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Methods for the validation of biotechnological, thermal and chemical processes for the treatment of animal by-products, sewage sludge and biowastes in order to determine the hygienic safety of the resulting fertilizers or comparable products by exposition of test organisms or test viruses – Part 1 : Validation with vegetative bacteria

Methoden zur Validierung von biotechnologischen, thermischen und chemischen Behandlungsprozessen für tierische Nebenprodukte, Klärschlamm und Bioabfall zur Gewährleistung der hygienischen Unbedenklichkeit der hergestellten Dünger oder vergleichbarer Produkte mittels Exposition von Prüforganismen oder Prüfviren – Teil 1 : Validierung mit vegetativen Bakterien

Méthodes pour validation des procès biotechnologiques, thermiques et chimiques pour traitement des sous-produits animaux, boues et des biodéchets pour atteindre la securitee hygienique des engrais ou produits comparable par exposition des organisme de test ou virus de test- Partie 1 : Validation avec des bacterie vegetative

Descriptors : *Salmonella Senftenberg*, *Enterococcus faecalis*, composting, thermophilic digestion, pasteurization, chemical treatment .

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Foreword

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

This document is a working document.

This standard is divided into five parts:

— *part 1 describes a validation procedure with vegetative test bacteria*

— *part 2 describes a validation procedure with test-viruses*

— *part 3 describes a validation procedure with parasites eggs*

— *part 4 describes a validation procedure with bacterial test spores*

— *part 5 describes a validation procedure with prions*

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Introduction

This document is developed in the framework of the project "Horizontal". It is the result of a desk study "*Prevention-Process control and process Validation*". After discussion with all parties concerned in CEN the standard has been developed further as a modular horizontal method. The capability of a process to inactivate pathogens causing raw-material dependant risks cannot be judged on by analysis of presence or absence of indicators (Bacterial, viral, fungal or parasitic) in the final product. Absence of all or one of the mentioned pathogens or indicators in the final product may be caused by several reasons. They may not be present in the raw material, they may be present in the raw material but in a low count (less than 5 log), the existing methods for reisolation of the target organism are insufficient in the investigated matrix due to ineffective enrichment procedures (e.g. bacteria) or the quantitative isolation of the indicator is technically not possible due to effects of the complicated matrix (e.g. viruses).

Even if the possibility of validating a process by input-output analysis of a certain indicator is generally given and will be covered in WIxxxx.., input- output analysis under practical conditions is depending on the microbiological properties of the input materials processed and therefore limited in feasibility under practical conditions. Therefore other strategies must be followed in such a case, e.g. process validation with one or more representative test–organism. Either if the thermophilic process itself or if a thermal treatment shall provide an inactivation of pathogens belonging to the indicated level of thermo- and chemoresistance, representative test-organisms must be exposed in a similar matrix as treated in a suitable test containment system (test-body) in a defined validation experiment. The relevant process parameters must be recorded during the exposure in order to define the technical conditions to be kept for save inactivation according to the results of the survival experiments and to define the critical control points in the application of a HACCP-concept.

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 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

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1 Scope

This part of the European Standard describes a procedure with vegetative bacteria for the validation of biotechnological, thermal and chemical processes for treatment of animal by-products, sewage sludge and biowastes deemed to provide hygienic safety of the resulting fertilizers or comparable products. The method includes the description of two different test containment systems (test bodies as mentioned in Regulation EC No.208/2006) suitable for the exposure in different treatment processes as well as the methods necessary for the preparation of the inoculum of test bacteria and for the sample processing after exposure.

The method is suitable for determination of the efficiency of biotechnological, thermal and chemical treatment process (CEN/TC 308 – doc. 525 (REVISION of Directive 86/278/EEC – 3rd Draft (1)) and Regulation (EC) No 208/2006 (2)) for the elimination of pathogens in untreated substrates. The treatment processes are validated through to a defined 5 Log_{10} reduction with a test organism such as *Salmonella* Senftenberg W775 negative and *Enterococcus faecalis*. If not otherwise determined by legislation in general *Salmonella Senftenberg* W775 negative shall be used for validation of aerobic thermophilic processes like composting and chemical processes, while predominantly thermophilic anaerobic processes and physical processes shall be validated with *Enterococcus faecalis*.

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 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.

REGULATION (EC) No 208/2006: amending Annexes VI and VIII to Regulation (EC) No 1774/2002 of the European Parliament and of the Council as regards processing standards for biogas and composting plants and requirements for manure.

CEN BT/TF 151 – prEN15215-1 2007: Soils, sludges and treated bio-wastes — Detection and enumeration of Salmonella spp. in sludges, soils, soil improvers, growing media and biowastes – Part 1 : Membrane filtration method for quantification resuscitation of sub-lethally stressed bacteria (to confirm efficacy of log drop treatment procedures)

CEN BT/TF 151 – prEN15215-2 2007: Soils, sludges and treated bio-wastes — Detection and enumeration of Salmonella spp. in sludge, soils, soil improvers, growing media and 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

3 Definitions

For the purposes of this European Standard, the following terms and definitions apply.

3.1
Salmonella spp.
Member of the family of *Enterobacteriaceae*, 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 for the detection of *Salmonella* Senftenberg
The principle of the detections method is described in Documents CEN BT/TF 151 – prEN15215-1, CEN BT/TF 151 – prEN15215-2 and in Annex A (A4).

3.3
Enterococcus faecalis

Enterococcus faecalis is gram-positive, catalase-negative, facultative anaerobes bacteria that grow as diplococci in short chains.

