Soils, sludges and treated bio-wastes — Detection and enumeration of *Salmonella* spp. in sludge, soils, and treated biowastes – 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, 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 biodéchets – 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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Foreword

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

This document is a working document.

The following TC has been involved in the preparation of the standard:

This Standard is divided in three parts:

- Part 1 describes a membrane filtration method,
- Part 2 is a liquid enrichment method and determination 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	Note yet validated	
Sludge	Note yet validated	
Biowaste	Note yet validated	
Soil improvers	Note yet validated	

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,and biowastes” and aims at evaluation of the latest developments in assessing *Salmonella* sp. 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 the project “Horizontal”.

Sludge, 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 this European Standard describes a method to detect and semi-quantitatively determine *Salmonellae* in sludge, soils, and biowastes in accordance with the requirements of the European Sewage Sludge Regulation Revision of Directive 86/278/EEC (3rd Draft, CEN/TC 308 – doc525).

The fully defined scope will be determined after the proposed validation trials have been agreed and carried out. This method has a detection limit (5 %) of 0,9 *Salmonella* spp. per g of wet weight, and a upper limit of detection of $4{,}65.10^4$ *Salmonella* spp. per g of wet weight, and a range of quantification of 4,71 Log.

NOTE The objective is to cover untreated and treated sludge, soils, soil improvers, growing media, biowastes and associated materials.

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 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 sludge from sewage and water treatment works (ISO 5667-13:1997)*.

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

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

3 Definitions

For the purposes of this document, 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. are capable of being enriched in selenite cystine broth at (36 ± 2) °C followed by growth in Rappaport-Vassiliadis medium at (42 ± 1) °C followed by characteristic growth on Rambach® agar or 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, 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 matter
the dry mass portion of the sludge obtained after the specified drying process. It is expressed as percent or in grams per kilogram (see EN 12880:2000, 3.1)

4 Symbols and abbreviations

cfu : colony forming unit

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 involved in the ISO 6340 : 1995. The main differences are the following:

— 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 Verification of interference

7 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 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 up to one month in conditions avoiding any alterations in their composition.

NOTE 1 The use of chemicals of other grades is permissible provided that they are shown to be of equivalent performance in the test.

NOTE 2 Ready to use media may also be used for the examination provided their compositions are equivalent to those specified in this sub clause.

7.1 Saline solution

Dissolve 0,85 g of sodium chloride in 100 mL of water and adjust the pH value of the solution to (7,0 ± 0,1) with sodium hydroxide or hydrochloric acid (0,1 mol L^{-1}). Pour the solution into suitable glass containers as required and sterilise in an autoclave (5.3) at (121 ± 3) °C for (15 ± 1) min.

7.2 Bromocresol purple solution

Dissolve 1 g of bromocresol purple $C_{21}H_{16}Br_2O_5S$ in 100 mL of water.

7.3 Kovac's reagent (indole reagent)

4-dimethylaminobenzaldehyde, $C_9H_{11}NO$	5 g
Isoamyl alcohol, $C_5H_{12}O$	75 mL
Hydrochloric acid (0,1 mol L^{-1})	25 mL

Dissolve the 4-dimethylaminobenzaldehyde, $C_9H_{11}NO$, in 75 mL of isoamyl alcohol, $C_5H_{12}O$, and heat in a water bath at 60 °C for 5 min. Then add 25 mL of hydrochloric acid (0,1 mol L^{-1}). The reagent will be ready for use after about 6 h to 7 h (indicated by a yellow colour).

Commercially available Kovac's reagent can be used according to the manufacturer's instructions.

7.4 Phenol red solution

Dissolve 1 g of phenol red in 1,25 mL of sodium hydroxide solution (0,1 mol L^{-1}) and make up to 250 mL with water.

7.5 Urea medium

7.5.1 Urea basal medium

a) 1,0 g of tryptone;

b) 1,0 g of D-glucose;

c) 5,0 g of sodium chloride;

d) 2,0 g of potassium di-hydrogen phosphate (KH_2PO_4);

e) 0,012 g of phenol red;

f) 12 g of agar.

Mix all the ingredients and heat the mixture in a boiling water bath until its components have dissolved. Adjust the pH value, using sodium hydroxide solution or hydrochloric acid (0,1 mol L^{-1}), so that after sterilization the pH will be (6,8 ± 0,1). Sterilize in an autoclave (7.3) at (121 ± 3) °C for (15 ± 1) min.

