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 3: Presence/absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport Vassiliadis medium

Boden, Schlamm und behandelte Bioabfälle — N*achweis* und Aufzählung *von Salmonella spp. aus Schlämmen, Böden, Düngemitteln und Bodenverbesserern, Kultursubstraten sowie Bioabfällen — Teil 3: Methode der Flüssiganreicherung in Peptonwasser mit Novobiocin in Kombination mit Rappaport-Vassiliadis — Medium zum qualitativen Nachweis des Vorkommens bzw. der Freiheit von Salmonellen in einer definierten Probenmenge*

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 3 : Présence/absence par enrichissement en milieu liquide peptone-novobiocine puis sur milieu Rappaport-Vassiliadis*

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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 presence/absence procedure to detect *Salmonella* spp. using a four-stage presence/absence method in up to 50g (wet weight) sample (BioAbfV, 1998 [1]) in sludges, soils and organic fertilisers of similar consistency to the matrices validated.

This method can be used irrespective of the dry residue content of the test material.

The method has a limit of detection of approximately *Salmonella* spp. 10 cfu in 50 g wet weight sludge [ENV ISO 13843: 2001].

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.*

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

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 Terms and definitions

For the purposes of this 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 enriched in buffered peptone water supplemented with Novobiocin and growth in RV medium (Rappaport Vassiliadis medium) followed by characteristic growth on BPLS agar (Brilliant green-phenol red-lactose-sucrose agar) or XLD agar (Xylose lysine deoxycholate agar) at (36 ± 2)°C

NOTE ***Salmonella typhi* and H_2S negative salmonellae (e.g. *Salmonella* Senftenberg H_2S negative) do not produce typical black colonies on XLD agar**

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
presumptive positives
isolates which are believed to be *Salmonella* spp., but not yet confirmed

3.8
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

BPLS agar: Brilliant green-phenol red-lactose-sucrose agar

XLD agar: Xylose lysine deoxycholate agar

cfu: Colony forming unit

RV medium: Rappaport Vassiliadis medium

5 Principle

This is a presence/absence method including recovery of sub-lethally damaged *Salmonella* spp. designed to process samples of up to 50 g wet weight. If less then 50 g wet weight is used the volume of primary recovery medium (buffered peptone water) should be adjusted in proportion.

In this method the detection of *Salmonella* spp. is undertaken in four steps:

a) pre-enrichment 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 inoculation on two different special solid media with subcultures;

d) serological and/or biochemical identification.

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 Buffered Peptone Water supplemented with 40 mg/L Novobiocin

6.1.1 Buffered Peptone Water

Peptone from casein	10 g
Sodium chloride	5 g
Potassium di-hydrogen phosphate	1.5 g
Di-sodium hydrogen phosphate dodecahydrate	9 g
Demineralised water	1000 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). Divided in 450 mL portions and sterilised by autoclave (15 ± 1) min at (121 ± 2)°C.

6.1.2 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, (7.14)). Store solution at 4°C, protected by light (e.g. aluminium foil). Solution can be stored for one week.

6.1.3 Buffered Peptone Water supplemented with 40mg/L Novobiocin

Buffered Peptone Water supplemented with 40 mg/L Novobiocin in 450 mL portions, was prepared transferring 180 µl of Novobiocin supplement solution into 450 mL (40 µg/mL) sterilized buffered peptone water immediately before use, mix by gentle shaking.

6.2 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 (7.9) and sterilized by autoclave (7.3) at (115 ± 2)°C for (15 ± 1) min.

6.3 Xylose-Lysine-Deoxycholate agar (XLD agar)

6.3.1 Phenol red solution

Phenol red	1 g
Sodium hydroxide solution (0.1 mol/ L)	1.25 mL
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.

6.3.2 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 the water bath for 45 min and pour into culture dishes (7.5).

6.4 Brilliant green-phenol red-lactose-sucrose agar, modif. (BPLS -Agar, modif.)

Peptone from meat	10.0 g
Yeast extract	5.0 g
Meat extract	5.0 g
Sodium dihydrogen phosphate	0.6 g
Di-sodium hydrogen phosphate	1 g
Lactose	10 g
Sucrose	10 g
Phenol red	0.09 g
Brilliant green	0.0047 g
Agar-agar	12.0 g
Demineralised water	1000 mL

Dissolve the ingredients and adjust the pH to (6.9 ± 0.1) using (1 mol/L) sodium hydroxide solution. Pour into culture dishes (7.5).

6.5 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 incubators regulated at (36 ± 2)°C (gyratory shaking and static) and (42 ± 1)°C (static)

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, of nominal capacities 1 and 10 mL

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

7.8 Apparatus for shaking the culture tubes

7.9 Culture tubes, 25 mL capacity, or equivalent containers

7.10 Vortex mixer suitable for 25 mL capacity culture tubes or equivalent containers

7.11 Laboratory spatula

7.12 pH meter, with temperature compensation and pH measuring cell

7.13 Membrane filtration equipment

7.14 Filter membrane, for media sterilisation (0.2 µm cellulose nitrate 47 mm diameter)

7.15 Adjustable micropipettor up to 200 µL capacity

7.16 Boiling water bath

7.17 Analytical balance

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 Pre - enrichment

Add 50 g (wet weight) of material (e.g. sewage-sludge, slurry, compost) to 450 mL of Buffered Peptone Water supplemented with Novobiocin (6.1), incubate for (21 ± 3) h at (36 ± 2)°C in a shaking incubator (150 rpm) (7.2). If less than 50 g of sample material are used, keep the relationship of 1:10 between weight of sample and volume of primary enrichment medium (6.1).