3.4
Method for the detection of *Enterococcus faecalis*
The principle of the detections method is described in Document CEN BT/TF CEN/TC 308: Isolation and enumeration of enterococci in sludges, soils, soil improvers, growing media and biowastes – Part 1: membrane filtration onto selective agars – Part 2: Miniaturised method (Most Probable Number) by inoculation in liquid medium, and in Annex A (A5).

3.5
Vegetative bacteria
Those bacteria which are capable of normal growth in broth or on agar media without pre-culture resuscitation.

3.6
cfu, colony forming unit
Growth of individual bacterial cells into visible colonies on agar media, including on membrane filters overlaying the agar media.

3.8
Spiking
Artificial contamination of a matrix to be exposed to the process with a defined amount of a bacterial suspension of the test strain.

3.9
Matrix

A matrix is a representative sample of the material deemed to be processed, that is spiked with the test strain of interest in order to evaluate the influence of its chemical, physical and biological properties to the inactivation of the exposed bacteria.

3.10
Biotechnological process
A process mainly characterized by the activity of organisms (e.g. microorganisms) under defined conditions for an intended purpose (e.g. composting).

3.11
Thermal process
A process mainly characterized by adding energy in the form of heat under defined conditions for an intended purpose (e.g. pasteurisation).

3.12
Chemical processes
A process mainly characterized by adding a certain amount of chemicals (e.g. lime) to a matrix under defined conditions for an intended purpose (e.g. disinfection).

3.13
Inactivation of bacteria
Reduction of infectivity of a bacteria during biochemical, thermal and chemical processes.

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4 Symbols and Abbreviations

SNA: Standard I nutrient agar

SNB: Standard I nutrient broth

TCS 1: test containment system type 1

TCS 2: test containment system type 2

5 Principle of the validation procedure

The procedure for the validation of various processes using suitable test containment system (TCS)

requires following stages:

1. Preparation of a defined suspension of the bacterial test strain in the laboratory

2. Determination of the initial bacterial count of the prepared suspension

3. Preparation of the substrate to be used as matrix

4. Preparation of test containment system

5.Filling of a defined volume of matrix into TCS and spiking with a certain amount of test bacteria (TCS 1) or spiking a defined volume of matrix with a certain amount of test bacteria and loading the TCS with the contaminated material (TCS 2)

6. Exposure of the TCS to the process at defined places in a suitable manner for the intended exposure time

7 Collecting of TCS and determination of residual bacterial count from the exposed matrix

8. Determination of the inactivation rate.

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 fit for purpose demineralised or distilled water free from substances capable of inhibiting growth under the test conditions. [ISO 8199:2005].

6.1 Standard I nutrient agar (SND) (pH7 ± 0.2)

Peptones	15.0 g
Yeast extract	3.0 g
Sodium chloride	6.0 g
Glucose	1.0 g
Agar agar	12.0 g
Demineralised water	1 000 mL

Dissolve the ingredients under stirring. If necessary, adjust the pH of the solution to 7±0.2 using hydrochloric acid (1 mol/L) or sodium hydroxide solution (1 mol/L). Sterilise in the autoclave (7.1) at 121 ± 3 °C for 15 ± 1 min and pour into culture dishes (7.5).

6.2 Standard I nutrient broth (SNB) (pH7 ± 0.2)

Peptones	15.0 g
Yeast extract	3.0 g
Sodium chloride	6.0 g
Glucose	1.0 g
Demineralised water	1 000 mL

Dissolve the ingredients under stirring. If necessary, adjust the pH of the solution to (7±0.2) using hydrochloric acid (1 mol/L) or sodium hydroxid solution (1 mol/L). Sterilise in the autoclave (7.1) at 121 ± 3 °C for 15 ± 1 min, and fill in 9 mL portions into culture tubes (7.9).

6.3 NaCl solution (0.9 % w/V)

Sodium chloride (NaCl)	9.0 g
Demineralised water	1000 mL

Mix the sodium chloride in water in a 2 000 mL flat bottom flask. Adjust the pH to 7.0 ± 0,2 à 25°C. Fill in 9 mL portions into culture tubes (7.9). Sterilize by autoclaving (7.3) at 121 ± 3 °C for 15 ± 2 min.

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 70 ± 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.

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

7.13 Filter membrane, (0,2 μm cellulose nitrate 47 mm diameter).

7.14 Adjustable micropipettor up to 200 μL capacity.

7.15 Boiling water bath.

7.16 Analytical balance.

7.17 Sterile plastic bottle for example 125 ml, 200 ml and 500 ml

7.18 Autoclave container

8 Procedure

8.1 Preparation of a defined suspension of the bacterial test strain in the laboratory

For the preparation of a defined suspension of the bacterial test strain, the following two lyophilised reference material shall be used:

1. *Salmonella Senftenberg* H_2S negative
   or/and
2. *Enterococcus faecalis*

The preparation of a defined bacterial suspension with *Salmonella Senftenberg* H_2S negative or *Enterococcus faecalis* requires the following three stages:

1. Preparing a re-constituted solution:
   Suspend the reference material in 9 ml Standard I nutrient broth (6.2). Incubate at (36 ± 2) °C (7.2) for (22 ± 2) h in the dark, with or without shaking.

2. Preparing a pure culture (stock culture) by inoculating solid media:
   Streak a loop full (10 µl) (7.7) from Standard I broth (6.2) onto Standard I nutrient agar (6.1). Incubate in the dark at (36 ± 2) °C (7.2) for (22 ± 2) h. After incubation, the plate culture can be stored at 4 °C (7.4) for up to 7 days.

