Soils, sludges and treated bio-wastes — Detection and enumeration of *Salmonella* spp. in sludges, soils, and biowastes — Part 3: Presence/absence method by liquid enrichment in peptone-novobiocin medium followed by Rappaport Vassiliadis medium

Boden, Schlamm und behandelte Bio-abfälle — N*achweis* und aufzählung *von Salmonella spp. aus Schlämmen, Böden, 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 biodéchets —Partie 3 : Présence/absence par enrichissement en milieu liquide peptone-novobiocine puis sur milieu Rappaport-Vassiliadis*

ICS:

Descriptors: *Salmonella*, sludges, soils, and biowastes

Document type: European Standard
Document subtype:
Document stage: Working Document
Document language: E

STD Version 2.2

Contents

Page

Foreword

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

This document is a working document.

This standard is divided into three parts:

- part 1 describes a membrane filtration method
- part 2 is a liquid enrichment method and determination by MPN
- part 3 is a presence / absence method by liquid enrichment.

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

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

Introduction

This document is developed in the framework of the project 'Horizontal'. It is the result of a desk study "Hygienic Parameters Feasibility of Horizontal Standards for *Escherichia coli* and *Salmonella* spp. In sludges, soils, and biowastes" and aims at evaluation of the latest developments in assessing *Salmonella* spp. in sludge, soil, treated biowaste and neighbouring fields. After discussion with all parties concerned in CEN and selection of a number of test methods described in this study the standard has been developed further as a modular horizontal method and has been validated within in the project 'Horizontal'.

Sludges, soils, and biowastes can contain pathogenic micro-organisms such as *Salmonella* spp. which occur mainly in the intestinal tract of humans and animals and are transmitted through faecal contamination. The use of such pathogen-contaminated materials in agriculture can cause outbreaks of infection due to the production of contaminated food or animal feedstocks and may also be transmitted to wild animals, consequently, there is a need to monitor rates to land. See Part 2.

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 8199:2001, have to be applied.

WARNING — "Waste and sludge samples can contain hazardous and inflammable substances. They can contain pathogens and be liable to biological action. Consequently, it is recommended that these samples should be handled with special care. The gases which can be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. National regulations should be followed with respect to microbiological hazards associated with this method"

The texts of the chapters 1 to 13 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

1 Scope

This part of the European standard describes a presence/absence procedure to detect *Salmonella* spp using a four-stage presence/absence method in up to 50g (wet weight) sample.

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

The method has a limit of detection of approximately 10 cfu/50 g wet weight sludge.

NOTE The objective is to cover untreated and treated sludges, soils, and biowastes.

2 Normative references

This Working document incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the 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 Working Document only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

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

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

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

3 Terms and 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. capable of being enriched in peptone water supplemented with Novobiocin and growth in RV medium followed by characteristic growth on BPLS agar or XLD 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
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
presumptive positives
isolates which are believed to be *Salmonella* spp., but not yet confirmed

3.8
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, 3.1]

4 Symbols and abbreviations

4.1
BPLS agar
Brilliant green-phenol red-lactose-sucrose agar

4.2
XLD agar
Xylose lysine deoxycholate agar

4.3
Cfu
Colony forming unit

4.4
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 lower sample quantities are processed, the relationship between the amount of sample and primary recovery medium shall be maintained

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 inoculation on two different special solid media with subcultures;

d) serological and/or biochemical identification

6 Verification of interferences

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

7.1 Buffered Peptone Water supplemented with 40mg/L Novobiocin

Buffered Peptone Water supplemented with 40 mg/L Novobiocin in 450 mL portions, prepared as follows:

— dissolve 1 g Novobiocin in 10 mL sterile distilled water and filter through membrane filter (pore size 0,2 µm, (8.14)).

— transfer 180 µl of this solution into 450 mL (40 µg/mL) sterilized buffered peptone water (prepared according to the manufacturer) immediately before use, mix by gentle shaking.

7.2 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 g malachite green;

Solve 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 (8.16).