7.5.2 Urea solution

Dissolve 400 g of urea, H_2NCONH_2, in water and make up to 1 000 mL. Filter sterilise the solution through a 0,2 µm sterile membrane filter.

7.5.3 Urea complete medium

Melt the Urea basal medium (6.5.1) and cool to 45 °C. Aseptically add 50 mL of urea solution (6.5.2) to 950 mL of Urea basal medium maintained at 45 °C. Distribute the medium into sterile culture tubes (7.8) in volumes of 10 mL portions allow the medium to solidify in order that the medium forms a slant in the tube.

7.6 Tryptophan-tryptone broth for the Indole formation test

Dissolve 10 g of tryptone, 1 g of DL-tryptophan for biochemical applications, and 5 g of sodium chloride in 1 000 mL of water while heating in a boiling waterbath.

Adjust the pH value using sodium hydroxide solution (0,1 mol L^{-1}) so that it will be (7,4 ± 0,1) after sterilization. Transfer 5 mL portions of the solution to culture tubes and sterilize in an autoclave (7.3) at (121 ± 3) °C for (15 ± 1) min.

7.7 Lysine decarboxylase test medium

Dissolve 5 g of L-lysine monohydrochloride, 3 g of yeast extract, 1 g of D-glucose, and 1,5 mL of bromocresol purple solution (6.2) in 1 000 mL of water while heating in a boiling water bath.

Adjust the pH value using sodium hydroxide solution (0,1 mol L^{-1}) so that it will be (6,8 ± 0,2) after sterilization. Transfer 5 mL portions of the solution to culture tubes and sterilize in an autoclave (7.3) at (121 ± 3) °C for (15 ± 1) min.

7.8 Peptone saline solution

Dissolve 1 g of casein peptone and 8,5 g of sodium chloride in 1 000 mL of water.

Sterilize the solution in an autoclave (7.3) at (121 ± 3) °C for (15 ± 1) min.

7.9 Selenite cystine broth

Aseptically dissolve in 900 mL of sterile distilled water:

a) 5 g of casein peptone;

b) 4 g of lactose;

c) 10 g of di-sodium phosphate ($Na_2HPO_4.12H_2O$);

d) 4 g of sodium hydrogen selenite (Na_2SeO_3);

e) 0,01 g of L-cystine.

Adjust pH to (7,0 ± 0.2) at 25°C using sodium hydroxide or hydrochloric acid (0,1 mol L^{-1}), and the final volume to 1 000 mL. Pour 90 mL fractions into 125 mL flasks (7.1) and 9 mL fractions into 25 mL tubes (7.8).

7.10 Rappaport-Vassiliadis medium

prepared according to the manufacturer or:

Dissolve the following in 1000 mL water in a 2000 mL flask, while heating in a boiling water bath:

— 4 g trypsin digested peptone from casein;

— 1 g trypsin digested peptone from meat;

— 29,0 g magnesium chloride hexahydrate;

— 8 g sodium chloride;

— 0,4 g di-potassium hydrogen phosphate;

— 0,6 g potassium di-hydrogen phosphate;

— 0,036 malachite green;

Dissolve the ingredients under stirring and gently heating and sterilize the solution in an autoclave at (115 ± 2) °C for (15 ± 1) min. If necessary, adjust the pH of the solution to 5,2 ± 0,1 using hydrochloric acid (1mol/l) or sodium hydroxide solution. Transfer 10 mL portions of the solution to culture tubes and reheat for ten minutes at 100 °C on a boiling water bath (7.16);

Normally, it is first filled into culture tubes and then sterilized.

7.11 Xylose/lysine/deoxycholate (XLD) agar

Mix the following ingredients in 1 000 mL of water in a 2 000 mL flat bottom flask :

a) 3 g of yeast extract;

b) 5 g of sodium chloride;

c) 5 g of L-lysine monohydrochloride;

d) 12,5 g of agar.

Heat the mixture in a boiling water bath until the components have dissolved. Sterilize the solution in an autoclave (7. 3) for (15 ± 1) min at (121 ± 3) °C, then add the following ingredients:

a) 7,5 g of lactose;

b) 7,5 g of D(+) – sucrose;

c) 3,75 g of D(+) – xylose;

d) 1 g of sodium deoxycholate;

e) 6,8 g of sodium thiosulfate ($5H_2O$) ;

f) 0,8 g of ammonium iron (III) citrate;

g) 20 mL of phenol red solution (6.4).

