SLOVENSKI STANDARD

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Karakterizacija blata ï Ugotavljanje prisotnosti in ġtevila Echerichia coli v blatu, zemljini, izboljġevalcih tal, rastnih substratih in bio-odpadkih - 2. del: Miniaturizirana metoda (najverjetnejġe ġtevilo ï MPN) s cepljenjem v tekoļ i medij

Characterization of sludges - 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

Charakterisierung von Schlämmen - Quantitativer Nachweis von Escherichia coli in Schlämmen, Böden, Bodenverbesserungsmitteln, Kultursubstraten sowie Bioabfällen - Teil 2: Miniaturisiertes Verfahren durch Animpfen in Flüssigmedium (MPN-Verfahren)

Caractérisation des boues - Détection et dénombrement des Escherichia coli dans les boues, les sols, les amendements du sol, les supports de culture et les biodéchets - Partie 2 : Méthode miniaturisée (nombre le plus probable) par ensemencement en milieu liquide

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Foreword

This Technical Report (CEN/TR 15214-2:2006) has been prepared by Technical Committee CEN/TC 308 “Characterization of sludges”, the secretariat of which is held by AFNOR.

This Technical Report does not replace any existing CEN standard.

The standard is divided into three parts, Part 1 gives a membrane filtration for quantification, Part 2 gives a miniaturised semi-quantitative MPN method and Part 3 gives a semi-quantitative macro method.

Introduction

Sludges, soils, soil improvers, growing media 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 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 sewage sludge. Suitable quality control procedures, at least those described in ISO 8199, 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".

1 Scope

This part of the CEN Technical Report describes a miniaturized most probable number (MPN) method for the quantitative detection of *Escherichia coli* in sludges, soils, soil improvers, growing media 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 is convenient for material with dry residue of more than 10 %. For materials with dry residue less than 20 %, the procedure specified in CEN/TR 15214-1 will be used.

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 12880:2000, *Characterisation of Sludges - Determination of dry residue and water content*

EN ISO 9308-3, *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 (ISO 9308-3:1998)*

ISO 8199, *Water quality - General guide to the enumeration of micro-organisms by culture*

3 Terms and definitions

For the purposes of this Technical Report, 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 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
β-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 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, 3.1].

4 Principle

This method is based on EN ISO 9308-3: “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 gram dry residue of sludge. In order to perform the analysis of *E. coli* in sludges, soils, soil improvers, growing media and biowastes, this document describes the whole procedure corresponding to the one of 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 tryptone salt diluent;

b) inoculation of the diluted sample in a row of microtitre plate 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 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 10 g sludge dry residue.

5 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:

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

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

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

5.4 Homogeniser (e.g. Stomacher[R], Seward Laboratories or equivalent).

5.5 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).

5.6 Ultraviolet observation chamber (Wood’s Lamp , 366 nm).

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

5.7 Portable refractometer (optional).

5.8 pH meter with an accuracy of ± 0.1.

5.9 Sterile test tubes of 25 ml volume, or flasks with similar capacity.

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

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

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

5.13 Sterile tips for multi-pipette.

5.14 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.

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

5.16 Sterile adhesive covering strips for sealing microtitre plates.

5.17 Sterile Petri dishes, 90 mm in diameter.

6 Sampling and hazards

6.1 Introduction

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.

6.2 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.

6.3 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.

6.4 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.

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. 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 Tryptone salt diluent

Per litre:

a) 1 g of casein peptone;

b) 8,5 g of sodium chloride.

Sterilise the solution in a steam steriliser (5.1) at (121 ± 3) °C for (15 ± 1) min.

7.2 Special diluent (SD)

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

Sterilize in the autoclave (5.1) 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 SD blue and avoid confusing with demineralised or distilled water.

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

NOTE 2 SD 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 (5.14).

Distribute in 96-well microtitre plates (5.15) 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 (5.3).

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

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

8 Procedure

8.1 Sample preparation

8.1.1 Determination of the dry residue content

The dry residue of the sample is measured using the method described in EN 12880.

8.1.2 Suspension preparation

Weigh a representative sub-sample of 10 g (dry residue) into a homogeniser bag (5.5) with an integrated mesh if large debris is to be excluded.

Add an appropriate volume of tryptone salt diluent (7.1) so that the final volume is 100 ml. Place the homogeniser bag (5.5) in a homogeniser (5.4) and homogenize for 1 min.

Process without delay.

NOTE For disinfectant (e.g. lime, peracetic acid) and treated sludges, a suitable neutralisation procedure must be used (see for example EN 1040:1997).

8.2 Analysis

8.2.1 Preparation of dilutions

Prepare serial dilutions in special diluent (SD, 7.2) from 1/20 to 1/2000000:

Vigorously stir the primary suspension (8.1.2) in order to obtain a homogeneous suspension and, using a sterile pipette, immediately transfer 18 ml of this homogenised suspension to a first tube (5.9) containing 18 ml of special diluent (1/2 dilution).

Using a fresh pipette (5.11), transfer 2 ml of this first dilution (homogenised) to a second 18 ml special diluent tube (1/20 dilution).

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

Continue as above until all the dilutions to 1/2000000 have been prepared.

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

8.2.2 Inoculation and incubation of microtitre plates

Inoculate a microtitre plate (5.15) 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 first dilution tube (1/20) to an empty sterile Petri dish of 90 mm diameter (5.17).

Using a multi-channel pipette (5.12) with 8 sterile tips (5.13), distribute 200 µl per wells into 16 wells of the microtitre plate (5.15) corresponding to this first dilution (use the 2 first columns on the left side of the microtitre plate).

For subsequent dilutions (1/200, 1/2000, etc.), operate in an identical manner, changing the Petri dish and the row of 8 sterile tips between each dilution, and using for each successive dilution the 2 following 8 wells columns of the microtitre plate. The two last 8 wells columns on the right of the microtitre plate should correspond to the 1/2000000 dilution.

Alternatively, any other suitable system (5.12) 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 (5.16) provided for this purpose.

Incubate the microtitre plate in an incubator (5.2) 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.

8.2.3 Reading

Place each microtitre plate with the adhesive on, in the UV observation chamber (5.6).

Consider all blue fluorescent wells as being positive.

9 Expression of results

9.1 Determination of the characteristic number

For each of the 6 inoculated dilutions, note the number of positive wells.

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