3. Preparing the final bacterial suspension (spiking suspension):
   Transfer aseptically five colonies using a loop (7.7) from Standard I nutrient agar (6.1) directly into 500 ml Standard I broth (6.2). Incubate at (36 ± 2) °C for (22 ± 2) h in the dark, with or without shaking. The resulting bacterial suspension contains approximately 10^7 to 10^9 bacterial colony forming units (CFU) per ml.

This bacterial suspension (spiking suspension) will be used for the artificially contamination of the matrix in the test containment system type 1 and type 2.

8.2 Determination of the initial bacterial count of the prepared suspension

Principe:

1. Take an aliquot (1 ml) out of the fresh prepared final spiking suspension. Prepare a serial tenfold dilution -1 ml of spiking suspension + 9 ml sterile NaCl solution (6.3) - up to 10^{-7}.

2. After mixing, transfer 0.1 ml from the last three dilutions (10^{-5}, 10^{-6} and 10^{-7}) on two Standard I nutrient agar plates (6.1) each.

3. For each plate, dispense inoculums over surface of agar using sterile laboratory spatula (7.11), by rotating the plate by hand or on a turntable.

4. Incubate at (36 ± 1) °C for (20 ± 2) h.

5. Count the number of grown colonies per agar plate. The ideal range of grown colonies is between 30 and 300 per plate. If the number of colonies exceeds the upper range, prepare additional 10^{-8} and 10^{-9} dilutions steps and transfer 0.1 ml per tube on two Standard I nutrient agar plates (6.1) each.

6. Store the spiking suspension and all dilution steps at 4 °C until the final number of colonies on the relevant agar plates is obtained.

Calculate the bacterial concentration (CFU/ml) in the undiluted spiking suspension according to the following equation:

$$CFU / ml = \frac{c}{nxdxv}$$

were:

- CFU: colony forming units
- n: number of plates taken into account in the dilution
- c: sum of CFU counted on the plates taken into account
- d: dilution factor corresponding to the lower dilution
- v: volume of the sample (0,1 ml)

	CFU on Standard I nutrient agar plate (duplicate analyses)		
Example	10^{-6} plates	10^{-7} plates	10^{-8} plates
	120, 104	73, 53	21, 30
Total count	224	126	51

(A): $CFU / ml = \frac{120 + 104}{2x10^{-6} x0{,}1}$ **(B)**: $CFU / ml = \frac{224}{2} x10^{7}$

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(C): CFU /ml = 112 x 10^7 **(D)**: CFU/ml = 1,12 x 10^9

8.3 Preparation of the substrate to be used as matrix

8.3.1 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.

Cleanliness when working is essential. When handling 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.

8.3.2 Substrate for biogas plants and pasteurisations units

Principle:

1. Collect approximately 1 l of the substrate in a plastic bottle (7.18).
2. Sterilize by autoclaving (7.3) at 121 ± 3 °C for 15 ± 2 min. to obtain a substrate free of *Salmonella* spp. and/or *Enterococcus* spp.
3. After autoclaving, adjust the pH value to 6.5 – 7.0.

8.3.3 Substrate for composting

Principle:

1. Collect as much substrate as required for filling the intended number of TCS 2 (calculate approximately 300g of substrate per TCS 2) in containers or sterile plastic bags.
2. After the substrate has been taken, put the substrate into a metal container (7.19)
3. Sterilize by autoclaving (7.3) at 121 ± 3 °C for 15 ± 2 min. to obtain a substrate free of *Salmonella* spp. and/or *Enterococcus* spp.

8.4 Preparation of test containment system

8.4.1 Test containment system type 1 (TCS 1)

This type of containment system will be used for more or less liquid substrates deemed to be processed in a biogas-plant or pasteurization unit (e.g. manure, catering waste, sludge). Several containments of this type may be exposed to the process depending on the mechanical forces to be expected at the localization of exposure either fixed to a rod without mechanical protection or in a suitable protection device, examples are given in Annex A.

Description:

The TCS 1 (see Fig. 1 in Annex A) consists of a hollow synthetic cylinder (polycarbonate base) covered on both sides with semi-permeable polycarbonate membranes (pore size 20 µm), which are fixed with two

tight screwable ring. The polycarbonate filter membrane allows access of soluble and gaseous compounds, from the substrate to the test organisms without contamination of the environment

8.4.2 Test containment system type 2 (TCS 2)

This type of containment system will be used for substrates deemed to be used in composting.

Description:

The TCS 2 consists of 225 g of matrix (compost) spiked with 25 ml of bacterial test strains. The thoroughly mixed sample is placed into a 30 x 30 cm bag made out of raschel bag material (see section 8.5.2).

8.5 Filling and inoculation of the containment system

TCS 1 and TCS 2 are treated different in filling and re-isolation of test bacteria.

8.5.1 Containment system type 1

Principle:

- Close one side of the test container with membrane support, membrane filter and lock-ring (see Appendix I Fig. 1). Place an empty Petri-dish on the scale and put the TCS 1 with the open side up into it. Weigh 9 g of substrate directly into TCS 1.
- Add 1 ml of spiking suspension (8.1) with *Salmonella Senftenberg* or *Enterococcus faecalis*-using sterile graduated pipettes.
- mix thoroughly using a sterile glass rod
- close the TCS 1 with membrane support, membrane filter and lock-ring (see Appendix I Fig. 1)
- The final bacterial count in the TCS 1 shall be between 10^7 and 10^8cfu/g.
- Prepare at least one TCS 1 more than required for exposure to the process to be validated as untreated control (UC)
- Store inoculated TCS 1 for a maximum of 15 h at temperatures between +4 °C and +8 °C until they are exposed in the biogas plant or pasteurisations unit,