Adjust the pH value to (7,4 ± 0,2) using a sodium hydroxide solution (0,5 mol L^{-1}). Heat in a boiling water bath for 45 min and pour into sterile Petri dishes.

7.12 Chromogenic medium: Rambach® agar (Merck or equivalent supplier)

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 to cool to (45 ± 2) °C and pour into sterile Petri dishes. Store at (5 ± 3) °C for a maximum of one month.

8 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199.Usual microbiological laboratory equipment and in particular:

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

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

8.3 Autoclave (steam sterilizer)

8.4 Refrigerator

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

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

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

8.8 Culture tubes, 25 mL capacity, or equivalent containers

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

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

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

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

8.13 Boiling water bath

8.14 Scale

8.15 Homogenizer Bag

8.16 Stirrer and magnetic bars

9 Sampling and hazards

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 biohazardous samples are followed.

See also the Warning note in 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, so all equipment used in the vicinity shall be flame proof to avoid any source of ignition.

See also the Warning note in Introduction.

9.4 Toxic chemicals

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

10 Procedure

10.1 Sample preparation

10.1.1 Determination of dry matter

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

10.1.2 Suspension preparation

Suspend a representative sub-sample of 25 g (wet weight) with an appropriate volume of peptone saline solution (7.8) so that the final weight is 250 g. Homogenize for 2 min and then process without delay.

For lime treated sludge adjust the pH to 7,0 ± 0,5 with 1 mol L^{-1} 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 neutralisation procedure must be used (see for example EN 1040:1997).

10.2 Analysis

10.2.1 Preparation of dilutions

— Take an aliquot of 1 mL out of the primary prepared suspension (10.1.2)

— Prepare a serial ten fold dilution (1 mL of primary suspension + 9 mL of peptone saline solution) up to 10^{-4}

10.2.2 Primary enrichment

— Inoculate 3 flasks containing 90 mL of selenite cystine broth (7.9) with 10 mL of the primary homogenised suspension prepared as described in 10.1.2.

— Inoculate 3 tubes containing 9 mL of selenite cystine broth (7.9) with 1 mL of the primary homogenised suspension prepared as described in 10.1.2.

— From each dilution step prepared as described in 10.2.1, transfer 1 mL per tube into 3 tubes containing 9 mL of selenite cystine broth (7.9)

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

10.3 Secondary selective enrichment

Aseptically transfer 0,1 mL of each pre-enrichment culture obtained as described in 8.2.1 to a culture tube containing 10 mL of Rappaport-Vassiliadis medium (7.11). Incubate at (41 ± 2) °C for (21 ± 3) h.

10.4 Plating out subculture

Remove culture liquid from the selective enrichment cultures obtained as described in 10.2.2 using a 10 µL sterile inoculation loop, and streak on both XLD agar medium (8.11) and Rambach® agar medium (7.12). Incubate aerobically at (36 ± 2) °C for (21 ± 3) h.

10.5 Identification of pure cultures

Identify the presumptive *Salmonella* spp. typical colonies on both XLD agar medium (7.9) and Rambach® agar medium (7.12). On XLD agar medium (7.9), typical colonies are pinkish red colonies with a black centre except for H_2S negative strains like *S.* Senftenberg H_2S-). On Rambach® agar medium (7.12) typical colonies are pink-coloured colonies.

Proceed to the serological confirmations on one of each type of typical identified colonies on each media as described in 10.5 and 10.6 and if necessary proceed to the biochemical confirmations.

NOTE 1 Colonies on XLD can appear completely black.

NOTE 2 Pure cultures should be prepared by the standard methods used in medical diagnostic laboratories. Only pure cultures should be used for the serological and biochemical tests.

10.6 Serological confirmation

The identity of the *Salmonella* suspect colonies may be confirmed by serological identification of their somatic and flagellar antigens (O- and H), according to manufacturer's instructions.

10.7 Biochemical confirmation

NOTE Commercial identification tests (e.g. API 20E from Biomérieux or equivalent) can be used instead of tests 10.5.1 to 10.5.3 for a greater range of biochemical tests.

