Soils, sludges and treated bio-wastes — Detection and enumeration of *Salmonella* spp. in sludges, soils and organic fertilisers of similar consistency to the matrices validated – Part 1 : Membrane filtration method for quantitative resuscitation of sub-lethally stressed bacteria (to confirm efficacy of log reduction treatment procedures)

Böden, Schlamm und behandelte Bio-abfälle – Nachweis von Salmonella spp. aus Schlämmen, Böden, Düngemitteln und Bodenverbesserern, Kultursubstraten 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 fertilisants organiques de consistance similaire aux matrices validées – 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 6 log 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.

The following TC's have been involved in the preparation of the standard: TC 308.

This standard is divided into three parts:

— part 1 describes a membrane filtration method for quantification,

— part 2 describes a semi-quantitative MPN method by liquid enrichment,

— part 3 describes 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
Mesophilic anaerobic digested sewage sludge		
Anaerobic treated biowaste		
Pelletised air dried sludge		
Digested sewage sludge presscake		
Composted sewage sludge		
Composted green waste		
Composted biowaste		

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, soil improvers, growing media and biowastes" and aims at evaluation of the latest developments in assessing *Salmonella* spp. in sludge, soil and organic fertilisers. 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 the project "Horizontal".

Sludges, soils and organic fertilisers 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 contaminated materials in agriculture can cause outbreaks of infection due to the production of contaminated food and animal feedstocks. They 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 12 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 of *Salmonella* spp., including potentially sub-lethally damaged *Salmonella* spp. in sewage sludges by culture of individual colonies on chromogenic agar media. It may be suitable for other sludges, soils and organic fertilisers of similar consistency to the matrices validated.

The method is suited to determine the efficiency of treatment process (CEN/TC 308 – doc 525 (Revision of Directive 86/278/EEC -3rd Draft [1]) and Regulation (EC) No 208/2006 [2]) for the elimination of pathogens in treated substrates. The treatment processes are validated through to a defined Log reduction with a test organism such as *Salmonella* Senftenberg W775.

NOTE ***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.**

The method has a limit of detection of approximately 3 *Salmonella* spp. cfu/g wet weight sample [ENV ISO 13843:2001], dependent on the solids content which at high concentrations (> 20 % (w/v)) may restrict filtration of the sample volume through the membrane if not first diluted.

2 Normative references

These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, only the edition cited applies. For undated references the latest edition of the publication referred to applies (including any 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*.

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

ISO 5725:1994, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*.

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*, 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)℃ followed by fermentation of propylene glycol and acid production on Rambach® agar [3] at (36 ± 2)℃. 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)℃

NOTE Some *Salmonella* (e.g. *S.* Typhi and *S.* Paratyphi) will not be detected as colonies appear colourless due to their inability to ferment propylene glycol.

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 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
quantitative resuscitation
recovery to vegetative growth of sub-lethally damaged bacteria isolated 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]

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 Reagents, diluents and culture media

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.

6.1 Phosphate buffered saline (PBS) (pH 7 ± 0.2)

Sodium chloride	8 g
Potassium chloride	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water	1 000 mL

Dissolve the ingredients under stirring. If necessary, adjust the pH of the solution to (7 ± 0.2) using hydrochloric acid (1 mol/L) or sodium hydroxide solution (1 mol/L). Sterilise in the autoclave (7.1) at (121 ± 3)°C for (15 ± 1) min. Store for a maximum of one month.

6.2 Modified tryptone soya broth (MTSB)

6.2.1 Novobiocin supplement

Novobiocin	1 g
Distilled water	10 mL

Aseptically, dissolve 1 g Novobiocin in 10 mL sterile distilled water and filter through membrane filter (pore size 0.2 µm, (7.5)). The solution should be stored at (-14 ± 2)°C for a maximum of 1 month.

6.2.2 Modified tryptone soya broth complete medium

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
Distilled water	1 000 mL

Dissolve the ingredients under stirring and adjust pH to (7.2 ± 0.2) if required using 0.1 mol/L sodium hydroxide or hydrochloric acid.