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NOTE: The bacterial count in the TCS 1 depend on the bacterial concentration in the spiking suspension and the type of spiked substrate and shall be verified immediately using method described in Annex A before exposure of the test containment to the process. (unexposed control)

Example:

CFU/ml in spiking suspension	Volume of spiking suspension used for artificially contamination	Substrate in TCS 1 (in g)	Final bacterial concentration in spiking substrate in TCS 1 (in CFU/ml)
10^9	1 ml	9 g	10^8
10^8	1 ml	9 g	10^7
10^7	1 ml	9 g	10^6

8.5.2 Containment system type 2

Principle:

- Weigh 225 g of substrate into a 1l sterile plastic bottle
- Inoculate this substrate with 25 ml of spiking suspension (8.1) with *Salmonella Senftenberg* or *Enterococcus faecalis*-using sterile graduated pipette by adding aliquots of 3ml in 5 steps
- After each step cap the flask and shake vigorously by hand, before adding a new aliquot. Ensure finally complete mixing
- this is the inoculated TCS 2 with final concentration of used bacteria between 10^7 and 10^8cfu/g.

NOTE: The bacterial count in the TCS 2 depend on the bacterial concentration in the spiking suspension and the type of spiked substrate and shall be verified immediately using on of the CEN methods (prEN15215-1 or prEN15215-2) before exposure of the test containment to the process. (unexposed control)

NOTE: The bacterial concentration in the TCS 1 depend on the bacterial concentration in the spiking suspension.

CFU/ml in spiking suspension	Volume of spiking suspension used for artificially contamination	Substrate in plastic beaker (in g)	Final bacterial concentration in spiking substrate in TCS 1 (in CFU/ml)
10^9	25 ml	225 g	10^8
10^8	25 ml	225 g	10^7
10^7	25 ml	225 g	10^6

- If sample was being spiked, a minimum of 1 h shall elapse between spiking and transfer into TSC 2
- After 1 h transfer spiked substrate into TSC 2 and store in the refrigerator at +4 °C until they placement into composting plant

8.6 Exposure of the TCS to the process at defined places in a suitable manner for the intended exposure time

The exposure of the TCS to the treatment process is carried out depending on the situation in the treatment process. The TCS must be exposed for a maximum of time according to the shortest amount of time that substrate can be expected to be exposed to the treatment process (e.g. pasteurisation at 70 °C for 60 min.).

TCS must be introduced at various locations within the process, being representative for the process and such can be regarded as critical control points. In processes where no even distribution of temperature or concentration of chemicals can be expected the sites of exposure shall represent the locations of lowest temperature or concentration, of the highest temperature or concentration and of a medium temperature or concentration. In general a minimum of 10 TCS must be introduced at such representative locations in the process. In processes in which an even distribution of temperature or chemicals can be expected (e.g. pasteurisation units) due to measurements previously carried out at least 4 TCS shall be exposed. At the places where the TCS are exposed the relevant process parameters (e.g. temperature or pH-value) shall be measured and recorded in parallel, those data are required for future steady supervision of the process at critical control points in routine operation.

8.7 Collecting of TCS and determination of residual bacterial count from the exposed matrix

Remove TCS after exposure and transport them stored at 4 °C to 8°C into laboratory and process them as soon as possible but not later than 12 h. If chemical processes are validated (e.g. lime-treatment) immediate neutralisation of the contaminated substrate from TCS is necessary. Start with microbiological analyses using methods prEN15215-1 or prEN15215-2 (TCS 2) and methods A4 and A5 given in Annex A (TCS 1).

8.8 Determination of the inactivation rate.

The validation of biotechnological, thermal and chemical processes must demonstrate that the process achieves the following overall reduction:

- reduction of 5 log10 of *Enterococcus faecalis* or *Salmonella* Senftenberg (775 H_2S negative)

If the reduction of *Enterococcus faecalis* or *Salmonella* Senftenberg (775 H_2S negative) after exposure does not comply with the above requirement, the process will be regarded as insufficient for hygienisation of substrate.

