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 2 : Liquid enrichment method in Selenite Cystine medium followed by enrichment in Rappaport – Vassiliadis medium for semi quantitative Most Probable Number (MPN) determination

Boden, Schlamm und behandelte Bio-abfälle — Quantitativen nachweis von Salmonella spp. aus Schlämmen, Böden, Dungemitteln und Bodenverbesserern, Kultusbstraten sowie Bioabfällen – Teil 2: Flüssiganreicherung in Selenit-Cystein – Bouillon in Kombination mit Rappaport Vassiliadis – Medium zur semiquantitativen Bestimmung der höchstwahrscheinlichen Keimzahl (MPN)

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 2 : méthode par enrichissement en milieu liquide sélénite-cystine puis en milieu Rappaport-Vassiliadis, pour la détermination semi-quantitative par la méthode du Nombre le Plus Probable (NPP)

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Contents

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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* sp. in sludge, 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”.

Sludge, 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 MPN procedure for the semi-quantitative enumeration of *Salmonellae* in sludge, soils and organic fertilisers of similar consistency to the matrices validated.

The method is suited to determine the efficiency of treatment process in accordance with the requirements of the European Sewage Sludge Regulation, Revision of Directive 86/278/EEC (3rd Draft, CEN/TC 308 – doc 525 [1]).

This method has a detection limit (5 %) of approximately 1 *Salmonella* spp. cfu per g of wet weight sample and a range of quantification of 4,71 Log [ENV ISO 13843].

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 sludge — Determination of dry residue and water content.*

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

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

EN 1040:1997, *Chemical disinfectants and antiseptics — Basic bactericidal activity — Test method and requirements (phase 1).*

ISO 6340:1995, *Water quality – Detection and enumeration of Salmonella.*

NF EN 12176:1998, *Characterization of sludge – Determination of pH-value.*

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 document, 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 enriched first in selenite cystine broth, at (36 ± 2)°C and then in Rappaport-Vassiliadis medium at (42 ± 1)°C) before being subsequently detected by characteristic growth on Rambach® agar and XLD agar 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

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
MPN
Every tube 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. The MPN is the number which makes the observed outcome most probable.

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]

4 Symbols and abbreviations

MPN: Most Probable Number

CN: characteristic number

XLD: Xylose Lysine Deoxycholate

5 Principle

The steps involved in this method have been made as close as possible to those to be followed in the ISO 6340:1995. The main differences are:

— sample preparation suitable for a solid matrix;

— a selective pre-enrichment step according to the possible high contamination of the sludge with interfering bacteria.

Six series of three flasks or tubes containing serial dilutions of the sludge suspension should be used for the Most Probable Number enumeration method.

The detection of *Salmonella* spp. requires four stages:

a) culturing of bacteria in a primary selective medium;

b) enrichment in a secondary selective medium which inhibits the growth of other micro-organisms but promotes that of *Salmonellae* (selective enrichment);

c) preparation of pure cultures by inoculating special solid media with subcultures;

d) biochemical and serological identification tests.

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 provided that they are shown to be of equivalent performance in the test.

6.1 Peptone saline solution

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

Dissolve the ingredients and adjust pH to (7 ± 0.2) using hydrochloric acid (1 mol/L) or sodium hydroxide solution (1 mol/L). Sterilize the solution in an autoclave (7.3) at (121 ± 3)°C for (15 ± 1) min.

6.2 Selenite cystine broth

Casein peptone	5 g
Lactose	4 g
Di-sodium phosphate ($Na_2HPO_4.12H_2O$)	10 g
Sodium hydrogen selenite (Na_2SeO_3)	4 g
L-cystine	0.01 g
Sterile distilled water	900 mL

Aseptically dissolve the ingredients and adjust pH to (7 ± 0.2) at 25℃ using sodium hydroxide or hydrochloric acid (0.1 mol L^{-1}). Pour 90 mL fractions into 125 mL flasks (7.1) and 9 mL fractions into 25 mL tubes (7.8).