Add supplements as follows:

Bile salts N° 3 (proprietary)	1.5 g
K_2HPO_4	1.5 g

Sterilise in the autoclave (7.1) at (121 ± 3)°C for (15 ± 1) min. Store at (5 ± 3)°C for a maximum of one month. Add filtered sterile novobiocin solution (6.2.1) to achieve a final concentration of 40 mg/L, immediately before use.

6.3 Resuscitation medium: Tetrathionate broth

'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	40.7 g
Distilled water	1 000 mL

Dissolve the ingredients under stirring 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 water	20 mL

Add freshly prepared, filtered sterile novobiocin solution (6.2.1) (to achieve a final concentration of 40 mg/L), immediately before use.

6.4 Chromogenic medium: Rambach® agar

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 water	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.

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.

6.5 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.

6.6 Peptone saline solution

Enzymatic digest of casein (peptone)	1 g
Sodium chloride	8.5 g
Distilled water	1000 mL

Dissolve the ingredient under stirring and adjust the pH to (7 ± 0.2). Sterilise in the autoclave (7.1) at (121 ± 3)°C for (15 ± 1) min. Store at (5 ± 3)°C for a maximum of 3 months.

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 - steam (autoclave)

7.2 Thermostatic incubator regulated at (36 ± 2)°C

7.3 Homogeniser

7.4 Centrifuge capable of centrifuging 50 mL at 200*g* to 300*g* for 3 min

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

7.6 Disposable filter units

7.7 Glass fibre pre-filter discs - 47 mm diameter, pore size 2.7 µm

7.8 Vacuum pump

7.9 Vacuum manifold

7.10 Stereo microscope fitted with × 10 eyepieces; use × 6 magnification

7.11 Cold light source - Illuminate membrane filters with dual fibre-optic light guides

7.12 UV observation lamp or chamber (366 nm)

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

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

WARNING — Avoid inhalation and ignition

7.14 Sterile homogeniser bags - 250 mL volume, with or without integrated mesh to exclude large particulate matter

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

7.16 Sterile bottles of 100 mL volume, or flasks with similar capacity

7.17 Automatic pipettes, capable of dispensing 0.1 mL to 1.0 mL and 1.0 mL to 5.0 mL volumes

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

7.19 Sterile tips for automatic pipettes

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

7.21 Vortex mixer

7.22 Analytical balance

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

7.24 pH meter

7.25 Laboratory spatula

7.26 Tweezers

7.27 Beakers and containers, 250 mL and 1000 mL

7.28 Filter funnels

7.29 Boiling bath

7.30 Sterile forceps

7.31 Bunsen burner

8 Sampling and hazards

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 h). In order to prevent propagation or inactivation of *Salmonella* 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 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 biohazardous samples are followed.

See also the Warning note in the Introduction.

8.2 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 no more than 72 h after receipt.

8.3 Handling

Cleanliness when working 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 is usually flammable, so all equipment in the vicinity shall be flame proof to avoid any source of ignition.

See also the Warning note in the introduction.

9 Procedure

9.1 Sample preparation

Weigh a representative 25 g (wet weight) of the as received sample into a 250 mL container (7.27).

Add an appropriate volume of PBS at pH 7.0 (6.1) so as to obtain a final weight of 250 g and mix thoroughly using a vortex mixer (7.21).

Perform a pH test on the sample in a separate aliquot using a pH meter (7.24).

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 [4]).**

NOTE: Centrifugation and Pre-filtration (Annex B - informative)

The variation in the level of solid material contained within the matrices applicable to this standard means that some samples will require centrifugation and pre-filtration before they can be processed by membrane filtration without blocking the membrane. Not all samples will require these optional steps, if it necessary then centrifugation and pre-filtration should be applied.

Transfer the contents of the container into a homogeniser bag (7.14) place in a homogeniser (7.3) and homogenise for 2 min.

Centrifugation

Transfer the homogeniser bag contents to 5 disposable centrifuge tubes (7.20) and centrifuge (7.4) the 5 50 mL aliquots at 200 g to 300 g for 3 min.

Pre-filtration

Decant the supernatant from the tubes into a beaker and filter through a glass-fibre pre-filter (7.7) using a filter funnel with receiver (7.28) to remove fine debris.

The filter funnels are sterilised in a boiling bath (7.29) prior to analysis, the filter funnels are removed from the boiling bath using sterile forceps (7.30) and attached to the vacuum pump (7.8).

