method

The validation has been carried out with different compost-samples from source separated biowastes artificially contaminated with a pre-diluted suspension of *Escherichia coli K12* (DSM; 498) containing about 10^1 cfu/ml, 10^2 cfu/ml and 10^3 cfu/ml. The compost has been heated at X°C for Xmin prior contamination and after verification of the absence of *E. coli*. Comparative analyses of the spiked samples had been performed by two different laboratories. A similar procedure had been applied to the different samples of municipal sewage sludge. Samples were pre-cooled to (5 ± 3) °C prior contamination and kept at this temperature until the analysis had been started. Time between contamination and analysis was 2 h ± 15 min. Statistical analysis was performed by calculating the mean value, the standard deviation and the 95 % confidence level using the SAS statistical package.

Table B.1 — Results of the comparative validation of 10 artificial contaminated compost samples in duplicate (n = 20)

	Bacterial content (*E. coli*) of the compost samples		
	10^1	10^2	10^3
Mean	3,13 E + 01	1,96 E + 02	3,63 E + 03
Standard deviation	2,85 E + 01	9,91 E + 01	2,88 E + 03
95 % confid. Interv.	1,8 – 4,46 E + 01	1,49 – 2,42 E + 02	2,33 – 5,02 E + 03

Table B.2 — Results of the comparative validation of 10 artificial contaminated sludge samples in duplicate (n = 20)

	Bacterial content (*E. coli*) of the sludge samples		
	10^1	10^2	10^3
Mean	1,45 E + 01	1,82 E + 02	9,92 E + 03
Standard deviation	1,23 E + 01	1,44 E + 02	6,38 E + 03
95 % confid. Interv.	8,7 E + 00 – 2,02 E + 01	1,15 – 2,49 E + 02	6,93 E + 02 – 1,29 E + 03

Bibliography

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