6.3 Rappaport-Vassiliadis medium

Trypsin digested peptone from casein	4 g
Trypsin digested peptone from meat	1 g
Magnesium chloride hexahydrate	29 g
Sodium chloride	8 g
Di-potassium hydrogen phosphate	0.4 g
Potassium di-hydrogen phosphate	0.6 g
Malachite green	0.036 g
Demineralised water	1000 mL

Dissolve the ingredients under stirring and gently heating. If necessary, adjust the pH of the solution to (5.2 ± 0.1) using hydrochloric acid (1 mol/L) or sodium hydroxide (1 mol/L) solution. Transfer 10 mL portions of the solution to culture tubes (7.8) and sterilized by autoclave (7.3) at (115 ± 2)℃ for (15 ± 1) min.

6.4 Xylose-Lysine-Deoxycholate agar (XLD agar)

6.4.1 Phenol red solution

Phenol red	1 g
Sodium hydroxide solution (0.1 mol/ L)	1.25 mL
Demineralised water	250 mL

Dissolve 1 g of phenol red in 1.25 mL of sodium hydroxide solution and make the solution up to 250 mL with water.

6.4.2 XLD agar complete medium

Yeast extract	3 g
sodium chloride	5 g
L-lysine monohydrochloride	5 g
Agar	12.5 g
Demineralised water	100 mL

Mix the following substances and heat the mixture until its components have dissolved. Sterilize the solution in an autoclave (7.3) for (15 ± 1) min at (121 ± 3)°C, and then add the following substances:

Lactose	7.5 g
D (+)-sucrose	7.5 g
D (+)-xylose	3.75 g
Sodium deoxycholate	1 g
Sodium thiosulfate pentahydrate	6.8 g
Ammonium iron (III) citrate	0.8 g
Phenol red solution	20 mL

Add aseptically the ingredients to the sterile solution prepared before and adjust pH to (7.4 ± 0.2) using sodium hydroxide solution (1 mol/L). Heat in a boiling water bath for 45 min and pour into culture dishes (7.5).

6.5 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 to cool 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.**

6.6 Nutrient agar

Meat extract	3 g
Trypsin digested peptone from casein	5 g
Sodium chloride	5 g
Agar	12 g
Demineralised water	1000 mL

Dissolve the ingredients by heating under stirring. Adjust the pH to (7.2 ± 0.2) using (1 mol/L) sodium hydroxide solution. Sterilize in an autoclave (7.3) for (15 ± 1) min at (121 ± 3)°C and pour into culture dishes (7.5).

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 Wide-mouth glass flasks or beakers for example, 125 mL, 200 mL, 500 mL and 2 000 mL

7.2 Thermostatic incubator regulated at (36 ± 2)°C and (42 ± 1)°C

7.3 Autoclave (steam sterilizer)

7.4 Refrigerator

7.5 Sterile plastics culture dishes, with lid of about 90 mm in diameter

7.6 Sterile graduated pipettes, glass or disposable plastic ware, capable of dispersing 0,1 mL, 1 mL and 10 mL

7.7 Inoculating loop (10 µL) (e.g. platinum-iridium wire), loop diameter approximately 3 mm

7.8 Culture tubes, 25 mL capacity, or equivalent containers

7.9 Vortex mixer suitable for 25 mL culture tubes or equivalent containers

7.10 pH meter, with temperature compensation and pH-measuring cell

7.11 Homogeniser

7.12 Filter membrane, for media sterilisation (0,2 µm cellulose nitrate 47 mm diameter)

7.13 Boiling water bath

7.14 Analytical balance

7.15 Homogenizer bag

7.16 Stirrer and magnetic bars Tunnel drier or vertical laminar airflow cabinet (preferably class II)

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 used in the vicinity shall be flame proof to avoid any source of ignition.

See also the Warning note in Introduction.

8.4 Toxic chemicals

Extreme care must be taken when handling sodium selenite and its solutions due to their high toxicity.

9 Procedure

9.1 Sample preparation

9.1.1 Determination of dry matter

The dry matter of the sludge is determined in accordance with EN 12880:2000.