The glass fibre pre-filter (7.7) is placed on the filter funnel using sterile tweezers (7.26) before the filter funnel 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 filter funnels 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 (Annex B - informative).*

9.2 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}.

NOTE Some spiking studies are undertaken with 10^7 or 10^8 cfu *S.* Senftenberg W775 per gram to demonstrate the efficacy of a treatment procedure for example giving a 6 Log decrease. They may require even further dilutions down to 10^{-6} and 10^{-7} [1].

Prepare the relevant number of sterile containers according to the number of selected dilutions; add 90 mL of MTSB (6.2) to each container.

Using a sterile pipette (7.18); transfer 10 mL of the filtrate into 90 mL of MTSB and mix thoroughly using a vortex mixer (7.21).

Using a fresh pipette; transfer 10 mL of the diluted filtrate into 90 mL of MTSB and mix thoroughly using a vortex mixer (7.21).

Continue as above until all the dilutions have been prepared.

9.3 Membrane filtration

Before the filtration step, prepare glass fibre filters saturated with Tetrathionate broth (6.5) in Petri dishes (7.15); for each filtered sample; place on a Petri dish a glass fibre pre-filter (7.7) and add (2 ± 0.5) mL of Tetrathionate broth (6.5).

Transfer the filter base (7.9) from the boiling water bath (7.29) to the manifold using sterilised forceps (7.30).

Membranes are removed from their packets with sterilised tweezers (7.26) 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 immersing in ethanol and then flamed off in a Bunsen burner (7.31).

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. 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.

Add a sufficient amount of peptone saline solution (6.6) (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.

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.

The membrane is carefully removed using sterile tweezers and transferred to the Tetrathionate broth 55 mm Petri dish (7.5). The membrane should be 'rolled' into the plate to prevent air bubbles becoming trapped between the growth medium and the membrane, and the lid of the Petri dish is replaced. Bubbles should be excluded so that the membranes are in intimate contact with the agar surface allowing unrestricted growth of viable bacteria present on the membrane surface.

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

Once filtration of samples is complete, the filter funnels are placed in 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.

Before filtering the first dilution (10^{-7}) filter a negative control; i.e. 10 mL MTSB. After the last dilution (10^{0}), filter a negative control (10 mL MTSB) and a positive control (i.e. 10 mL suspension of *Salmonella spp.* containing about 10^{2} cfu/ml target organisms).

9.4 Resuscitation and enumeration of colonies on chromogenic agar

Incubate at (36 ± 2)℃ for (21 ± 3) h in accordance with ISO 8199:2005. Plates must be incubated in an open container.

Remove the filters from the Tetrathionate broth-soaked filter pads using sterile tweezers and transfer to the surface of a 55 mm diameter Rambach® agar (6.4) plate. Incubate plates at (36 ± 2)℃ for 24 h and 48 h.

Enumerate typical pink colonies using a stereo microscope (7.10) and cold light source (7.11). *Salmonellae* such as *Salmonella* Enteritidis, *S.* Typhimurium and *S.* Senftenberg can be detected reliably at 24 h. However, 48 hours maybe required to enumerate more fastidious strains such as *Salmonella* Dublin.

Only plates within the range 10-100 colonies should be considered for the expression of results. If no counts are in this range it may be appropriate to consider counts outside this range provided that an accurate enumeration is possible.

9.5 Confirmation of colony identity

The typical pink 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 let them dry to allow excess C8 esterase solution to evaporate off. Fluorescent and typical pink colonies are confirmation of *Salmonellae*.

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 adhering to good Health & Safety practice with regard to personal protective equipment (PPE).**

9.6 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.

10 Expression of results

Calculation of the total number of *Salmonellae* (present per g wet weight of the original sample) is by multiplying the total number of typical pink and fluorescent colonies on the filter membrane of selected plates (9.4) by the overall dilution factor.

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 *Salmonella* colonies on the filter membranes : $n = n1 + n2 + \ldots$

v is the total volume filtered through the selected filter membrane (from 10 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 10 mL of dilution A and the following counts are obtained at the respective dilutions, there are two counts for the sample in the 10-100 range:

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

Then:

n= number of colonies (81+15 = 96)

v= dilution factors (0.1 x 10 x 0.01) + (0.1 x 10 x 0.001)

$c = 96 / 0.011$ = 8.7 10^3 *Salmonella* concentration per g wet weight of original sample (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.

