Soils, sludges and treated bio-wastes — Detection and enumeration of *Salmonella* spp. in sludges, soils, and biowastes – Part 1 : Membrane filtration method for quantification resuscitation of sub-lethally stressed bacteria (to confirm efficacy of log drop treatment procedures)

Böden, Schlamm und behandelte Bio-abfälle – Nachweis von Salmonella spp. aus Schlämmen, Böden, sowie Bioabfällen – Teil 1 : Membranfiltrations methode zur quantitativen Miterfassung vorgeschädigter Salmonellen

Sols, boues et bio-déchets traités – Détection et dénombrement de Salmonella spp. dans les boues, les sols, et les biodéchets – Partie 1 : Méthode par filtration sur membrane permettant la resuscitation quantitative des bactéries stressées de manière sub-léthale (pour confirmer l'efficacité de l'abattement de 6 logs lors des procédés de traitement)

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Foreword

This document has been prepared in the framework of the project Horizontal.

This document is a working document.

This standard is divided into three parts:

— *part 1 describes a membrane filtration method*

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

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

This standard is applicable and validated for several types of matrices. The table below indicates which ones.

Material	Validated	Document
Soil	Not validated yet	
Sludge	Not validated yet	
Biowaste	Not validated yet	
Soil improvers	Not validated yet	

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 *Escherichia coli* and *Salmonella* spp. in sludges, soils, and biowastes" and aims at evaluation of the latest developments in assessing *Salmonella* spp. in sludge, soil, treated biowaste and neighbouring fields. 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 modular horizontal method and has been validated within in the project 'Horizontal'.

Sludges, soils, 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.

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 8199:2005, 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 13 are normative; annexes are normative or informative, as stated in the top lines of the annexes

1 Scope

This part of the European Standard describes 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, 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 The objective is to cover untreated and treated sludges, soils, 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 3 cfu/g wet weight sample, 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 1 *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 2 This method is not suitable for treated sludges containing less than 1 viable *Salmonella* spp. per 1 g wet weight.

NOTE 3 This method is not suitable for untreated sludges containing low levels of *Salmonella.*

NOTE 4 This method is only suitable for materials with dry residues less than 20 %.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

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

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

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

3 Definitions

For the purposes of this European Standard, 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

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 sample obtained after the specified drying process. It is expressed as percent or in grams per kilogram [EN 12880:2000, 3.1].

4 Symbols and Abbreviations

DS – Dry Solid

MTSB – Modified Tryptone Soya Broth

PBS – Phosphate Buffered Saline

PPE – Personal Protective Equipment

5 Principle

The homogenised diluted 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 the more fastidious *S.* Dublin) and positive colonies are quantified. The presence of *Salmonella* spp. is indicated by presumptive 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 the 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.

6 Verifications of interference

7 Reagents, diluents and culture media

7.1 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 fit for purpose demineralised or distilled water free from substances capable of inhibiting growth under the test conditions (ISO 8199:2005). If the media are not used immediately, preserve them in the dark at (5 ± 3) °C for a maximum of one month in conditions avoiding any alterations in their composition.

NOTE The use of chemicals of other grades is permissible provided that they are shown to be of equivalent performance in the test.

7.2 Modified tryptone soya broth (MTSB)

Tryptone Soya Broth Base (Oxoid CM 129, Basingstoke, UK, or equivalent supplier):

Pancreatic digest of casein (tryptone)	17 g
Papaic digest of soya bean meal (soya peptone)	3 g
Dextrose	2.5 g
Sodium chloride	5 g
K_2HPO_4	2.5 g
Demineralised or distilled water,	to 1 000 mL

The final pH should be adjusted to 7.2 ± 0.2 if required using 0.1 mol/L sodium hydroxide or hydrochloric acid.

Make up following manufacturer's instructions. Add supplements as follows:

Bile salts No 3 (proprietary)	1.5 g

K_2HPO_4	1.5 g

Sterilise in the autoclave (8.1) at (121 ± 3) °C for (15 ± 1) min. Store at (5 ± 3) °C for a maximum of one month. Add filtered (0.2 µm, 8.5) sterile novobiocin solution (to achieve a final concentration of 40 mg/L), immediately before use.

7.3 Demineralised or distilled water

Free from substances inhibiting growth under the test conditions. (ISO 8199:2005)

Sterilise in the autoclave (8.1) at (121 ± 3) °C for (15 ± 1) min unless sterilised in the preparation process.

