Soils, sludges and treated bio-wastes — Detection of *Clostridium perfringens* in sludges, soils and treated bio-wastes – Part 2: Macromethod (Most Probable Number) by inoculation into selective liquid medium

Boden, Schlamm und behandelte Bio-abfälle —Nachweis von Clostridium perfringens aus Schlämmen, Böden und Behandelte Bioabfällen – Teil 2: Makromethode (MPN) in selektiven Flüssigmediums

S*ols, boues et bio-déchets traités — Détection de Clostridium perfringens dans les boues, les sols et les biodéchets traités – Partie 2 : Macrométhode (NPP) par ensemencement en milieu liquide*

ICS:

Descriptors: *Clostridium pefringens*, 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 *Clostridium perfringens* 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.

Clostridium perfringens is a spore-forming bacteria commonly found in soil, faeces and in the intestines of healthy people and animals. The toxin produced by *Clostridium perfringens* causes food-borne illness. Consequently, it can be used as an indicator of faecal contamination occurring in the past. It can also be used to monitor the effectiveness of pasteurisation or disinfection treatments as its spores are resistant to treatments (*e.g.* heat, high pH).

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 most probable number (MPN) procedure for the analysis, by culture of *Clostridium perfringens* in selective broth, in sludge, soils and biowastes.

The user should, prior to analysis, validate the method for the particular type of sample they wish to analyse: sludges, soils and 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 9 MPN/g wet weight [ENV ISO 13843:2001].

2 Normative references

The following normative references are cited at appropriate places in the text. They were referred to extensively and offer indispensable advice for the application of this method. For dated references, only the edition cited applies. For undated references the latest edition of the publication referred to applies (including any amendments).

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

EN 12880:2000, *Characterisation of sludges — Determination of dry residue and water content*.

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

ENV ISO 13843:2001, *Water Quality — Guidance on validation of microbiological methods.*

3 Terms and definitions

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

3.1
Clostridium perfringens
Clostridium perfringens are anaerobic (or micro-aerobic) gram-positive bacillus (rods), spore forming, non-motile, reduces sulphite, reduces nitrates to nitrites, produces acid and gas from lactose, metabolises 4-methylumbelliferyl phosphate (MUP) to 4-methylumbelliferone and liquefies gelatin within 48h

3.2
method definition
C. perfringens metabolises 4-methylumbelliferyl phosphate (MUP) using the enzyme acid phosphatase to produce 4-methylumbelliferone, which fluoresces when placed under long-wavelength (365-nm) ultraviolet light

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

The essence of the MPN method is to dilute the sample to such a degree that inocula in the tubes will sometimes but not always contain viable organisms. The "outcome", *i.e.*, the number of tubes and the number of tubes with growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions incubating tubes at several dilutions

3.4
vegetative bacteria
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
spores
endospores formed by *Clostridium perfringen*s which are heat resistant

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 *Clostridium perfringen*s, 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

MUP 4-methylumbelliferyl disodium salt

mTSC Modified Tryptose sulphite cycloserine

QSR Quarter Strength Ringers

MPN Most Probable Number

CN Characteristic Number

UV Ultra violet

5 Principle

Homogenised samples are diluted as appropriate, inoculated into selective broth media and incubated anaerobically at (43 ± 1)℃ for (22 ± 2)h. *Clostridium perfringens* is indicated by the presence of fluorescence when visualised under UV light.

The method has a limit of detection of 100 cfu g^{-1} wet weight and is suitable for all dry weight samples (high and low).

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

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride $6H_2O$	0.12 g
Sodium bicarbonate	0.05 g
Deionised water	1000 mL

Dissolve reagents 1 L deionised or distilled water by heating if necessary.

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

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

6.3 4-methylumbelliferyl (MUP) disodium salt

4-methylumbelliferyl (MUP) disodium salt	100.0 mg
Deionised or distilled water	5 mL

Add MUP to water and filter sterilise through a 0.2 µm Millipore filter.

6.4 D-cycloserine

D-cycloserine	400.0 mg
Deionised or distilled water	5 mL

Add D-cycloserine to the water, and filter sterilise through a 0.2 µm filter.

NOTE **both supplements (6.3 and 6.4) are commercially available as a single supplement in vial form. One vial is suspended in 5 mL sterile water and the contents added to 500 mL media.**

6.5 Modified Tryptose sulphite cycloserine broth (mTSC broth)

Tryptose	15 g
Meat extract	5.0 g
Soy peptone	10.0 g
Yeast extract	5 g
Sodium acetate	5 g
L-cysteine hydrochloride	2 g
Deionised water	1 L

Add reagents to one litre of water and heat to boiling. Autoclave at (121 ± 3)℃ for (15 ± 1) min. Once cooled to 50ºC add 2.5 mL (**per 500 ml**) of TSC supplement or 2.5 mL (**per 500 ml**) D-cycloserine (6.4) and swirl to mix and 2.5 mL 4-methylumbelliferyl phosphate disodium salt (6.3). Check the pH 7.0 ± 0.2.