11 Performance data

Performance data in terms of repeatability and reproducibility of the procedure, obtained by interlaboratory tests at the European scale in the frame of the validation study of the FP6 EU Horizontal-Hyg project 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)

Performance data of the interlaboratory comparison

A.1 Objective of the interlaboratory comparison

In a European wide laboratory comparison study according to ISO 5725-2, the performance characteristics of the standard “Detection and enumeration of *Salmonella* spp. in sludges, soils and treated biowaste. Part1: Membrane filtration method for quantification” were established.

A.2 Material used in the interlaboratory comparison study

The interlaboratory comparison of the membrane filtration method for quantification of *Salmonella* spp. in soil, sludge and treated biowaste took place from May to July 2007. It was carried out with 14 European laboratories on 7 different matrices. The matrices selected for the interlaboratory comparison were chosen to represent soil, sludge and biowaste as broad as possible, because the standard will find general application across different types of soil and soil related materials (detailed information can be found in the final report on the interlaboratory comparison study [5]).

Table A.2-1 provides a list of the type of matrices chosen for *Salmonella* spp. detection.

Table A.2-1 Matrices types tested in the interlaboratory comparison trial

Matrice type	Short name
Mesophilic anaerobic digested sewage sludge	MAD
Anaerobic treated biowaste	ATB
Pelletised air-dried sludge	PADS
Digested sewage sludge presscake	DSSP
Composted sewage sludge	CSS
Composted green waste	CGW
Composted biowaste	CBW

In the interlaboratory comparison study the following starting points were used:

The laboratory samples were all taken from a large batch of the different matrices according to the normal practice. The choice was made to analysed only spiked samples so as to obtain positive results. The spiking, the mixing and the sub-sampling were carried out as needed to prepare representative laboratory samples of approximately 150 g from the large batch sample. These were sent out by courier to each of the participating laboratories.

The experimental plan designed by project Horizontal-Hygiene on the basis of each laboratory being given 3 laboratory samples of each of the 2 batches of the seven matrices to be tested.

A.3 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.3-1 — Summary of components of the *Salmonella* spp. Membrane filtration method precision

Limit of detection (5%) *Salmonella* spp. /g wet weight	**Upper limit of quantification (5%)** *Salmonella* spp. /g wet weight	**Range of quantification** Log10 unit	**Results of dispersion U^2**
2.70	$1.32\ 10^{10}$	9.68	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).

A.4 Interlaboratory comparison results

The statistical evaluation was conducted according to ISO 5725-2. The average values, the repeatability (r) and the reproducibility (R) were obtained (Table A.4-1).

The repeatability corresponds to the maximum difference that can be expected (with a 95% statistical confidence) between one test result and another, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present standard by the same laboratory using its own facilities and testing laboratories samples obtained from the same primary field sample and prepared under identical procedures.

The repeatability limit was calculated using the relationship: $r_{test} = f.\sqrt{2}.s_{r.test}$ with the critical range factor f = 2.

NOTE The above relationship refers to the difference that may be found between two measurement results performed each on two laboratory samples obtained under the same conditions. The value f = 2 used in the factor $f.\sqrt{2}$ corresponds to the theoretical factor of 1.96 for a pure normal distribution at 95% statistical confidence. Also, this value f = 2 corresponds to the usual value k = 2 of the coverage factor recommended in the Guide to the expression of Uncertainty in Measurement (GUM). However, it may be necessary to a use a larger value for f in situations as described clause 12.

The reproducibility, like repeatability corresponds to the maximum difference that can be expected (with a 95% statistical confidence) between one test result and another test result obtained by another laboratory, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present standard by two different laboratories using their own facilities and testing laboratory samples obtained from the same primary field sample and prepared under identical procedures.

This reproducibility limit was calculated using the relationship: $R = f.\sqrt{2}.s_R$ with the critical range factor f = 2.

