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 determination of the count of selected endogenous organisms in the substrate before and after processing and calculation of the reduction rate (Input-Output-Analysis)

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 durch Ermittlung der Anzahl ausgewählter endogener Organismen im Substrat vor und nach der Behandlung zur Errechnung der Reduktionsrate (Input-Output Analyse)

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 enombrement des organisme selecté du substrate avant et aprés traitment pour calculer la rate de recuction (Analyse Input-Output)

Descriptors : *Escherichia coli*, *Enterococci*,Coliphages 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.

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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 this standard, 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 (see WI ……).. 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 endogenous vegetative bacteria and bacteriophages 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 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 Log_{10} reduction with selected organisms present in the untreated substrate such as *Enterococci, Escherichia coli and* somatic coliphages. *Enterococci* and *E. coli* can be regarded within certain limitations as being representative for non sporulating bacterial pathogens and pathogenic viruses with moderate chemo- and thermoresistance while somatic coliphages can be used as substitute for thermoresistant pathogenic viruses. If not otherwise determined by legislation in general *E. coli* and *Enterococci* shall be used for validation of aerobic thermophilic processes like composting and chemical processes, while predominantly thermophilic anaerobic processes and thermal processes shall be validated with *Enterococci* only and if reduction of thermoresistant viruses is required with somatic coliphages.

Note: If the bacterial count in the untreated substrate (input material) is below 10^6 cfu/ml or g or the phage-count is below 10^5 pfu/ml or g this validation method cannot be applied. (see 9).

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 – prEN15214-1 2007: Soils, sludges and treated bio-wastes — Detection and enumeration of *Escherichia coli* in sludges, soils, soil improvers, growing media and biowastes – Part 1 , Part 2 and Part 3.

CEN BT/TF CEN/TC 308: Isolation and enumeration of enterococci in sludges, soils, soil improvers, growing media and biowastes – Part 1 and Part 2.

CEN BT/TF CEN/TC: Isolation and enumeration of bacteriophages in sludges, soils, soil improvers, growing media and biowastes

CEN BT/TF 151 – WP 3-Part I 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, 2 and 3:

3 Definitions

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

3.1
Enterococci

Enterococci are a group of gram-positive, catalase-negative, facultative anaerobic bacteria that grow in short chains.

3.2
Method for the detection of *Enterococci*
The principle of the detection 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.

3.3
Escherichia coli

Escherichia coli, belongs to the family of *Enterobacteriaceae*, is Gram-negative, non-sporulating, rod-shaped, lactose positive and able to grow at 44 °C. Most *E. coli* strains are able to produce indole from tryptophane and are β-glucuronidase-positive

3.4
Method for the detection of *Escherichia coli*
The principle of the detections method is described in Documents CEN BT/TF 151 – prEN15214-1, 2 and 3.

3.5
Somatic coliphages

Somatic coliphages are bacteriophages which consist of a capsid containing DNA as the genome.

3.6
Method for the detection of bacteriophages
The principle of the detections method is described in Documents CEN BT/TF 151 (Extraction of bacteriophages from sludge, soils and treated biowaste).

3.7
Vegetative bacteria
Those bacteria which are capable of normal growth in broth or on agar media without pre-culture resuscitation.

3.8
cfu, colony forming unit
Growth of individual bacterial cells into visible colonies on agar media, including on membrane filters overlaying the agar media.

3.9
pfu, plaque forming unit
A plaque forming unit is a measure of the number of particles capable of forming plaque per unit volume.

3.10
Spiking
Artificial contamination of a matrix to be exposed to the process with a defined amount of a bacterial suspension of the test strain.

3.11
Matrix

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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.

3.12
Biotechnological process
A process mainly characterized by the activity of organisms (e.g. microorganisms) under defined conditions for an intended purpose (e.g. composting).

3.13
Thermal process
A process mainly characterized by adding energy in the form of heat under defined conditions for an intended purpose (e.g. pasteurisation).

3.14
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).

3.15
Inactivation of microorganisma
Reduction of infectivity of a microorganisms during biochemical, thermal and chemical processes.

4 Symbols and Abbreviations

TGB: Tryptone-glucose-broth

MYA: Yeast extract agar

5 Principle of the validation procedure by input-output analysis

The procedure for the validation of various processes using input-output analysis requires following stages:

1. Determination of the minimum retention time of feeding substrate in a tracer experiment with *Bacillus globigii* spores (biological tracer)[1]
2. Determination of the initial count of the selected endogenous organism in untreated substrate
3. Determination of the final count of the selected endogenous organism in treated substrate
4. Calculation of the inactivation rate.

Note 1: Any other method for determination of the minimum retention time may be used, if validated with the involved matrix.

The validation by input-output analysis shall be carried out twice at separate periods of time. If the process to be validated is influenced by climatic factors at least one validation shall be done in the warm and one in the cold season.