9.1.2 Suspension preparation

Suspend a representative 25 g wet weight sub-sample with an appropriate volume of peptone saline solution (6.1) so that the final weight is 250 g. Homogenize for 2 min and then process without delay.

For lime-treated materials :

adjust the pH to (7.0 ± 0.5) with 1 mol L^{-1} hydrochloric acid. The sample is mixed by shaking between each addition of hydrochloric acid to ensure the correct pH is achieved. The sample is transferred to a sterile 250 mL container and tested using a pH meter (7.10).

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 neutralisation procedure must be used (see for example EN 1040:1997).

9.2 Analysis

9.2.1 Preparation of dilutions

Take an aliquot of 1 mL out of the primary prepared suspension (9.1.2).

Prepare a serial ten fold dilution (1 mL of primary suspension + 9 mL of peptone saline solution) up to 10^{-5}.

9.2.2 Primary enrichment

Inoculate 3 flasks containing 90 mL of selenite cystine broth (6.2) with 10 mL of the primary homogenised suspension prepared as described in 9.1.2.

Inoculate 3 tubes containing 9 mL of selenite cystine broth (6.2) with 1 mL of the primary homogenised suspension prepared as described in 9.1.2.

From each dilution step prepared as described in 9.2.1, transfer 1 mL per tube into 3 tubes containing 9 mL of selenite cystine broth (6.2).

Incubate the 3 flasks and the 15 tubes at (36 ± 2)°C for (21 ± 3) h.

9.3 Secondary selective enrichment

Aseptically transfer 0,1 mL of each pre-enrichment culture obtained as described in 9.2.2 to a culture tube containing 10 mL of Rappaport-Vassiliadis medium (6.3). Incubate at (41 ± 1)°C for (21 ± 3) h.

9.4 Plating out

Remove culture liquid from the selective enrichment cultures obtained as described in 9.3 using a 10 µL sterile inoculation loop, and streak on both XLD agar medium (6.4) and Rambach® agar medium (6.5). Incubate aerobically at (36 ± 2)°C for (21 ± 3) h.

Identify the presumptive *Salmonella* spp. typical colonies on both XLD agar medium (6.4) and Rambach® agar medium (6.5). On XLD agar medium, typical colonies are pinkish red with a black centre except for H_2S negative strains like *S.* Senftenberg H_2S-. On Rambach® agar medium typical colonies are pink.

9.5 Serological and biochemical confirmation

The *Salmonella* colonies may be confirmed by serological identification of their somatic and flagellar antigens (O- and H), according to manufacturer's instructions. Biochemical tests may be perform using for example commercial identification tests.

For serological and/or biochemical confirmation subculture at least one typical colony from each medium on Nutrient agar (6.6) and incubate the plates at (36 ± 2)°C for (21 ± 3) h to obtain a pure culture.

10 Expression of results

10.1 Determination of the Most Probable Number (MPN)

For each of the 6 dilutions (from the primary suspension (9.1.2) to 10^{-5}), note the number of flasks and/or tubes giving a positive culture on XLD and Rambach® plates.

Identify the corresponding characteristic number (CN) according to the instructions given in ISO 8199:2005 for NPP calculation:

The CN corresponds to the number of positive tubes of the 3 last dilutions giving a number of positive tubes > 0.

When it is possible, choose 3 serial dilutions for which results are neither totally positive nor totally negative. If it is not possible, it is better to choose the 3 serial dilutions with positive results than negative results.

If less than 3 serial dilutions give positive results select the dilution corresponding to the higher concentration of the sample and the 2 next dilutions.

If positive tubes are present only at one dilution, select this dilution, the previous and the next dilution to determine the CN.