NOTE The above relationship refers to the difference that may be found between two measurement results performed each on two laboratory samples obtained under the same conditions. The value f = 2 used in the factor $f.\sqrt{2}$ corresponds to the theoretical factor of 1.96 for a pure normal distribution at 95% statistical confidence. Also, this value f = 2 corresponds to the usual value k = 2 of the coverage factor recommended in the Guide to the expression of Uncertainty in Measurement (GUM). In the case when reference is made to the dispersion of the values that could reasonably be attributed to the parameter being measured, the dispersion limit is equal to $k.s_R$ with the usual value k = 2, resulting in a dispersion limit lower than the reproducibility limit (i.e. a ration of $\sqrt{2}$). However it may be necessary to use a larger value $f.\sqrt{2}$ (or k) in situation as described in clause 9.

In case of relatively heterogeneous materials, the repeatability and the reproducibility limits may be larger than the values given in Table A.4-1 (this means that the value chosen for the critical range factor f is larger than 2 as well as for the coverage factor k for dispersion). This is because the extreme results may have been obtained in accordance with the present standard and/or be caused by the variability within, or in between, the laboratory samples.

For the calculations, as the test results were expressed on a log scale, the standard deviations in repeatability and Reproducibility conditions, respectively s_r and s_R were also expressed on a log scale. The expression of repeatability and Reproducibility in terms of maximum difference that can be expected between one test and another is given then by the limit of repeatability and Reproducibility respectively $r = 2\sqrt{2}.s_r$ and $R = 2\sqrt{2}.s_R$.

In order to make easier the interpretation, the values of repeatability and reproducibility are expressed in terms of maximal difference between two independent measurements on log scale, with a confidence level of 95%:

Example

Assuming r_1 and r_2 two independent measurements observed for a given method in repeatability conditions with $r_1 > r_2$:

$\log(r_1) - \log(r_2) \leq 0.9$ (95% of the cases), corresponding to almost 1 log of difference between results

The deviations between test results obtained under repeatability and reproducibility conditions can also be expressed by the maximal ratio between two independent measurements on natural scale (number of germs), with a confidence level of 95%

Example

$\log(r_1) - \log(r_2) \leq 0.9$ (95% of the cases)

then $r_1/r_2 \leq 10^{0.9}$

then $r_1/r_2 \leq 7.9$

thus *r_1 is significantly higher than r_2 if $r_1/r_2 > 7.9$.*

Table A.4-1 — Summary of *Salmonella* spp. Membrane filtration method results of inter-laboratory comparison

Matrix	Overall mean (*Salmonella* spp. /g wet weight)	Repeatability (ratio)	Reproducibility (ratio)	Discarded outliers (statistical fitness)	Removed data (other reasons)	Total number of data	Total number of labs
Mesophilic anaerobic digested sewage sludge	< 14[2]	5.7[2]	103.3[2]	-	-	12	11
	18	16.1	263.9	-	-	24	12
Anaerobic treated biowaste	390886	5.1	9.2	2	-	33	13
	259263	1.9	111.0	-	-	36	12
Pelletised air dried sludge	< 2.70[1]	-	-	-	-	15	14
	191740	5.2	7121.3	-	-	39	13
Digested sewage sludge presscake	1030	2.8	29.1	2	-	30	12
	6384	3.3	158.2	-	-	33	11
Composted sewage sludge	1850642	11.2	285.4	-	-	21	10
	19616	3.5	198.9	-	-	39	13
Composted green waste	40327	6.4	9.9	1	-	33	13
	13302	5.5	326.5	-	-	36	13
Composted biowaste	1046	221.0	7794.8	-	-	27	13
	1399819	7.3	30.0	2	-	33	13

[1] Theoretical limit of detection with a probability of 95% calculated for the method

[2] Few data were finally available for data processing. Estimation to be considered carefully

NOTE In judging the results it is important to consider the concentrations levels, at which measurements have been carried out.

