

**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)

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English Version

**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)**

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  
vorgeschiedigter 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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## 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 (3<sup>rd</sup> Draft, CEN/TC 308 – doc 525). Treatment type A processes are initially to be validated through a to be defined  $\text{Log}_{10}$  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  $(36 \pm 2)$  °C followed by fermentation of propylene glycol and acid production on Rambach agar at  $(36 \pm 2)$  °C. Most serovars are unable to ferment lactose and are  $\beta$ -galactosidase negative, but capable of fermenting propylene glycol and producing acid on Rambach agar when incubated at  $(36 \pm 2)$  °C (See also 8.5)

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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  $(36 \pm 2)^\circ\text{C}$  on a sterile glass fibre disk soaked with resuscitation medium (Tetrathionate broth). After 24 h the membrane is recovered aseptically and incubated at  $(36 \pm 2)^\circ\text{C}$  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  $\beta$ -galactosidase which hydrolyses colourless X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) in the medium to produce a blue chromophore.