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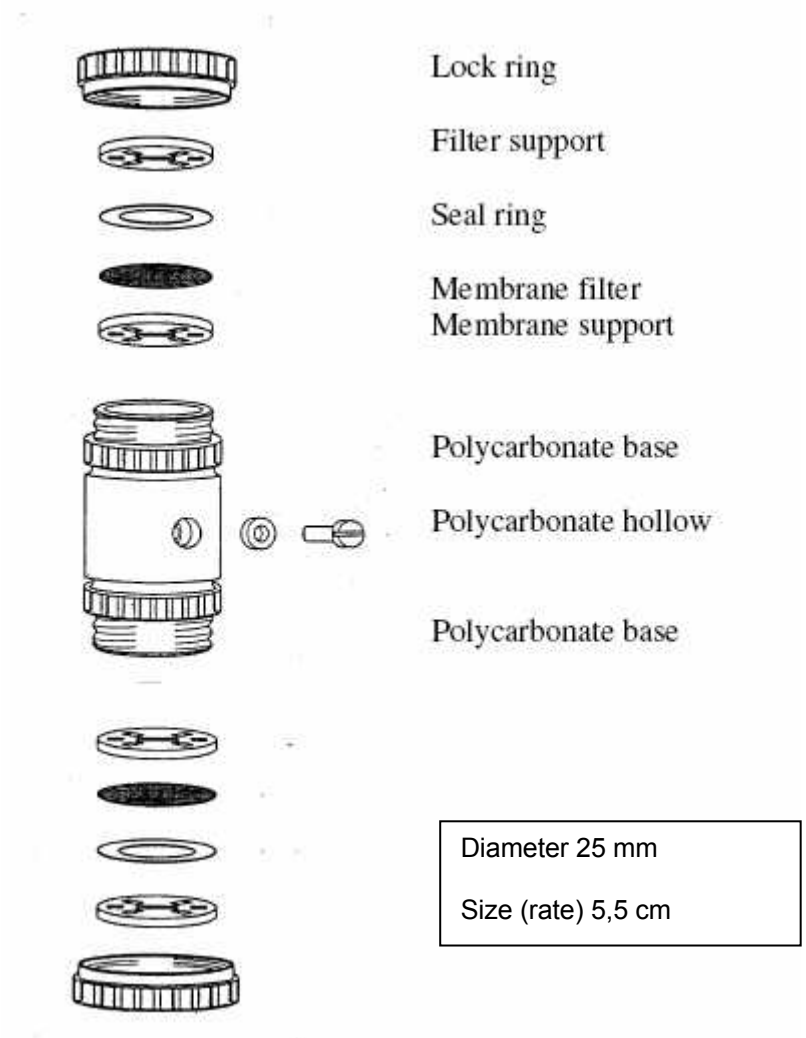
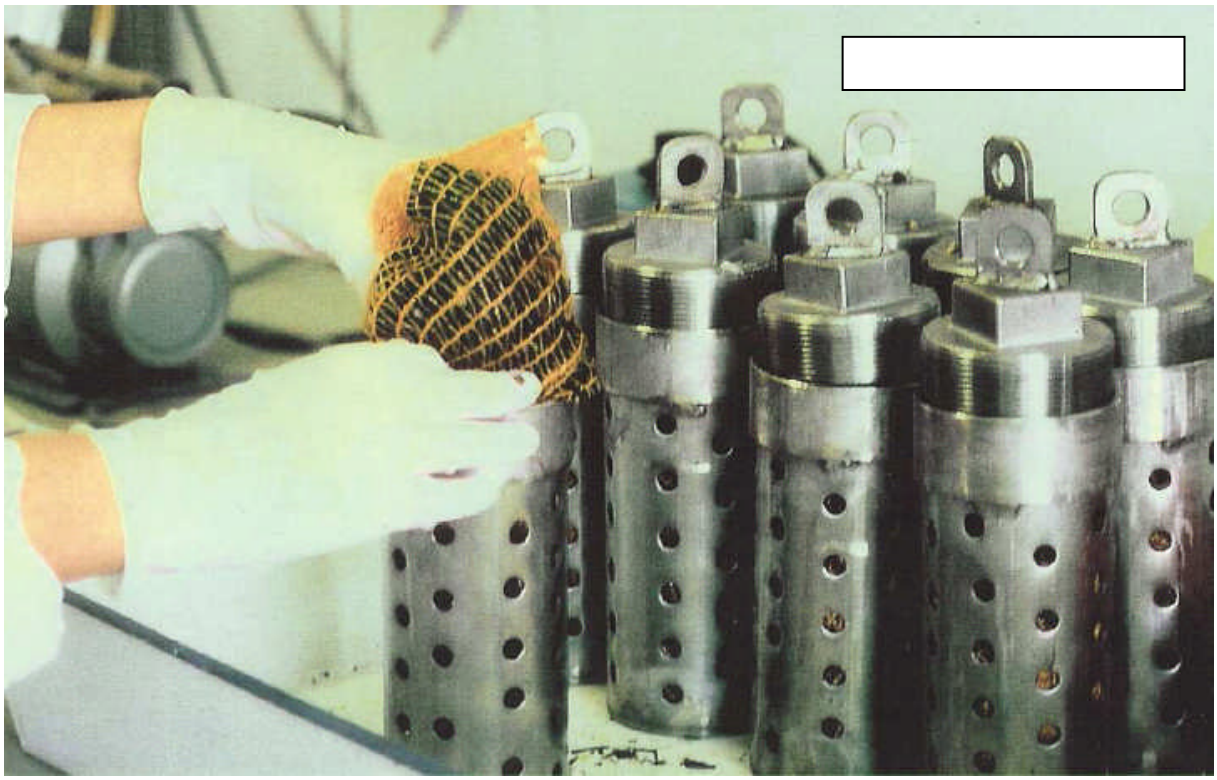
Annex A
(informative)