Annex B
(informative)

Prefiltration and centrifugation: comparison tests

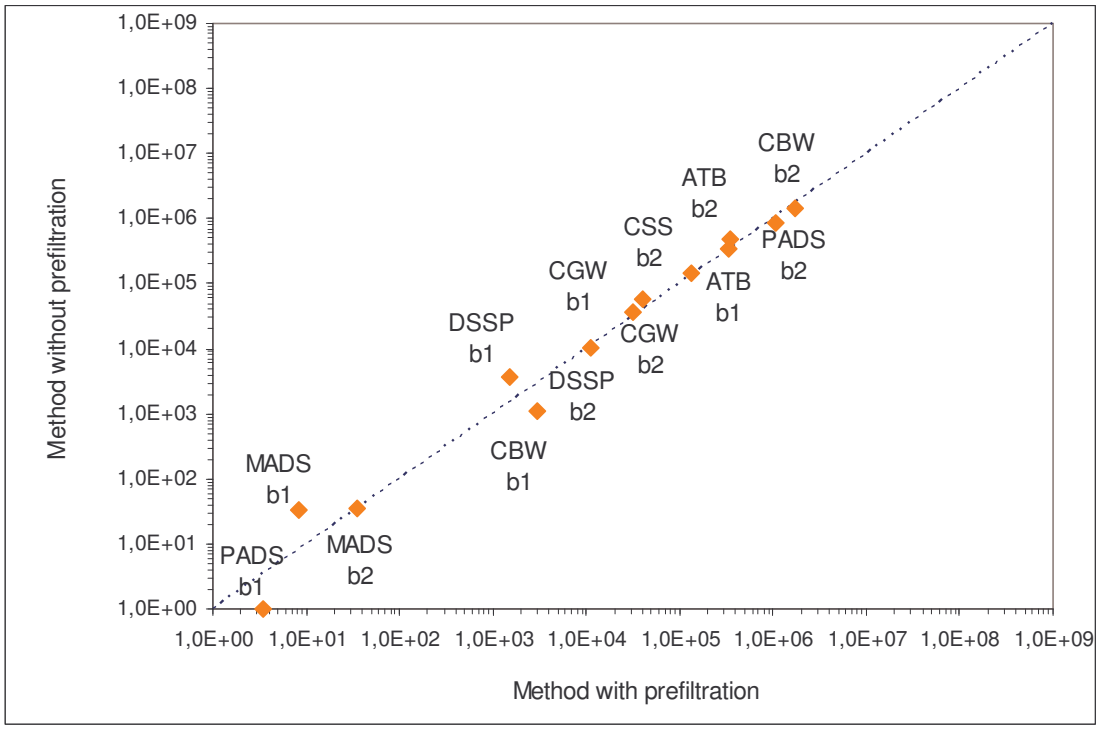
In parallel with the validation study, a short comparative study was performed by two labs to evaluate the interest of the prefiltration and centrifugation step according to various matrices to be analysed (see Table A.2-1: the matrices tested during the validation study).

Following the experimental plan of the validation study, two batches of three samples of each matrix were analysed by each of the two laboratories.

The results of the method applied with and without prefiltration and centrifugation step were plotted on the same graph. The statistical processing was carried out with all data using paired comparisons. Any dot represents the results of the 6 analyses of the same matrix batch with and without the prefiltration and centrifugation step. The position of the dots on the biplot related to the straight line of equivalence allows the detection of any trend.

A non parametric statistical test, Wilcoxon test – paired signed rank test, was used to determine whether the trend was statistically significant.

The paired comparisons of results with and without prefiltration and centrifugation step of each set of matrix are shown on Graph B.1.



Graph B.1: Biplot representations of *Salmonella* spp. filtration method results with and without prefiltration; b1= first batch analysed; b2 = second batch analysed.

Wilcoxon test p-value:

p-value = 0.5525

The comparison of the paired results for the *Salmonella* spp. filtration method with and without the prefiltration and centrifugation did not show obvious significant trend through the different matrices batches.

Bibliography

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[3] Rambach, A., (1990) New plate medium for facilitated differentiation of *Salmonella* spp. from *Proteus* spp. And other enteric bacteria. Applied and Environmental Microbiology, 56, 301 – 303.

[4] EN 1040:1997, Chemical disinfectants and antiseptics - Basic bactericidal activity - Test method and requirements (phase 1)".

[5] Maux, M., Molinier, O. and Guarini, P. 2007. Validation Study Report - Interlaboratory trial to evaluate the performances of the 6 draft *E. coli* and *Salmonella* spp. Horizontal-Hygiene standards (DL 2/1.10), EC-FP6-project Horizontal-Hyg contract n° SSPI-CT-2003-502411.