Soils, sludges and treated bio-wastes — Isolation and enumeration of intestinal enterococci in sludges, soils and treated bio-wastes – Part 1: Membrane filtration method onto selective agar

Boden, Schlamm und behandelte Bio-abfälle — Quantitativer Nachweis von Enterococci aus Schlämmen, Böden und Behandelte Bioabfällen – Teil 1: Membranfiltrationsverfahren

S*ols, boues et bio-déchets traités — Détection et dénombrement des enterococci intestinaux dans les boues, les sols et les biodéchets traités – Partie 1 : Méthode par filtration sur membrane*

ICS:

Descriptors: Enterococci, sludges, soils, biowastes

Document type: European Standard
Document subtype:
Document stage: Ruggedness trial
Document language: E

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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 membrane filtration procedure for the quantitative analysis, by culture of individual colonies on selective agar, of intestinal enterococci species in sewage sludge, compost and biowaste samples. The user should, prior to analysis, validate the method for the particular type of sample they wish to analyse: sludges, soils and treated biowastes.

This method is of particular use if determination of treatment efficiency is required as outlined in the revision of Directive 86/278/EEC (3rd draft, CEN/TC 308-doc 525 [1]).

The method has a limit of detection of approximately 27 cfu/g wet weight [ENV ISO 13843], dependent of the solids content which at high concentrations (>20% (w/v)) can block the filtration of the sample volume through the membrane if not diluted prior to filtration. Therefore if the sludge sample has or is expected to have high solids content or the numbers present are expected to be lower than the detection limit this method may not be suitable.

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 Definitions

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

3.1
enterococci
Enterococci are gram-positive, catalase-negative, facultative anaerobic cocci that grow in pairs (diplococci) or in short chains, and possess Lancefield's Group D antigen. The organisms can be differentiated from other catalase-negative gram-positive cocci by the ability to grow in the presence of bile salts and sodium azide, 6.5% sodium chloride, at 44°C, reduce 2,3,5-triphenyltetrazolium chloride (TTC) to formazan and produce pyroglutamateaminopeptidase (*i.e.* PYR reaction).

3.2
method definition
Reduction of TTC to formazan producing red/maroon to pink colonies when grown on Slanetz and Bartley (m-Enterococcus) agar.

The chromagen indoxyl-β-D-glucoside is cleaved by β-D-glucosidase positive enterococci producing an insoluble blue dye which diffuses into the agar surrounding the colony.

Hydrolysis of aesculin to produce dextrose and aesculetin and dextrose when grown on aesculin azide agar. The aesculetin combines with ferric citrate in the media producing a black/brown precipitate in the media

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

3.4
vegetative bacteria
bacteria 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

SBA Slanetz and Bartley agar

QSR Quarter Strength Ringers

BAA Bile aesculin agar

5 Principle

The homogenised diluted sample is filtered. The membrane filter is then recovered and incubated aerobically at (36 ± 1)℃ for (4 ± 0.5) h, then at (43 ± 1)℃ for (40 ± 4) h onto SBA plate. Enterococci is indicated by the presence of pink to maroon colonies on SBA plate, when visualised by eye.

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 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
Distilled water	1 000 mL

Dissolve all reagents by heating in 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) min. Check the pH at (7.0 ± 0.2).

6.3 Slanetz and Bartley agar (m-Enterococcus agar) (SBA)

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Di-potassium hydrogen phosphate	4.0 g
Sodium azide	0.4 g
Tetrazolium chloride	0.1 g
Agar	10.0 g

Add reagents to 1 L of water and heat to just boiling. **DO NOT AUTOCLAVE**. Once cooled to 50ºC pour into Petri dishes (do not store media to re-heat). Media appears straw coloured. If a pink colour appears in media it should be discarded as it has overheated. Check the pH at (7.2 ± 0.2).

NOTE Media is commercially available in powder form or as pre-prepared plates.

6.4 Bile aesculin agar (BAA)

Peptone	8.0 g
Bile salts	20.0 g
Ferric citrate	0.5 g
Aesculin	1.0 g
Agar	15.0 g
Demineralised or distilled water	1 000 mL

Mix all ingredients and bring to the boil whilst stirring continuously.

Add reagents to the water and heat to boiling and steam sterilise (autoclave) at (121 ± 3)ºC for (15 ± 1) min. Prepared media appears as opaque green brown. Check the pH at (7.1 ± 0.2).

NOTE Media is commercially available in powder form or as pre-prepared plates

6.5 3% hydrogen peroxide solution (catalase test)

Hydrogen peroxide	3 mL
Distilled water	100 mL

Prepare aseptically the solution and store at room temperature for one week.

7 Apparatus

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

Usual microbiological laboratory equipment and in particular:

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

7.2 Thermostatic incubator(s) either one cyclic regulated at (36 ± 1)ºC and (43 ± 1)ºC or two one regulated at (36 ± 1)ºC and one at (43 ± 1)ºC

7.3 Homogeniser

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

7.5 Membrane filtration manifold

7.6 Membrane filters 47 mm diameter, cellulose nitrate based, gridded 0.45µm nominal pore size

7.7 Membrane filter units, 150 mL capacity either disposable or sterilisable

7.8 Sterile forceps.

7.9 Sterile Petri dishes, 50 mm in diameter, for holding SBA medium

7.10 Sterile universals plastic or glassware capable of holding up to 20 mL

7.11 Sterile graduated pipettes, capable of dispensing 2-10 mL

7.12 Sterile measuring cylinder capable of measuring 250 mL

7.13 Pipettors capable of dispensing 100 µL and 1 mL

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

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

7.16 Sterile tips

7.17 Analytical balance

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

7.19 Vortex mixer

7.20 pH meter with an accuracy of ± 0.1

7.21 Laboratory spatula

7.22 Boiling water bath, if sterilisible filter units are used

7.23 Bunsen burner or working within a Class II safety cabinet

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 QSR as appropriate for the expected concentration of enterococci in sample. For example raw sludges may have up to 10^6-10^7 enterococci per g but treated sludges may only require dilutions to 10^{-1}-10^{-2}.