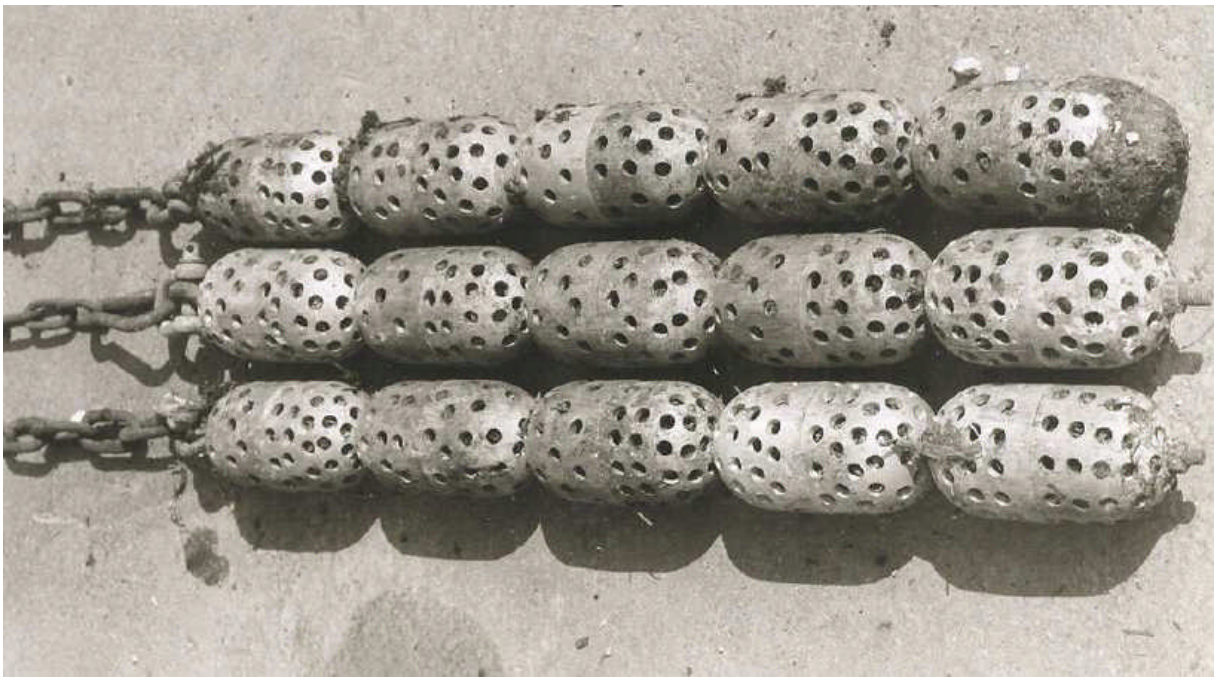
A1: Fig. 1



A2: Fig. 2



A3: Fig. 3



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Most Probable Number (MPN) - method for quantitative resuscitation of *Salmonella* Senftenberg H_2S negative (to confirm efficacy of log reduction treatment procedures) in 1 g of spiked sample

1. Scope

This method describes a most probable number (MPN) method for the semi-quantitative detection of *Salmonella* Senftenberg H2S negative in 1 g of spiked samples.

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

2. Definitions

For the purposes of this document, the following terms and definitions apply.

2.1
method definition
Salmonella spp. capable of being enriched in buffered peptone water and growth in RV medium (Rappaport Vassiliadis medium) followed by characteristic growth on BPLS agar (Brilliant green-phenol red-lactose-sucrose agar) at (36 ± 2) °C

2.2
Most probable number (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.

2.3
cfu, colony forming unit
growth of individual bacterial cells into visible colonies on agar media

2.4
sub-lethally damaged bacteria
those bacteria which have been stressed but not killed in treatment processes or storage

2.5
resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria previously incapable of growth on agar media

2.6
presumptive positives
isolates which are believed to be *Salmonella* spp., but not yet confirmed

3. Symbols and abbreviations

MPN: Most Probable Number

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

cfu: colony forming unit

RV medium: Rappaport Vassiliadis medium

TCS 1: test containment system type 1

4. Principle

This is a MPN method including designed to process samples of 1 g wet weight from the spiked sample.

In this method the detection of *Salmonella* Senftenberg H_2S negative is undertaken in seven steps.

a) suspension of the sample in 0.9 % m/V sodium chloride;

b) serial dilution of this suspension in the same diluent (from 10^{-1} up to 10^{-8})

c) pre-enrichment of bacteria in a primary medium;

d) enrichment in a selective medium (selective enrichment);

e) preparation of pure cultures by inoculation on one special solid media with subcultures;

f) serological confirmation;

g) Quantification by the MPN technique.

5. Reagents, diluents and culture media

5.1 General instructions

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].

5.2 NaCl solution (0.9 % w/V)

- Sodium chloride (NaCl) 9.0 g
- Demineralised water 1000 mL

Mix the sodium chloride in water in a 2 000 mL flat bottom flask. Adjust the pH to 7.0 ± 0,2 à 25°C. Fill in 9 mL portions into culture tubes (6.9).

Sterilize by autoclaving (6.3) at 121 ± 3 °C for 15 ± 2 min.

5.3 Buffered Peptone Water

- Peptone from casein 10 g
- Sodium chloride 5 g

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- Potassium dihydrogen 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 hydroxid solution (1 mol/L). Divided in 9 mL portions into culture tubes (6.9) and sterilised by autoclave (15±1) min at (121±2) °C.

5.4 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 (1mol/L) or sodium hydroxide (1mol/L) solution. Transfer 10 mL portions of the solution to culture tubes (6.9) and sterilized by autoclave (6.3) at 115±2 °C for 15±1 min.

5.5 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. Sterilize in an autoclave (6.3) for 15 ± 1 min at 121 ± 3 °C and pour into culture dishes (6.5).

5.6 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 (6.3) for 15 ± 1 min at 121 ± 3 °C and pour into culture dishes (6.5).

6. 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:

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

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

6.3 Autoclave (Steam sterilizer).

6.4 Refrigerator.

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

6.6 Sterile graduated pipettes, of nominal capacities 1 and 10 mL.

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

6.8 Apparatus for shaking the culture tubes.

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

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

6.11 Laboratory spatula.

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

6.13 Adjustable micropipettor up to 200 µL capacity.

6.14 Boiling water bath.

6.15Analytical balance.

7. Procedure

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7.1 Sample preparation

- Place 1 g (wet weight) of spiked sample from the TCS 1 into 9 mL sterile 0.9 % NaCl solution.
- Shake at on vortex mixer (6.10) for 3 min.

7.2 Analysis

7.2.1 Preparation of the serial tenfold dilution

- Take an aliquot of 1 mL out of the primary prepared suspension (7.1) from sample preparation.
- Prepare a serial tenfold dilution up to 10^{-8}: 1 mL of prepared suspension (7.1) + 9 mL of sterile 0.9 % NaCl solution.