7.4 Phosphate buffered saline (PBS) (pH7 ± 0.2) – commercially available

Sodium chloride	8 g
Potassium chloride	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Demineralised or distilled water	to 1 000 mL

Sterilise in the autoclave (8.1) at (121 ± 3) °C for (15 ± 1) min. Store for a maximum of one month.

7.5 Resuscitation medium: Tetrathionate broth

Tetrathionate Broth Base (Oxoid CM 29, or equivalent supplier):

'Lab-Lemco' powder	0.9 g
Peptone	4.5 g
Yeast extract	1.8 g
Sodium chloride	4.5 g
Calcium carbonate	25 g
Sodium thiosulphate pentahydrate	40.7g
Demineralised or distilled water,	to 1 000 mL

Make up following manufacturer's instructions and adjust pH to 8.0 ± 0.2, heat to 100 °C, then allow to cool slowly to (45 ± 2) °C. Store at (5 ± 3) °C for a maximum of one month. Just before use add 20 mL of iodine-iodide solution:

Iodine	6 g
Potassium iodide	5 g
Demineralised or distilled water	20 mL

Add freshly prepared, filtered (0.2 µm) (8.5) sterile novobiocin solution (to achieve a final concentration of 40 mg/L), immediately before use.

7.6 Chromogenic medium: Rambach® agar or equivalent

NOTE: Rambach® agar may cause irritation to skin and breathing problems if inhaled, as a precaution it is recommended that a flow cabinet is used whilst working with this medium.

Opaque agar (proprietary product)	15 g
Propylene glycol	10.5 g
Peptone/Yeast extract	8 g
Chromogenic + selective supplements (Proprietary product, or equivalent)	1.5 g
Sodium deoxycholate	1,0 g
Sodium chloride	5,0 g
Demineralised or distilled water	to 1 000 mL

Make up following manufacturer's instructions at 100 °C, allow cooling to (45 ± 2) °C and pour into sterile Petri dishes. Store at (5 ± 3) °C for a maximum of one month.

7.7 C_8 esterase confirmation solution

Dissolve 4-methylumbelliferyl caprylate in absolute ethanol (1 mg/mL). Store in the dark at (5 ± 3) °C for a maximum of six months.

7.8 Peptone saline solution

Bacteriological peptone (Oxoid L37 or equivalent)	1 g
Sodium chloride	8.5 g
Demineralised or distilled water	to 1000 mL

Make up following manufacturer's instructions. Sterilise in the autoclave (8.1) at (121 ± 3) °C for 15 ± 1 min. Store at (5 ± 3) ^{o}C for a maximum of 3 months.

8 Apparatus

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

It should be ensured that microbiological laboratory equipment of proven performance and suitability for the method should be used. Where ever possible the product specified should be used, if this is not possible a product of proven equivalent performance must be used.

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

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

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

8.4 Centrifuge capable of centrifuging 50 mL at 200*g* to 300*g* for 3 mins.

8.5 Membrane filters (0.45 μm gridded, cellulose nitrate, 47 mm diameter or equivalent).

8.6 Disposable filter units

8.7 Glass fibre pre-filter discs (47 mm diameter) (e.g. Whatman GF/D pore size 2.7 μm or equivalent).

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

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

8.10 Stereo microscope fitted with × 10 eyepieces; use × 6 magnification).

8.11 Cold light source fitted with a Schott KG 1.45 × 45 filter (blue) or equivalent. Illuminate membrane filters with dual fibre-optic light guides**.**

8.12 UV observation lamp or chamber (366 nm).

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

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

WARNING — Avoid inhalation and ignition

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

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

8.16 Sterile bottles of 100 mL volume, or flasks with similar capacity**.**

8.17 Automatic pipettes, capable of dispensing 0.1 mL to 1.0 mL and 1.0 mL to 5.0 mL volumes**.**

8.18 Sterile graduated pipettes, glass or disposable plastic ware, capable of dispensing 10 mL volumes.

8.19 Sterile tips for automatic pipettes**.**

8.20 Sterile conical centrifuge tubes, 50 mL volume, disposable plastic.

8.21 Vortex Mixer, Heidolph REAX top 100-2400 tr/min or equivalent.

8.22 Scale, Ohaus precision standard balance or equivalent

8.23 Refrigerator, capable of maintaining 5 ± 3°C

8.24 PH Meter, Jenway pH meter 3320 or equivalent

8.25 Laboratory spatula

8.26 Tweezers, capable of sterilisation by immersion in ethanol and subsequent flaming**.**

8.27 Beakers and containers, 250mL and 1000mL

8.28 Nalgene filter holder with receiver Cat no: 300-4000 or equivalent

8.29 Boiling Bath – Clifton or equivalent

8.30 Sterile forceps

8.31 Bunsen Burner

9 Sampling

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 hours). In order to prevent propagation or inactivation of *Salmonella* during transport to the laboratory and subsequent storage, the necessary precautions depending upon the matrix shall be taken.

