SLOVENSKI STANDARD

SIST-TP CEN/TR 15215-1:2006

01-julij-2006

Karakterizacija blata ï Ugotavljanje prisotnosti in ġtevila Salmonela sp. v blatu, zemljini, izboljġevalcih tal, rastnih substratih in bio-odpadkih - 1. del: Metoda membranske filtracije za kvantitativno obnovo (ponovno oġivljanje) bakterij, ki so pod subletalnim stresom (za potrjevanje uļ inkovitosti postopka logaritemskega dodajanja po kapljicah)

Characterization of sludges - Detection and enumeration of Salmonella spp. in sludges, soils, soil improvers, growing media and biowastes - Part 1: Membrane filtration method for quantitative resuscitation of sub-lethally stressed bacteria (to confirm efficacy of log drop treatment procedures)

Charakterisierung von Schlämmen - Quantitativer Nachweis von Salmonella spp. in Schlämmen, Böden, Bodenverbesserungsmitteln, Kultursubstraten sowie Bioabfällen - Teil 1: Membranfiltrationsverfahren zur quantitativen Miterfassung vorgeschädigter Bakterien (zur Bestätigung der logarithmischen Verminderung durch ein Behandlungsverfahren)

Caractérisation des boues - Détection et dénombrement de Salmonella spp. dans les boues, les sols, les amendements du sol, les supports de culture et les biodéchets - Partie 1 : Méthode par filtration sur membrane permettant la ressuscitation quantitative des bactéries stressées de maniere sub-léthale (pour confirmer l'efficacité de l'abattement de logs lors des procédés de traitement)

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TECHNICAL REPORT

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English Version

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Caractérisation des boues - Détection et dénombrement de Salmonella spp. dans les boues, les sols, les engrais, les amendements organiques et les biodéchets - Partie 1 : Méthode par filtration sur membrane permettant la ressuscitation quantitative des bactéries stressées de manière sub-léthale (pour confirmer l'efficacité de l'abattement de logs lors des procédés de traitement)

Quantitativer Nachweis von Salmonella spp. in Schlämmen, Böden, Düngemitteln und Bodenverbesserern, Kultursubstraten sowie Bioabfällen - Teil 1: Membranfiltrationsverfahren zur quantitativen Miterfassung vorgeschädigter Bakterien (zur Bestätigung des logarithmisch-tropfenweisen Behandlungsverfahrens)

This Technical Report was approved by CEN on 3 September 2005. It has been drawn up by the Technical Committee CEN/TC 308.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: rue de Stassart, 36 B-1050 Brussels

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Foreword

This Technical Report (CEN/TR 15215-1:2006) has been prepared by Technical Committee CEN/TC 308 “Characterization of sludges”, the secretariat of which is held by AFNOR.

This document does not replace any existing CEN method.

This standard is divided into three parts:

— *part 1 gives a membrane filtration method*

— *part 2 is a liquid enrichment method and determination by MPN and*

— *part 3 is a presence / absence method by liquid enrichment.*

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 can cause outbreaks of infection due to the production of contaminated food or animal feedstocks and may also be transmitted to wild animals, consequently, there is a need to monitor rates to land. See CEN/TR 15215-2.

Examination for *Salmonellae* should only be carried out in laboratories competent for carrying out work involving pathogens. Suitable quality control procedures, at least those described in ISO 8199, 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".

1 Scope

This part of the CEN Technical Report specifies a membrane filtration procedure for the quantitative resuscitation and enumeration, by culture of individual colonies on chromogenic agar media, of *Salmonella* spp. including potentially sub-lethally damaged *Salmonella* spp. in sewage sludges. It may be suitable for other sludges, soils, soil improvers, growing media and biowastes but the user shall validate the method using these materials. The fully defined scope will be determined after the proposed validation trials have been agreed and carried out.

NOTE 1 The objective is to cover untreated and treated sludges, soils, soil improvers, growing media and biowastes.

The method is particularly suited to determining the efficiency of treatment procedures for the elimination of pathogens in sewage sludge as outlined in the Revision of Directive 86/278/EEC (3rd Draft, CEN/TC 308 – doc 525). Treatment type A processes are initially to be validated through a to be defined Log_{10} reduction with a test organism such as *Salmonella senftenberg* W775.

The method has a limit of detection of approximately 1 cfu/g wet weight sludge, dependent on the solids content which at high concentrations (> 20 % (w/v)) can restrict filtration of the sample volume through the membrane if not first diluted.

NOTE 2 *Salmonella* spp. can be present in biosolids including untreated and treated sewage sludge as both vegetative and sub-lethally damaged cells; the latter require resuscitation to enable colony growth for accurate enumeration on agar media.

NOTE 3 This method is not suitable for treated sludges containing less than 1 viable *Salmonella spp.* per 1 g wet weight.