10.7.1 Urea hydrolysis

— Streak the agar slope surface of the Urea complete medium (7.5.3) ;

— Incubate at (36 ± 2) °C for (21 ± 3) h and examine at intervals.

If the reaction is positive, splitting of the urea liberates ammonia which changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h.

The presence of *Salmonella* spp*.* leads to a negative reaction, i.e. absence of colouring.

10.7.2 L-lysine decarboxylation

— Inoculate just below the surface of the liquid Lysine carboxylase test medium (7.7);

— Overlay the medium with sterile liquid paraffin or oil;

— Incubate at (36 ± 2) °C for (21 ± 3) h.

The presence of *Salmonella* spp. leads to a positive reaction, i.e. a purple colouring after incubation.

10.7.3 Indole formation

— Inoculate the tryptophan-tryptone broth (7.6) and incubate at (36 ± 2)°C for (21 ± 3) h ;

— Reveal the formation of indole in the culture tube with Kovacs reagent (7.3).

The presence of *Salmonella* spp. leads to a negative reaction for indole formation, i.e. absence of red colouring.

11 Expression of results

11.1 Determination of the Most Probable Number (MPN)

For each of the 6 dilutions (from 10^0 corresponding to primary suspension 10.1.2 to 10^{-5}), note the number of flasks and/or tubes giving a positive culture on XLD and/or Rambach® plate after enrichment cultures (10.1 and 10.2), after confirmation tests.

Identify the corresponding characteristic number (CN) according to the instructions given in ISO 8199 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, neither 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 use the dilution containing the higher concentration in sample and the 2 next dilutions.

If one only wells serial gives positive results, use this dilution and the previous and the next dilution (table 1 example d).

Dilution of sample	10^0 (primary suspension)			10^0 (primary suspension)			10^{-1}			10^{-2}			10^{-3}			10^{-4}		
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®	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XLD	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
CN	**3**			**2**			**0**						0			0		

CN : characteristic number

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^{-2}, 10^{-3}, 10^{-4}) are positive, prepare 10^{-5} and 10^{-6} dilutions and transfer 1 mL per tube into 3 tubes containing 9 mL Selenite Cystine broth each.

11.2 Calculation (See also 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}
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	1.5					
Dilution Factor (DF)	10^{0}					
Calculation (MPN/DF)	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	15					
Dilution Factor (DF)	10^{0}					
Calculation (MPN/DF)	15×10^{0}					
Result : MPN *Salmonella* g^{-1} wet weight of sample	15					
Example c						
CN	3	**3**	**2**	**1**	0	0
MPN table	15					
Dilution Factor (DF)	10^{-1}					
Calculation (MPN/DF)	15×10^{1}					
Result : MPN *Salmonella* g^{-1} wet weight of sample	150					
Example d						
CN	3	3	**3**	**2**	**1**	0
MPN table	15					
Dilution Factor (DF)	10^{-2}					
Calculation (MPN/DF)	15×10^{2}					
Result : MPN *Salmonella* g^{-1} wet weight of sample	$1.5\ 10^{3}$					
Example e						
CN	3	3	3	**3**	**2**	1
MPN table	15					
Dilution Factor (DF)	10^{-3}					
Calculation (MPN/DF)	15×10^{3}					
Result : MPN *Salmonella* g^{-1} wet weight of sample	$1.5\ 10^{4}$					

Result should be express as MPN g^{-1} of wet weight and low and upper limits should always be given into brakets.

12 Performance data

Performance data in terms of repeatability and reproducibility 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

13 Test report

The test report shall contain the following information:

1. A reference to this European Standard including its date of publication;
2. Sampling report including precise identification of the sample;
3. specific reporting on the considered quantification standard;
4. expression of results according to clause 10;
5. any deviation from this standard, and any facts which may have influenced the result.

Annex A
(informative)

MPN Statistical Table for 3-Tubes MPN procedure (de Man *et al*., 1983)

Characteristic number			MPN index	Confidence limits			
1st digit	2nd digit	3rd digit		[3] 95 %	[3] 95 %	[3] 99 %	[3] 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	3	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

Characteristic number			MPN index	Confidence limits			
1st digit	**2nd digit**	**3rd digit**		**³ 95 %**	**³ 95 %**	**³ 99 %**	**³ 99 %**
3	3	2	110	20	400	10	570
3	3	3	> 110				

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