Mix the primary suspension, dilution A (1/10 dilution), and using a sterile pipette aseptically transfer 1 mL into a sterile tube containing 9 mL QSR. Mix this dilution and using a new sterile pipetted transfer 1 mL of this dilution to a tube containing 9 mL QSR.

Continue this procedure until the required number of dilutions has been prepared.

9.3 Membrane Filtration

Filter 1 mL aliquots of each diluted sample through a 0.45 µm pore size cellulose nitrate filter. To ensure even distribution of the sample over the filter surface, prior to addition of the sample add approximately 10-20 mL of diluent (QSR solution or equivalent) to the membrane in the filter housing.

NOTE Keep the vacuum in the off position until the sample has been added.

As soon as the sample has filtered through, using a low vacuum pressure (not exceeding 65 kPa 500 mm of mercury), switch off the vacuum so as little air as possible passes through the filter.

With each analysis undertaken the following controls should be included:

Positive control – *e.g. Enterococcus faecalis* NCTC 775

Negative control – *e.g. E. coli* NCTC 9001

Blank – diluent used *i.e.* QSR

Analysis of the control samples should be conducted at the start of analysis and at the end of sample analysis by filtration of each control through a filter and placing onto selective media being used.

9.4 Resuscitation and enumeration of colonies on selective agar

Remove the filters from the filter housing using sterile tweezers, holding the filter at the edge of the filter. Place the filters face upwards onto 50 mm diameter plates containing pre-dried SBA (6.3). Use a 'rolling' action to prevent the formation of air bubbles under the media which would prevent the media coming into contact with the membrane. Invert the plates and incubate SBA (m-Enterococcus) plates initially at (36 ± 1)°C for (4 ± 0.5) h, then increase the temperature to (43 ± 1)°C for (40 ± 4) h.

Enumerate maroon/pink colonies (including pale pink colonies) over 0.5 mm diameter that are smooth and convex in shape by eye on SBA plates.

Note Some species of *Bacillus*, *Aerococcus* and *Staphylococcus* can also grow on Slanetz and Bartley (m-Enterococcus) agar. *Bacillus* spp. produce pink colonies but are usually rough, flat

and sometimes spreading. *Staphylococcus* species produce red colonies but are catalase positive. *Aerococcus* species produce red colonies, are catalase negative but usually PYR (pyroglutamateaminopeptidase) negative.

The maximum number of colonies that should be counted on a single membrane is approximately 80 cfu. If no other plates are available with less colonies present an estimate of the number present can be made.

9.5 Confirmation

The membrane filters are transferred onto BAA plates (6.4) and incubating for 1 - 4 h at (43 ± 1)°C. Enumerate all colonies producing a brown blackening of the agar. All confirmed colonies (at least 10 colonies where >10 are present on plates) on BAA plates should also be confirmed as catalase negative by picking a BAA positive colony on a loop and suspending in 3% hydrogen peroxide. An absence of bubbles indicates a negative reaction.

Confirmed colonies are bile aesculin positive, catalase negative.

Additionally, a biochemical tests can be applied such as test strips or equivalent can be used.

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

Calculation of the number of enterococci present (per g wet weight of the original sample) is by dividing the total number of typical maroon/pink colonies (n) on the filter membrane of the selected plates (9.4) by the total volume filtered of the initial sample. The result of the confirmation steps (BAA and catalase tests) must be taken into account to estimate the total number of typical colonies and calculate the final result [ISO 8199:2005].

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

where:

c = enterococci concentration per g wet weight of original sample

n = total number of typical enterococci colonies on the selected filter membranes: $n = n1 + n2 + \ldots$

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

NOTE **The dilution factor of the dilution A taken for filtration step should not be forget in the final calculation.**

Example:

If the volume of the test dilution used (v_i) is 1 mL of dilution A and the following counts are obtained at the respective dilutions:

Dilution	Counts
10^{-2}	81 colonies
10^{-3}	15 colonies

Then:

n= 81+15 = 96

v= (0.1 x 1 x 0.01) + (0.1 x 1 x 0.001)

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

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

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

e = % dry residue of the original wet sample.

11 Performance data

First performances data of the procedure, following the ruggedness study (European scale intralaboratory trial) performed during the FP6 EU Horizontal-Hyg project [5] is given in Annex A (informative).

12 Test report

The test report shall contain the following information:

a) reference to this part of this European Standard;

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

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

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

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

Annex A
(informative)

First assessment of the precision of the method

The statistical evaluation was conducted according to ISO 13843. The limit of detection, the upper limit of quantification, the range of quantification and the results of dispersion U^2 were obtained (Table A.3-1).

The limit of detection corresponds to the number of particles (germs per test portion) when the probability of a negative result is 5% (superior limit of the confidence interval of the null result).

Poisson distribution corresponds to the random distribution of the number of particles at the moment of sampling a perfectly homogenised suspension.

The relative variance U^2 corresponds to the relative standard deviation squared ratio of the standard deviation squared and the mean squared as:

$$U^2 = s^2/m^2$$

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

Table A.1 — Summary of components of the precision of the intestinal enterococci filtration 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**
26.96	$1.32\ 10^{11}$	9.7	Less than 0.05

NOTE **In judging the results it is important to consider that they do not depend on the experimental data but only on the design of the measurement protocol (random variation).**

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