NOTE Generally chilling the sample to (5 ± 3) °C is recommended.

9.1 General

Samples are liable to ferment 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 bio-hazardous samples are followed.

See also the Warning note in the introduction.

9.2 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.

9.3 Handling

Cleanliness when working is essential. When handling sludge samples, it is necessary to wear gloves, a face and eye protection, and ensure adequate protection against bottles bursting. The gas evolved is flammable.

See also the Warning note in the introduction.

10 Procedure

10.1 Sample preparation

— Weigh a representative 25 g (wet weight) of the as received sample into a 250 mL container (8.27).

— Add an appropriate volume of PBS at pH 7.0 (7.4) so as to obtain a final weight of 250g and mix thoroughly using a vortex mixer (8.21).

— Perform a pH test on the sample in a separate aliquot using a pH meter (8.24). 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 during the neutralisation process, start a new analysis with a fresh test portion.

NOTE 2 For other relevant sludge treatment chemicals (e.g. peracetic acid), a suitable oxidant neutralisation procedure must be used (see for example EN 1040:1997).

— Transfer the contents of the container into a homogeniser bag (8.14) place in a homogeniser (8.3) and homogenise for 2 min.

— Transfer the homogeniser bag contents to 5 disposable centrifuge tubes (8.20) and centrifuge (8.4) the 5 50 mL aliquots at 200*g* to 300*g* for 3 min.

— Decant the supernatant from the tubes into a beaker and filter through a glass-fibre pre-filter (8.7) using a Nalgene holder with receiver (8.28) to remove fine debris.

The Nalgene filter holder should have been sterilised in a boiling bath (8.29) prior to analysis, the filters are removed from the boiling bath using sterile forceps (8.30) and attached to the vacuum pump (8.8).

The glass fibre pre-filter (8.7) is placed on the Nalgene filter holder using sterile tweezers (8.26) before the Nalgene filter cup is secured in position. The vacuum may now be used to draw the sample through the filter; it is recommended that the sample is not all introduced to the filter cup at the same time because blockages may occur.

Once pre-filtration has finished the filtrate should be transferred to a sterile 250 mL container, the Nalgene filters should be returned to the boiling bath and be sterilised for a minimum of 5 minutes before being used again.

NOTE 3 For samples with <20% (m/V) DS the above procedure has been shown not to significantly reduce the recovery efficiency of *Salmonellae.*

10.2 Sample dilution

The number of dilutions to subsequently filter varies according to the presumed level of *Salmonella* contamination of the sample to be tested. Typically, dilution A (the filtrate) should be serially diluted 10^{-1} to 10^{-3} with MTSB. This will permit the enumeration of up to 10^5 *Salmonellae* per gram wet weight sample. Higher *Salmonella* contamination levels will require additional dilutions of the filtrate of 10^{-4} to 10^{-5}, (for example, if spiking studies are undertaken with 10^7 or 10^8 *S.* Senftenberg W775 per gram to demonstrate the efficacy of a treatment procedure for example giving a 6 Log_{10} decrease).

Some spiking studies may require even further dilutions down to 10^{-6} and 10^{-7} respectively.

Prepare the relevant number of sterile container according to the number of selected dilutions; add 90 mL of MTSB (7.1) to each container.

Using a sterile pipette (8.18); transfer 10 mL of the filtrate to the first container containing 90 mL of MTSB and mix thoroughly using a vortex mixer (8.21).

Using a fresh pipette; transfer 10 mL of the diluted filtrate to the second container containing 90 mL of MTSB and mix thoroughly using a vortex mixer (8.21).

Continue as above until all the dilutions have been prepared.

10.3 Membrane filtration

10.3.1 Before the filtration step, prepare glass fibre filters saturated with Tetrathionate broth (7.5) in Petri dishes (8.15); for each filtered sample; place on a Petri dish a glass fibre pre-filter (8.7) and add 2 ± 0.5 mL of Tetrathionate broth (7.5).