NOTE 4 This method is not suitable for untreated sludges containing low levels of *Salmonella.*

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applied. 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*.

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
Salmonella spp.
member of the family of *Enterobacteriaceae*, these are Gram-negative, non-sporulating, rod-shaped bacteria, most of which are motile. They can be distinguished from other genera of the *Enterobacteriaceae* family by biochemical methods and serologically identified by their somatic or flagellar antigens (O and H-antigens)

3.2
method definition
Salmonella spp. capable of being resuscitated on Tetrathionate broth at (36 ± 2) °C followed by fermentation of propylene glycol and acid production on Rambach agar at (36 ± 2) °C. Most serovars are unable to ferment lactose and are β-galactosidase negative, but capable of fermenting propylene glycol and producing acid on Rambach agar when incubated at (36 ± 2) °C (See also 8.5)

NOTE Some *Salmonella* (e.g. *S. typhi* and *S. paratyphi*) will not be detected

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
those bacteria which are capable of normal growth in broth or on agar media without pre-culture resuscitation

3.5
sub-lethally damaged bacteria
those bacteria which have been stressed but not killed in treatment processes or storage

3.6
resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria previously incapable of growth on agar media

3.7
quantitative resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria recovered discretely on a membrane filter, prior to transfer to chromogenic medium for growth of individual colonies

3.8
presumptive positives
isolates which are believed to be *Salmonella* spp., but not yet confirmed

3.9
dry residue
the dry mass portion of the sludge obtained after the specified drying process. It is expressed as percent or in grams per kilogram

[EN 12880:2000, 3.1]

4 Principle

The homogenised diluted sludge sample is centrifuged and filtered, the membrane filter recovered aseptically and incubated at (36 ± 2) °C on a sterile glass fibre disk soaked with resuscitation medium (Tetrathionate broth). After 24 h the membrane is recovered aseptically and incubated at (36 ± 2) °C on chromogenic medium (Rambach agar). The membranes are examined after 24 h and 48 h (the latter to detect more fastidious *S. dublin*) and positive colonies are quantified. The presence of *Salmonella* spp. is indicated by bright red colonies resulting from fermentation of propylene glycol; other coliforms appear blue, green, violet or colourless due to their inability to ferment propylene glycol while some produce β-galactosidase which hydrolyses colourless X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the medium to produce a blue chromophore.

To distinguish *Salmonella* spp. from occasional *Citrobacter* spp., spray an aerosolised solution of 4-methylumbelliferyl caprylate (1 mg/ml) in ethanol directly onto the filters on the Rambach agar. The presence of *Salmonella* spp. is indicated by fluorescence of the colonies under UV light at 366 nm, resulting from the production of C_8 esterase activity. The colonies may also be confirmed using biochemical test strips or serological identification of their somatic and flagellar antigens (O- and H- antigens).

5 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:

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

5.2 Thermostatic incubator regulated at (36 ± 2) °C.

5.3 Homogeniser (e.g. Stomacher®, Seward Laboratories or equivalent).

5.4 Centrifuge capable of centrifuging 50 ml at 200 × g to 300 × g for 1 min.

5.5 Disposable filter units (0,45 µm gridded, cellulose nitrate, 47 mm diameter and 0,2 µm cellulose nitrate, 25 mm diameter).

5.6 Coarse glass fibre filter discs (47 mm diameter) (e.g. Whatman GF/D pore size 2,7 µ or equivalent).

5.7 Vacuum pump (e.g. Neuberger Model N726-3FT-18 or equivalent).

5.8 Vacuum manifold (e.g. Millipore or equivalent) to hold filter units.

5.9 Stereo microscope fitted with × 10 eyepieces, use × 6 magnification).

5.10 Cold light source, fitted with a Schott KG 1,45 × 45 filter (blue) or equivalent. Illuminate membrane filters with dual fibre-optic light guides.

5.11 UV observation lamp or chamber (366 nm).

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

5.12 Nebuliser spray (to spray ethanolic solution of 4-methylumbelliferyl caprylate over the filters on agar media).

WARNING — Avoid inhalation and ignition

5.13 Sterile homogeniser bags (e.g. Seward Laboratories or equivalent), 250 ml volume, with or without integrated mesh to exclude large particulate matter.

5.14 Sterile Petri dishes, 50 mm in diameter, for incubating soaked glass fibre discs and also holding Rambach agar medium.

5.15 Sterile bottles of 100 ml volume, or flasks with similar capacity.

5.16 Automatic pipettes, capable of dispensing 0,1 ml to 1,0 ml and 1,0 ml to 5,0 ml volumes.

5.17 Sterile graduated pipettes, glass or disposable plastic ware, capable of dispensing 10 ml volumes.