7.2.2 Preenrichment

- From each dilution step, transfer 1 mL per tube (7.1) into 3 tubes containing 9 mL of buffered peptone water (5.3) each.
- Incubate for 21 ± 3 h at 36 ± 2 °C.

NOTE 1- The number of dilution steps:

- for untreated spiked sample a dilution up to 10^{-8} should be carried out.
- for treated spiked sample, dilution up to 10^{-5} should be sufficient.

NOTE 2-The primary solution (described in 7.1) is already diluted as 10^{-1}. From this primary dilution, transfer 1 mL into three tubes each containing 9 mL buffered peptone water (5.3), as a first dilution step.

7.2.3 Enrichment

Aseptically transfer 0,1 mL of each pre-enrichment culture obtained as described in 8.2.2 to a culture tube containing 10 mL of Rappaport-Vassiliadis medium (5.4). Incubate at 41 ± 1 °C for 21 ± 3 h.

7.2.4 Plating out

Remove culture liquid from the selective enrichment cultures obtained as described in 8.2.3 using a 10 µL sterile inoculation loop, and streak on BPLS (5.5) culture plates Incubate aerobically at 36 ± 2 °C for 21 ± 3 h.

NOTE 1- one agar plate is divided into 3 equal parts

Typical colonies on BPLS medium appear pink.

7.2.5 Serological confirmation

Pure cultures should be prepared by the standard methods used in medical diagnostic laboratories. Only pure cultures should be used for the subsequent serological test.

For serological confirmation subculture at least two typical colony from BPLS medium (5.5) on standard I nutrient agar plates (5.6) and incubate the plates at (36 ± 2) °C for (21 ± 3) h to obtain a pure culture.

8. Determination of the Most Probable Number (MPN)

- For each of the 8 dilutions (from 10^{-1} to 10^{-8}), note the number of positive fields with *Salmonella* Senftenberg H2S negative on BPLS plates (5.5).

- Identify the corresponding characteristic number (CN). The CN corresponds to the number of positive tubes of the 3 last dilutions giving a number of positive tubes $>$ 0.

- Calculate the MPN corresponding to the identified characteristic number using the De Man table (1983) by multiplying the MPN index by the dilution factor. The result corresponds to a MPN per mL of primary prepared suspension.

For example:

Dilution step	Positive fields (*Salmonella* colonies)		
10^{-1}	+	+	+
10^{-2}	+	+	+
10^{-3}	+	+	+
10^{-4}	+	-	-
10^{-5}	-	+	-
10^{-6}	-	-	-
10^{-7}	-	-	-
10^{-8}	-	-	-
Characteristic number	3	1	1
MPN table	7.5		
Dilution factor	10^{-3}		
Result: MPN *Salmonella* Senftenberg H_2S negative /g (wet weight) of original spiked sample	7.5×10^3		

The result per gram of sample material is MPN *Salmonella* Senftenberg H_2S negative /g (wet weight) of original spiked sample from the TCS 1.

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A5

Most Probable Number (MPN) - method for quantitative resuscitation of *Enterococcus faecalis* (to confirm efficacy of log reduction treatment procedures) in 1 g of spiked sample

1. Scope

This method describes a most probable number (MPN) method for the semi-quantitative detection of *Enterococcus faecalis* in 1 g of spiked samples.

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

2. Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
method definition
for the purpose of the present method, the following *Enterococci* definition shall apply: Enterococci capable of being aerobically enriched in Azide dextrose broth at (36 ± 1) °C followed by characteristic growth on Kanamycin esculin agar at (36 ± 1) °C.

2.2
Most probable number (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.

2.3
cfu, colony forming unit
growth of individual bacterial cells into visible colonies on agar media

2.4
sub-lethally damaged bacteria
those bacteria which have been stressed but not killed in treatment processes or storage

2.5
resuscitation
stimulation to vegetative growth of sub-lethally damaged bacteria previously incapable of growth on agar media

2.6
presumptive positives
isolates which are believed to be *Enterococcus faecalis*, but not yet confirmed.

3. Symbols and abbreviations

MPN: Most Probable Number

AD broth: Azide dextrose broth

KEA agar: Kanamycin esculin azide agar

cfu: colony forming unit

TCS 1: test containment system type 1

4. Principle

This is a MPN method including designed to process samples of 1 g wet weight from the spiked sample.

In this method the detection of *Enterococcus faecalis* is undertaken in four steps.

h) suspension of the sample in 0.9 % m/V sodium chloride;

i) serial dilutions of this suspension in the same diluent (from 10^{-1} up to 10^{-8});

j) enrichment of bacteria in a selective liquid medium

k) preparation of pure cultures by inoculation on one special solid media with subcultures;

l) serological confirmation;

m) Quantification by the MPN technique;

5. Reagents, diluents and culture media

5.1 General instructions

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].

5.2 Azide dextrose broth

Mix the following substances in 1 000 ml water in a 2 000 ml flat bottom flask:

- 20.0 g peptone from casein
- 4.8 g meat extract
- 7.5 g D (+) - glucose
- 7.5 g sodium chlorure
- 0.2 g sodium azide

Adjust pH to (7.2 $\pm$ 0.2) at 25°C using sodium hydroxide or hydrochloric acid (0,1 mol L^{-1}mol/l), and the final volume to 1 000 mL. Pour 9 ml fractions into 25 ml tubes (6.8). Sterilize the solution in an autoclave (6.3) at (121 $\pm$ 3) °C for (15 $\pm$ 1) min

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WARNING – Sodium azide mixture is toxic if swallowed. Contact with acids liberates very toxic gas.

5.3 NaCl solution (0.9 % m/V)

Mix the following substance in 1 000 ml water in a 2 000 ml flat bottom flask.