10.3.2 Transfer the filter base (8.9) from the boiling water bath (8.29) to the manifold using sterilised forceps (8.30). Take care not to contaminate its surface by touch or clothing.

10.3.3 Membranes are removed from their packets with sterilised tweezers (8.26) and placed grid side up onto the filter base. Membranes that have torn are dropped or which touch any object must be discarded. Membranes should only be handled by the edge with tweezers designed for that purpose. The tweezers must be sterilised first heating in a Bunsen burner (8.31), and then dipping directly into boiling water.

10.3.4 The filter cup is then removed from the water bath and attached to the base taking care not to wrinkle the membrane. Filter funnels should only be removed from the water bath with disinfected forceps and placed directly onto bases. Hands may be used to transfer funnels back to the water bath. Neither forceps nor tweezers should be placed directly onto the bench. When not in use they should be placed on a specified rack to prevent contamination. If the filtration equipment is left for any significant length of time, the bases must be returned to the water bath. Bases left unused for short periods can be covered with the base or lid of a sterile Petri dish until filtration recommences.

10.3.5 Add a sufficient amount of peptone saline solution (7.8) (15 ± 5 mL) into the filter cup; pipette 10mL of the diluted sample into the filter cup. Replace the top on the sample container. Place the used container back into the rack. The container must not be placed on the filtration bench.

10.3.6 The sample may now be drawn through the filter by vacuum and only when filtration is complete should the vacuum be turned off. The magnetic filter cup is lifted off, and returned to the boiling water bath.

10.3.7 The membrane is carefully removed using sterile tweezers and transferred to the Tetrathionate broth 50mm Petri dish (7.5). The membrane should be 'rolled' into the plate to prevent air bubbles becoming trapped between the resuscitation medium and the membrane, and the lid of the Petri dish is replaced. Air bubbles will prevent growth medium getting to the surface of the membrane and thus restrict the resuscitation of any isolated bacteria.

10.3.8 Any wrinkled or torn membranes discovered after filtration must be discarded. The filter base must then be re-sterilised and the dilution filtered again.

10.3.9 Once filtration of samples is complete, the filter funnels are placed in to the boiling water bath for disinfection. They must be totally immersed in boiling water for at least two minutes before being removed to continue filtration.

10.3.10 Before filter the first dilution (10^{-7}) add a negative control; i.e. 10 mL MTSB. After the last dilution (10^{0}), add a negative control (10 mL MTSB) and a positive control (For example: 10 mL suspension of *Salmonella* Enteritidis NCTC 6676). Other strains are permitted for use.

10.4 Resuscitation and enumeration of colonies on chromogenic agar

Incubate at (36 ± 2) °C for (21 ± 3) h. in accordance with ISO 8199 (2005). Plates must be incubated in an open container in stacks no greater than six plates high.

Remove the filters from the Tetrathionate broth-soaked filter pads using sterile tweezers and transfer to the surface of a 50 mm diameter Rambach® agar (7.5) plate. Incubate plates at (36 ± 2) °C for 24 h and 48 h, ensuring the plates are again incubated in an open container and plates are stacked no greater than six plates high.

Enumerate positive typical pink colonies using a stereo microscope (8.10) and Cold light source (8.11). *Salmonellae* such as *Salmonella* Enteritidis, *S*. Typhimurium and *S.* Senftenberg can be detected reliably at 24 h. However, *Salmonella* Dublin shall be enumerated at 48 h.

NOTE *Salmonella* Enteritidis, Typhimurium, Senftenberg and Dublin are also described as *Salmonella enterica*, serovars, Enteritidis, Typhimurium, Senftenberg and Dublin.

10.5 Confirmation of colony identity

The typical presumptive positives colonies are confirmed at 48h by spraying the plates with C8 esterase confirmation solution (7.7) containing 1 mg/mL 4-methylumbelliferyl caprylate dissolved in absolute ethanol. Prior to enumeration under UV light at 366 nm (8.12) open the plates and dry them in the incubator at (36 ± 2) °C for 5 ± 1min to allow excess C8 esterase solution to evaporate off. Fluorescent colonies are confirmation of *Salmonellae*. The confirmation rate of the colonies must be taken into account when calculating the final result according to ISO 8199 (2005).

NOTE When using a chemical aerosol spray precautions should be taken to avoid inhalation and contact with the skin. Please ensure the confirmation is carried out in a flow cabinet and the analyst is wearing all the necessary personal protective equipment (PPE).