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

7.3 Xylose-Lysine-Deoxycholate agar (XLD agar)

prepared according to the manufacturer or:

Mix the following substances in 1000 mL water in a 2000 mL flat bottom flask:

— 3 g yeast extract;

— 5 g sodium chloride;

— 5 g L-lysine monohydrochloride;

— 12,5 g agar.

Heat the mixture in a boiling water bath until its components have dissolved.

Sterilize the solution in an autoclave (steam sterilizer) (8.3) for (15 ± 1) min at (121 ± 3) °C, then add the following substances:

— 7,5 g lactose;

— 7,5 g D (+)-sucrose;

— 3,75 g D (+)-xylose;

— 1 g sodium deoxycholate;

— 6,8 g sodium thiosulfate pentahydrate;

— 0,8 g ammonium iron (III) citrate;

— 20 mL phenol red solution (prepared by dissolving 1 g of phenol red in 1,25 mL of sodium hydroxide solution (0,1 mol/ L) and making the solution up to 250 mL with water.);

Adjust the pH to 7,4 ± 0,2 using (1 mol/L) sodium hydroxide solution. Heat on a boiling water bath for 45 minutes and pour into culture dishes.

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

prepared according the manufacturer or:

Dissolve the following in 1 000 mL water in a 2 000 mL flat bottom flask, while heating in a boiling water bath:

— 10.0 g peptone from meat;

— 5.0 g yeas extract;

— 5.0 g meat extract;

— 0.6 g sodium dihydrogen phosphate;

— 1.0 g di-sodium hydrogen phosphate;

— 10.0 g lactose;

— 10.0 g sucrose;

— 0.09 g phenol red;

— 0.0047 g brilliant green;

— 12.0 g agar-agar

Adjust the pH to 6,9 ± 0,1 using (1 mol/L) sodium hydroxide solution. Pour into culture dishes.

7.5 Nutrient agar

prepared according the manufacturer or:

Dissolve the following in 1 000 mL water in a 2 000 mL flat bottom flask, while heating in a boiling water bath:

— 3,0 g meat extract;

— 5,0 g trypsin digested peptone from casein;

— 5,0g sodium chloride;

— 12,0 g agar.

Adjust the pH to 7,2 ± 0,2 using (1 mol/L) sodium hydroxide solution. Sterilize in an autoclave (steam sterilizer) (8.3) for (15 ± 1) min at (121 ± 3) °C and pour into culture dishes.

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

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, of nominal capacities 1 and 10 mL.

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

8.8 Apparatus for shaking the culture tubes.

8.9 Culture tubes, 25 mL capacity, or equivalent containers.

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

8.11 Laboratory spatula.

8.12 pH meter, with temperature compensation and pH measuring cell.

8.13 Membrane filtration equipment.

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

8.15 Adjustable micropipettor up to 200 µL capacity.

8.16 Boiling water bath.

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 particularly if not treated, and may 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.

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, 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.

10 Procedure

10.1 Primary enrichment

Add 50 g (wet weight) of material (e.g. sewage-sludge, slurry, compost) to 450 mL of Buffered Peptone Water supplemented with Novobiocin (7.1), incubate for (21 ± 3) h at (36 ± 2) °C in a shaking incubator (150 rpm) (5.2). If less than 50g of sample material are used, keep the relationship of 1:10 between weight of sample and volume of primary enrichment medium (7.1).

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 (see for example EN 1040:1997).

10.2 Secondary enrichment

Transfer 0,1 mL of the primary enrichment culture (10.1) into two tubes, each containing 10 mL of Rappaport-Vassiliadis medium (7.2), incubate one at (36 ± 2) °C and the other one at (41 ± 2) °C for (21 ± 3) h (both in static conditions) (8.2).

10.3 Plating out

Streak a loopfull (10 µL) from each tube onto XLD (7.3) and BPLS (7.4) culture plates. Incubate at (36 ± 2) °C for (21 ± 3) h

Subculture at least three typical black colonies from XLD and at least three typical pink colonies from BPLS culture plates each on nutrient agar plates (7.5) and incubate at (36 ± 2) °C for (21 ± 3) h to obtain a pure culture for confirmation steps.

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.