EXAMPLE

Dilution of sample	10^{-1} (primary suspension)			10^{-1} (primary suspension)			10^{-2}			10^{-3}			10^{-4}			10^{-5}		
Inoculum (mL of enrichment broth)	10	10	10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Selenite Cystine Broth (mL)	90	90	90	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Rambach® plate	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XLD plate	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
CN	**3**			**2**			**0**			0			0			0		

NOTE **Store all dilution steps (Peptone Saline Solution) of the sample at (5 ± 3)°C until the final result of *Salmonella* spp. is obtained. If all agar plates in the last three dilution steps (10^{-3}, 10^{-4}, 10^{-5}) are positive, prepare 10^{-6} and 10^{-7} dilutions and transfer 1 mL per tube into 3 tubes containing 9 mL Selenite Cystine broth each.**

10.2 Calculation [ISO 8199:2005]

Refer to the table in Annex A (informative) to determine the Most Probable Number (MPN) of *Salmonella* spp. in the suspension (9.1.2).

EXAMPLE

Dilution of the sample	10^{-1}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Inoculum volume	10 mL	1 mL	1 mL	1 mL	1 mL	1 mL
Dilution factor	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}

Example a

CN	**2**	**1**	**0**	0	0	0
MPN table	2/1/0 = 1.5					
Dilution Factor	10^{0}					
Calculation	1.5×10^{0}					
Result : MPN *Salmonella* g^{-1} wet weight of sample	1.5					

Example b

CN	**3**	**2**	**1**	0	0	0
MPN table	3/2/1 = 15					
Dilution Factor	10^{0}					
Calculation	15×10^{0}					
Result : MPN *Salmonella* g^{-1} wet weight of sample	1.5×10^{1}					

Example c

CN	3	**3**	**2**	**1**	0	0
MPN table	3/2/1 = 15					
Dilution Factor	10^{-1}					
Calculation	15×10^{1}					

Result : MPN *Salmonella* g^{-1} wet weight of sample	1.5×10^2

Example d

CN	3	3	**3**	**2**	**1**	0
MPN table	3/2/1 = 15					
Dilution Factor	10^{-2}					
Calculation	15×10^2					
Result : MPN *Salmonella* g^{-1} wet weight of sample	1.5×10^3					

Result should be expressed as *Salmonella* spp. MPN g^{-1} of wet weight and low and upper limits should always be given into brackets.

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 B (informative).

12 Test report

The test report shall contain the following information:

a) reference to this European Standard;

b) all information necessary for complete identification of the sludge 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 [2])

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)

Performance data of the interlaboratory comparison

B.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. Part 2: Liquid enrichment in Selenite Cystine medium followed by enrichment in Rappaport-Vassiliadis medium for semi quantitative (MPN) determination” were established.

B.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 [3]).

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

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

Mesophilic anaerobic digested sewage sludge
Anaerobic treated biowaste
Pelletised air-dried sludge
Digested sewage sludge presscake
Composted sewage sludge
Composted green waste
Composted biowaste

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.

B.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 B.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.3-1 — Summary of components of the *Salmonella* spp. MPN 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**
0.90	$4.65\ 10^4$	4.71	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).

B.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 B.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 B.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 B.4-1 — Summary of *Salmonella* spp. MPN 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	9	24.8	104.8	-	-	27	13
	43	94.1	401.4	-	-	30	14
Anaerobic treated biowaste	> 4.65 10^{4} (1)	-	-	-	-	3	13
	> 4.65 10^{4} (1)	-	-	-	-	3	13
Pelletised air dried sludge	- (4)	-	-	-	-	27	14
	> 4.65 10^{4} (1)	-	-	-	-	9	14
Digested sewage sludge presscake	1559	27.1	172.0	-	-	27	13
	6476	148.3	1281.1	-	-	24	13
Composted sewage sludge	> 4.65 10^{4} (1)	-	-	-	-	0	13
	48693 (2)	4.4 (2)	4.4 (2)	-	-	18	13
Composted green waste	9244 (3)	31.2 (3)	214388.3 (3)	-	-	15	13
	12589	14.1	81.0	-	-	30	13
Composted biowaste	136	8936.7	102600.0	-	-	30	13
	> 4.65 10^{4} (1)	-	-	-	-	39	14

(1) Theoretical upper limit of quantification with a probability of 95% calculated for the method

(2) Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully

(3) Few data were finally available for data processing. Estimation to be considered carefully

(4) No statistical processing was carried out because numerous results were close to the theoretical limit of detection (with a probability of 95%) calculated for the method

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

Bibliography

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