10.6 Determination of the dry residue content

The numbers of *Salmonellae* can 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.

11 Expression of results

Calculation of the number of *Salmonellae* (present per g wet weight of the original sample) is by multiplying the number of fluorescent colonies on the filter by the overall dilution factor. Plates should be selected in the range of between 10 and 100 typical colonies. If quantification of colonies within the range is not possible, counts outside the range 10-100 are considered for the expression of results.

Numbers present per g wet weight are calculated according to:

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

where

c is the original wet weight concentration of *Salmonellae* no. per g;

n is the total number of typical *Salmonella* colonies on the selected membranes

v is the total volume filtered through the selected membrane (from 10 ml);

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 10 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 10 x 0.01) + (0.1 x 10 x 0.001)

$c = 96 / 0.011$ = 8.7 10^3 cfu/g ww

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

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

e = the dry residue (%) of the original wet sample.

12 Performance data

Information concerning the repeatability and reproducibility of the procedure, obtained using interlaboratory tests, is given in Annex A (informative).

This annex A will be completed by the results of the validation study (European scale Interlaboratory trial) that will be performed during the FP6 EU Horizontal-Hyg project.

See Annex A.

13 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)

Performance data of the interlaboratory comparison

Full validation data with a minimum of eight laboratories in at least three different countries will be produced once the proposed validation trials have been agreed, financed and carried out

Performance data for quantification of viable *Salmonella* spp. in untreated and pasteurised sewage sludge (to demonstrate $\log_{10}$ 4 or $\log_{10}$ 6 reduction efficiencies).

Raw sludge samples were spiked with the *Salmonellae* by an independent laboratory, some were treated by pasteurisation for 30 s at 70 °C, and all replicates were sent as randomised, anonymous samples to another independent laboratory. Data were reported back to the original independent laboratory for decoding as colony forming units per gram wet sludge.

The immunological and esterase confirmatory tests on random colonies picked from the membranes on the chromogenic agar medium confirmed that there was no evidence of false positives in either the pre- or post-treatment samples. (10 % of colonies were tested.)

Table A.1 — *Salmonella* Senftenberg

Treatment	Dilution	Rep 1 (n=3) cfu/g	Rep 2 (n=3) cfu/g	Mean cfu/g
Pre-	10^4	$1{,}42 \times 10^6$	$1{,}18 \times 10^6$	$1{,}30 \times 10^6$
	10^5	$1{,}25 \times 10^6$	$1{,}60 \times 10^6$	$1{,}43 \times 10^6$
Post-	Undiluted	< 1	< 1	< 1
	Undiluted	< 1	< 1	< 1
Inocula mean		$1{,}34 \times 10^6$	$1{,}39 \times 10^6$	$1{,}37 \times 10^6$

Table A.2 — *Salmonella* Typhimurium

Treatment	Dilution	Rep 1 (n=3) cfu/g	Rep 2 (n=3) cfu/g	Mean cfu/g
Pre-	10^3	$2{,}65 \times 10^4$	$2{,}90 \times 10^4$	$2{,}78 \times 10^4$
	10^4	$3{,}00 \times 10^4$	$2{,}50 \times 10^4$	$2{,}75 \times 10^4$
Post-	Undiluted	< 1	< 1	< 1
	Undiluted	< 1	< 1	< 1
Inocula mean		$2{,}83 \times 10^4$	$2{,}70 \times 10^4$	$2{,}77 \times 10^4$

Table A.3 — *Salmonella* Enteritidis

Treatment	Dilution	Rep 1 (n=3) cfu/g	Rep 2 (n=3) cfu/g	Mean cfu/g
Pre-	10^5	$4{,}00 \times 10^6$	$5{,}30 \times 10^6$	$4{,}65 \times 10^6$
	10^6	$1{,}00 \times 10^7$	$6{,}00 \times 10^6$	$8{,}00 \times 10^6$
Post-	Undiluted	< 1	< 1	< 1
	Undiluted	< 1	< 1	< 1
Inocula mean		$7{,}00 \times 10^6$	$5{,}65 \times 10^6$	$6{,}33 \times 10^6$

Errors between replicates were generally less than 15 %, except for highly diluted samples of sludge with a high concentration of *S.* Enteritidis.

NOTE Zero data indicate below level of detection (i.e. < 1 cfu/ g wet weight).

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