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 neutralisation procedure must be used [EN 1040:1997].**

9.2 Enrichment

Transfer 0.1 mL of the primary enrichment culture (9.1) into two tubes, each containing 10 mL of Rappaport-Vassiliadis medium (6.2), incubate one at (36 ± 2)°C and the other one at (41 ± 1)°C for (21 ± 3) h (both in static conditions) (7.2).

9.3 Plating out

Streak a loopfull (10 µL) from each tube onto XLD (6.3) and BPLS (6.4) culture plates. Incubate at (36 ± 2)°C for (21 ± 3) h.

Typical colonies on XLD medium appear black (except *Salmonella* typhi and H_2S negative salmonellae colonies (e.g. *Salmonella Senftenberg* H_2S negative)), and typical colonies on BPLS medium appear pink.

Subculture at least three typical colonies from each medium on nutrient agar plates (6.5) and incubate the plates at (36 ± 2)°C for (21 ± 3) h to obtain a pure culture for the confirmation step.

NOTE if none of the subcultivated colonies can be confirmed as *Salmonellae* (10.4) the procedure shall be repeated until a positive result is obtained or no typical colonies remain.

9.4 Serological and biochemical confirmations

Further confirmation of *Salmonellae* can also be achieved by serological identification of their somatic and flagellar antigens (O- and H-antigens), and if necessary by biochemical tests using for example commercial identification tests.

9.5 Determination of the dry residue content

The result can be expressed as presence or absence of *Salmonella* spp. in a certain amount of the investigated material (wet weight). If the results refer to dry matter, 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

A positive response will be reported as: “Presence of *Salmonella* spp. in up to 50 g material (as received)”;

A negative response will be reported as “Absence of *Salmonella* spp. in up to 50 g material (as received)”.

NOTE 1 If the result is required in relation to dry weight rather than as received weight then carry out a dry residues analysis (EN 12880:2000).

NOTE 2 If less than 50 g are analysed expression of results must be modified indicating the actual weight of 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 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)

Performance data from laboratory tests

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. Part3: Presence/Absence method by liquid enrichment in peptone-novobiocin followed by Rappaport-Vassiliadis medium” were established.

A.2 Material used in the interlaboratory comparison study

The interlaboratory comparison of the Presence/Absence method for detection 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 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

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.

A.3 First assessment of the precision of the method

The statistical evaluation was reduced to the statistical determination of confidence intervals (Table A.3-1).

Table A.3-1 — Summary of components of the Salmonella spp. Presence/Absence method precision

Result	Confidence internal (Error of first kind α=5%)
Presence	at least 5.13 *Salmonella* spp. in 50 g wet weight
Absence	less than 300 *Salmonella* spp. in 50 g wet weight
Limit between presence (50%) and absence (50%)	69.3 *Salmonella* spp. in 50 g wet weight

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 not conducted, only qualitative data were obtained (Table A.4-1).

Table A.4-1 — Summary of *Salmonella* spp. Presence/Absence method results of inter-laboratory comparison

Matrix	Final result	Number of laboratory with at least one result "Absence"	Total number of labs	Total number of data
Mesophilic anaerobic digested sewage sludge	Presence	1 lab (3 replicates)	13	39
	Presence	1 lab (1 replicate)	14	41
Anaerobic treated biowaste	Presence	-	12	36
	Presence		13	39
Pelletised air dried sludge	Presence	1 lab (3 replicates)	14	41
	Presence		14	41
Digested sewage sludge presscake	Presence		12	36
	Presence		13	39
Composted sewage sludge	Presence	1 lab (3 replicates)	13	39
	Presence	1 lab (1 replicate) & 1 lab (3 replicates)	13	39
Composted green waste	Presence		14	41
	Presence		13	39
Composted biowaste	Presence	1 lab (1 replicate)	13	39
	Presence	1 lab (3 replicates)	14	41

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

Annex B
(informative)

Performance data with field samples

B.1 Results of the comparative validation with and without Novobiocin in the first enrichment obtained with waste water and compost samples collected under practical conditions

In these experiments 112 waste water samples and 130 compost samples randomly collected from different plants had been investigated by comparing the performance of a primary enrichment with and without Novobiocin. Secondary enrichment had been done wit Rappaport medium (see also A.1) incubated at 37 °C and 43 °C in parallel. It can be taken from Table B.1, that addition of Novobiocin to the primary enrichment resulted in detection of more positive samples in all the investigated combinations of sample material and incubation temperature for the secondary enrichment. Significance had been confirmed by using the SAS package of Sigma Plot.

Table B.1 — Results of comparative analysis of performance of primary enrichment medium with and without Novobiocin with two types of samples

Primary enrichment (peptone water)	Enrichment	Selective medium	Material			
			Waste water		Compost	
			Detection		Detection	
			37 °C	43 °C	37 °C	43 °C
without	1. Rappaport-Vassiliadis	XLD/BPLS	24 %	46 %	3 %	8 %
Novobiocin 40µg/l	1. Rappaport-Vassiliadis	XLD/BPLS	37 %	62 %	5 %	14 %
			n =112		n =130	

XLD = Xylose - Lysine - Deoxycholate – agar.

BPLS = Brilliant green - Phenol red - Lactose - sucrose – agar.

Bibliography

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