NOTE **Alternatively add one vial of Perfringens supplement per 500 mL media.**

7 Apparatus

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

Usual microbiological laboratory equipment and in particular:

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

7.2 Thermostatic incubator(s) at (37 ± 1)ºC

7.3 Homogeniser

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

7.5 pH meter with an accuracy of ± 0.1

7.6 Bunsen burner

7.7 Pipettors – capable of dispensing 100 µL and 1 mL

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

7.9 Sterile 100 mL containers – plastic or glassware

7.10 Sterile universals – plastic or glassware

7.11 Sterile tips

7.12 Anaerobic condition generators anaerobic jars with anaerobic packs or anaerobic incubator

Note Indicator strips should be included to ensure anaerobic conditions have been achieved

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

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

7.14 Sterile Petri dishes – 90 mm diameter

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

7.16 Vortex mixer

7.17 Laboratory spatula

8 Sampling

Take samples of at least 500 g wet weight and deliver them to the laboratory as quickly as possible (within 24 h). In order to prevent propagation or inactivation of *Clostridium perfringens* 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 solution as appropriate for the expected concentration of *Clostridium perfringens* in sample. For example raw sludges may have up to 10^{6}-10^{9} *Clostridium perfringens* per g but treated sludges may only require dilutions to 10^{-1}-10^{-6}. If the concentration is not known all dilutions should be retained (at 5 ± 3℃) until analysis is complete. If the sample is highly contaminated the first dilution(s) can be omitted from analysis.

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

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

9.3 Analysis

Pipette 1 mL of each sample to 9 mL mTSC broth. To reduce addition of air to media pipette into the bottom of the tube avoiding formation of bubbles. Replace the lid loosely if incubating in anaerobic jar or carefully add a layer (0.5 – 1.0 mL) of sterile mineral or paraffin oil and replace lid. For each dilution to be tested perform three replicates.

With each analysis perform a positive control culture, with for example *C. perfringens* NCTC 102.

9.4 Resuscitation and enumeration of positive tubes in selective broth

Place in anaerobic gas generating jars with appropriate gas generator, in anaerobic incubator or overlay with sterile mineral oil and incubate at (43 ± 1)℃ for (22 ± 2) h and (44 ± 2) h if tubes are negative after initial incubation. Positive reaction is the presence of light blue fluorescence under UV light in the broth.

9.5 Confirmation

The media used in the described method is confirmatory but if additional confirmation is required biochemical tests can be applied such as test strips or equivalent.

9.6 Determination of the dry residue content

The numbers of *Clostridium perfringens* may be calculated per wet weight or dry weight. For the latter, it is necessary to determine the dry residue of the sample using the method described in EN 12880:2000. This shall be performed in parallel with the microbiological analysis.

10 Expression of results

10.1 Calculation of MPN

Clostridium perfringens are deemed present if the samples are fluorescent under UV light. For each of the dilutions, note the number of positive tubes (fluorescent - between 0 and 3). Identify the characteristic number (CN) 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 [2] – Annex A). The result corresponds to a MPN per mL of primary prepared suspension.

To calculate the number in the original sample (wet weight) account of the dilution used in analysis needs to be taken.

Example:

CN = 3/1/0

MPN/mL = 4.3

Dilutions used = 10^{-3}

MPN/g = $(4.3/10^{-3})$ x 250 g /25 g

$\therefore$ = 43,000 g^{-1} wet weight

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

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

Where

N_d = bacterial count in MPN g dry weight

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

N_w = bacterial count in MPN *Clostridium perfringen*s/g wet weight

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

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

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 are given in Annex C (informative).

12 Test report

The test report shall contain the following information:

a) reference to this part of this European Standard;

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

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

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

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

Annex A
(informative)
MPN Statistical Table
for 3-Tubes MPN procedure (de Man *et al.*, 1983 [4])A.

Characteristic number			MPN index	Confidence limits			
1st digit	2nd digit	3rd digit		≥ 95 %	≥ 95 %	≥ 99 %	≥ 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	0	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
3	3	2	110	20	400	10	570
3	3	3	> 110				

Annex B
(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 B.1 — Summary of components of the precision of the *Clostridium perfringens* MPN method

Limit of detection (5%) *C. perfringens* /g wet weight	**Upper limit of quantification (5%)** *C. perfringens* /g wet weight	**Range of quantification** Log10 unit	**Results of dispersion U^2**
8.99	$4.65\ 10^6$	5.7	0.8

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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