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 Yeast extract agar (MYA) (pH7 ± 0.2)

Meat peptones	10.0 g
Yeast extract	2.0 g
$MnSO_4$, H_2O	0.04 g
Glucose	1.0 g
Agar agar	15.0 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).

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6.2Standard 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).

6.3 Tryptone glucose broth (TGB) (pH7 ± 0.2)

Tryptone	5.0 g
Yeast extract	2.5 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 10 mL and 100 ml portions into culture tubes (7.9).

6.4 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.

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

7.13 Adjustable micropipettor up to 200 μL capacity.

7.14 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

7.19 **Centrifuge**

8 Determination of the minimum hydraulically retention time and pas through curve with *Bacillus globigii* spores suspension

The preparation of a defined *Bacillus globigii* spores suspension shell be done according to CEN Draft prEN 14347.

8.1 Preparation of the *Bacillus globigii* spores suspension

The volume of spore sustension to be prepared depends on the dilution factor to be expected in the process. The spore count to be expected after dilution in the process shall be at least 10^3 cfu/ml .

Principle:

- Inoculate TGB broth (6.2) agar with approximately 10^6 cfu/ml (1 ml) bacterial spores from defined *Bacillus globigii* spores suspension
- Incubate at (30 ± 2) °C (7.2) for (22 ± 2) h in the dark, with or without shaking.
- Transfer aseptically 2-3 ml of prepared suspension to MYA agar
- Incubate at (30 ± 2) °C for (72 ± 2) h
- Control the growth of typical pink colonies on the MYA agar and verify sporulation by microscopic examination of a bacterial smear every day
- Incubate until the maximum sporulation rate is reached and remaining vegetative cells are getting autolytic but not longer than 10 days
- Wash off spores from the agar surface with approximately 10 ml of water

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- Transfer the spores suspension from the MYA agar using a pipette into the centrifuge tubes and centrifuge at 2000 g at 4 °C for 20 minutes
- Transfer the centrifuged suspension into the sterile glass flask, heat for 10min ± 1 min at 75 °C ± 2 °C and adjust the bacterial count to 1x 10^9 to 5x10^9 cfu/ml
- This is the *Bacillus globigii* spores suspension

8.2 Exposure of the *Bacillus globigii* spores to the process

The spore suspension shall be added direct to the process by mixing it into the feeding material directly before entering the process. A sample for the determination of the initial count shall be taken immediately after mixing. In processes which are operating in a semi-batch mode the first sample has to be taken at the first harvesting period and at least five subsequent samples have to be taken at least for the next 48h.

If the process is run continuously, the first sample have to be taken immediately but not later than 5 min after feeding the reactor with the spiked material. If no other technical informations are available concerning the through flow of material at least every hour a sample have to be taken

20 ml/g of every sample are transferred into 180 ml 0.9 % sterile NaCl solution.and shake at a minimum of 150 rpm for 30 min. up to 20 h at (5 ± 3) °C.

Principe:

1. Take an aliquot (1 ml) out of this suspension. Prepare a serial tenfold dilution -1 ml of suspension + 9 ml sterile NaCl solution (6.4) - up to 10^{-3}.

2. After mixing, transfer 0.1 ml from the dilutions on two Standard I nutrient agar plates (6.2) 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. Identify the plates on which first grow of *Bacillus globigii* occurs and count the number of typical pink grown colonies per agar plate.

6. Store the 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 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)

9 Determination of the initial microorganisms count in untreated substrate (Input)

If not otherwise determined by legislation, 10 samples have to be taken from the input material (untreated substrate) and the count of the selected organisms has to be determined by the corresponding method (see 3).

Note 1: Prior any further preparation of materials and the determination of the minimal retention time a preliminary determination of the initial count of the targeted microbial parameters has to be done. If the bacterial count is below 10^6 cfu/ml or g or the phage-count is below 10^5 pfu/ml or g this validation method cannot be applied. In this case 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 has to be done.

10 Determination of the microbial count in treated substrate (Output)

Samples have to be taken in relation to the samples deemed for the determination of the initial count and by taking into account the results of the tracer experiments. The time between the taking of the input samples and taking of the output samples shall resemble the minimum exposure time.

If not otherwise determined by legislation, 10 samples have to be taken from the output material (treated substrate) and the count of the selected organisms has to be determined by the corresponding method (see 3).

11 Determination of the inactivation rate.(IR)

Calculate the median value from the results determined from the input samples (med I) and of those fom the output samples (med O). The inactivation rate in the process is calculated by the following equation:

lg IR =lg med I – lg med O

If not otherwise determined by legislation lg IR shall be 5 with bacterial parameters and 4 with bacteriophages. If the reduction after exposure does not comply with the above requirements, the process will be regarded as insufficient for hygienisation of substrate.

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