10.4 Serological and biochemical confirmations

Further confirmation of *Salmonellae* can also be achieved by serological identification of their somatic and flagellar antigens (O- und H-antigens), and if necessary by biochemical testing using for example API 20E (Biomérieux) test strips or equivalent, according to manufacturer's instructions.

10.5 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.

11 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.

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;

e) any detail not specified in this part of this European Standard and any other factor which may have affected the results.

13 Performance data

See Annex A and Annex B.

Annex A
(informative)

Performance data from laboratory tests

Results of comparative studies for examination of *Salmonella*

A.1 Confirmation of sensitivity of several combinations of primary enrichment with Novobiocin and selected secondary enrichment media in combination with different types of samples

Samples of municipal sewage sludge, catering waste and liquid manure had been artificially contaminated with about 10^1 *Salmonella* Senftenberg (DSM 10062) per 50 g of sample (1mL out of the corresponding tube from a serial dilution of a 18 h broth culture in 0,9 % NaCl – solution). Catering waste, sewage sludge and liquid manure were heated at 70°C for 3h and absence of *Salmonella* was confirmed prior contamination. The combination of primary enrichment with all tested secondary enrichment media incubated at 37 °C and 43 °C were sensitive enough to detect this contamination-level in all types of tested samples on XLD and BPLS agars according to the procedure described in this standard under 8. Comparative experiments were carried out by two independent investigators with 10 samples of each type. Results show, that Rappaport medium without Novobiocin is sufficient in secondary enrichment.

NOTE Tested with a contamination level of 10 *Salmonella* Senftenberg / 50 g (Similar results were obtained with contamination levels of 10^2, 10^3 and 10^5 *Salmonella* Senftenberg*)*.

Table A.1

Primary / enrichment	**Secondary enrichment**	**Selective medium**	***Salmonella* Senftenberg H_2S^+ (black)**					
			Set 0 Detection-qualitative					
			Sewage sludge		**Catering waste**		**Liquid manure**	
			37 °C	43 °C	37 °C	43 °C	37 °C	43 °C
Peptone water with Novobiocin	1. Rappaport – Vassiliadis	XLD/BPLS	+	+	+	+	+	+
	2. Tetrathionate with Novobiocin	XLD/BPLS	+	+	+	+	+	+
	3. Rappaport-Vassiliadis with Novobiocin	XLD/BPLS	+	+	+	+	+	+
XLD agar = Xylose - Lysine - Deoxycholate – agar.								
BPLS agar = Brilliant green - Phenol red - Lactose -sucrose– agar.								

A.2 Validation data of a 50 g primary/secondary enrichment method for *Salmonella* from compost in the frame work of a ring – trial in 2002

Salmonella Senftenberg (DSM 10062) on different contamination levels ($10^{1,}$ $10^{2,}$ 10^3 cfu/sample). The compost was heated to 70 °C for 3 days followed by verification of absence of *Salmonella* prior artificial contamination. Samples were sent to 41 different laboratories together with corresponding control samples. Survival of *Salmonella* used for and keeping of the range of contamination during the transport had been verified with the help of selected control samples sent back by the involved laboratories immediately after receiving the samples. The percentage of negative and positive samples in the different contamination range is listed in the Table A.2.

Table A.2 — Results of the ring test with 41 laboratories (comparison in percentages)

(Two laboratories included here were not able to detect *Salmonella* in any sample and did not pass the ring trial)

Results (mean value)	10^1 PFU/50g compost	10^2 PFU/50g compost	10^3 PFU/50g compost	no contamination
Detected	38	37	38,5[a]	39,5[a]
Not detected	3	4	2,5[a]	1,5[a]
Percent laboratories Detected/not detected	92,7 %	90,2 %	93,9 %	96,3 %

[a] Every set of samples contained two different labelled samples with the same spike and with no *Salmonella* respectively.

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

CEN/TC 308 - doc 525:2001, Revision of Directive 86/278/EEC (3rd Draft).

ISO/CD 6340-2:2000, *Water quality – Detection and enumeration of Salmonella*.

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

DIN 38414-13:1992, *German standard methods for the examination of water, waste water and sludge - sludge and sediments (group S) – Detection of salmonellae in disinfected sewage sludge (S 13)*.