— 9,0 g sodium chloride

sterilize in an autoclave (steam sterilizer) (6.3) for (15 ± 2) min at (121 ± 3) °C and fill into culture tubes (6.9) in 9 ml portions under sterile conditions.

5.4 Kanamycin esculin azide agar

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

- 20.0 g peptone from casein
- 5.0 g yeast extract
- 1.0 g sodium citrate
- 0.15 g sodium azide
- 0.02 g kanamycin sulfate
- 1.0 g esculine
- 0.5 g Ammonium iron (III) citrate
- 15.0 g agar-agar

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

Sterilize the solution in an autoclave (steam sterilizer) (6.3) for (15 ± 1) min at (121 ± 3) °C. Adjust the pH to 7.1 ± 0.2 at 25 °C using (1 mol/ l) sodium hydroxide solution and pour into sterile Petri dishes (6.5).

5.5 Standard I nutrient agar

prepared according the manufacturer or:

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

- 15.0 g peptones
- 3.0 g yeast extract
- 6.0 g sodium chloride
- 1.0 g D (+) - glucose
- 12.0 g agar-agar

Heat the mixture in a boiling water bath until its components have dissolved. Sterilize the solution in an autoclave (steam sterilizer) (6.3) for (15 ± 1) min at (121 ± 3) °C. Adjust the pH to 7.1 ± 0.2 at 25 °C using (1 mol/ l) sodium hydroxide solution and pour into sterile Petri dishes (6.5).

6. 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:

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

6.2 Thermostatic incubators regulated at (42 ± 1) °C (static) for anaerobic jar or anaerobic chamber regulated at (42 ± 1) °C

6.3 Autoclave (Steam sterilizer).

6.4 Refrigerator.

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

6.6 Graduated pipettes, of nominal capacities 1 and 10 ml.

6.7 Apparatus for shaking the glass flask..

6.8 Culture tubes, 25 ml capacity, or equivalent containers.

6.9 Vortex mixer suitable for of 25 ml capacity culture tubes or equivalent containers.

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

6.11 Boiling water bath.

6.12 **Inoculating loop** (10μl) (e.g. platinum-iridium wire), loop diameter approximately 3 mm

6.13 Laboratory spatula

6.14 Stirrer and magnetic bars

7. Procedure

7.1 Sample preparation

Place 1 g (wet weight) of spiked sample from the TCS 1 into 9 mL sterile 0.9 % NaCl solution. Shake at on vortex mixer (6.10) for 3 min.

7.2 Analysis

7.2.1 Preparation of the serial tenfold dilution

- Take an aliquot (1 ml) out of the primary prepared suspension (PS) (7.1) from sample preparation.
- Prepare a serial tenfold dilution (1 ml of PS (7.1) + 9 ml 0.9 % sterile NaCl solution) up to 10^{-8}

7.2.2 Enrichment in the selective liquid media

- From each dilution step, transfer 1 ml per tube into 3 tubes containing 9 ml AD broth (5.2) each.
- Incubate at (36 ± 2) °C for (40 ± 2) h.

NOTE 1- The number of dilution steps:

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- for untreated spiked sample a dilution up to 10^{-8} should be carried out.

- for treated spiked sample, dilution up to 10^{-5} should be sufficient.

NOTE 2-The primary solution (described in 7.1) is already diluted as 10^{-1}. From this primary dilution, transfer 1 mL into three tubes each containing 9 mL AD broth (5.2), as a first dilution step.

7.2.3 Plating out

Remove culture from the selective enrichment cultures obtained using a sterile inoculation loop, and streak on KEA agar (5.4) and incubate aerobically at (36 ± 2) °C for (40 ± 2) .

NOTE 1- one agar plate is divided into 3 equal parts.

Typical colonies on KEA (5.4) agar are white or grey surround by black yards.

7.2.4 Serological confirmation

Pure cultures should be prepared by the standard methods used in medical diagnostic laboratories. Only pure cultures should be used for the subsequent serological test.

Subculture at least three typical black colonies from KEA agar on Standard I nutrient agar plates (5.5) and incubate at (36 ± 2) °C for (22 ± 2) h to obtain a pure culture for confirmation steps. Further confirmation of *Enterococci* can also be achieved using co – agglutination technique (Phadebact ® Streptococcus test or equivalent), according to manufacturer's instructions.

8. Determination of Most Probable Number (MPN)

- For each of the 8 dilutions (from 10^{-1} to 10^{-8}), note the number of positive fields with *Enterococci* on KEA agar (between 0 and 3).

- Identify the characteristic number corresponding to the number of positive fields of the 3 last dilutions giving a number of positive tubes >0.

- Calculate the MPN corresponding to the identified characteristic number using DE MAN table (1983 – European Journal Applied Microbiol. Biotechnol. 17, 301-305). The result correspond to a MPN per ml of primary prepared suspension.

For example:

Dilution step	Positive fields (*Enterococci* colonies)		
10^{-1}	+	+	+
10^{-2}	+	+	+
10^{-3}	+	+	+
10^{-4}	+	-	-
10^{-5}	-	+	-
10^{-6}	-	-	-
10^{-7}	-	-	-
10^{-8}	-	-	-
Characteristic number	**3**	**1**	**1**
MPN table	7.5		
Dilution factor	10^{-3}		
Result: MPN *Enterococci* /g (wet weight) of original sample	7.5×10^{3}		

The result per gram of sample material is MPN *Enterococci* /g (wet weight) of original sample f :

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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		≥ 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	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		≥ 95 %	≥ 95 %	≥ 99 %	≥ 99 %
3	3	2	110	20	400	10	570
3	3	3	> 110				

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