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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 3 : Validation with parasites eggs

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 3 : Validierung mit Parasiteneiern

Méthodes pour validation des procès biotechnologiques, thermiques et chimiques pour traitement des sous-produits animaux, boues et des bio déchets pour atteindre la securitee hygienique des engrais ou produits comparable par exposition des organisme de test ou virus de test- Partie 3 : Validation avec des oefs des parstites

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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 parasites eggs 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 one suitable test containment system (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 inoculums of test viruses 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. In general thermal processes validated with vegetative bacteria require no additional validation with parasites because according to the state of science their thermoresistance is lower than that of the test bacteria described in Part 1. Application of this standard is generally required for the validation of chemical processes. The treatment processes shall provide a reduction of viable eggs of *Ascaris sp.* by at least 99,9 % (3 log10).

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.

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.

3 Definitions

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

3.1
helminth ova
helminths are parasitic worms. Helminths typically enter the body through the mouth and lodge in the intestines where they hatch, grow into adult worms, and begin producing eggs or ova. The ova are then passed out of the body with feces. Some helminths can infect areas of the body other than the intestines. Helminth ova were commonly found in relatively large numbers in environment.

There are three main classes of helminths that are important from a human health standpoint: nematodes (roundworms), cestodes (tapeworms), and trematodes (flukes).
The helminth ova are among the more particularly targeted in Europe for the sludge monitoring, the nematodes ova as Trichuridae (Trichuris, Capillaria) and as Ascaridiae (Ascaris, Toxocara) and the cestodes ova as Taeniidae (Taenia) and as Hymenolepidiae (Hymenolepis).

3.2
viable ova
ova are considered as being viable or potentially viable when the integrity of their structure can be observed or if a larval development is detected inside

3.3
non viable ova
Any absence or disorganisation of internal/external structures is criteria attesting the non viability of ova.

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3.4
inactivation of parasites ova
reduction of infectivity of a parasites ove during biochemical, thermal and chemical processes

3.5
parasites ova suspension
suspension of a defined parasites ova strain that is used for the validation of biochemical, thermal and chemical processes

3.6
biotechnological process
A process mainly characterized by the activity of organisms (e.g. microorganisms) under defined conditions for an intended purpose (e.g. composting)

3.7
thermal process
A process mainly characterized by adding energy in the form of heat under defined conditions for an intended purpose (e.g. pasteurization)

3.8
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)

4 Symbols and Abbreviations

TCS 3: test containment system type 3

5 Principle of the validation procedure

The procedure for the validation of processes using suitable test containment system (TCS 3) requires following stages:

1. Preparation of the parasites ova suspension

2. Preparation of test containment system type 3 (TCS 3)

4. Exposure of the TCS 3 to the process at defined places in a suitable manner for the intended exposure time

5. Collecting of TCS 3 and determination of residual parasites ova from the exposed TCS 2

6. Determination of the inactivation rate.

6 Reagents, diluents and culture media

Normal tap water is used for all parasitological investigations described here.

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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 Apparatus for sterilization for moist heat sterilization, an autoclave capable of being maintained at (121 ±3) °C for a minimum holding time of 15 min;

7.2 Wide-mouth glass flasks or beakers

7.3 Inverted microscope

7.7 Containers: sterile test tubes, bottles or flasks of suitable capacity.

7.8 Petri dishes, (**plates**) of size 30 mm to 100 mm.

7.9 Graduated sterile pipettes, of nominal capacities 10 ml and 1 ml and 0,1 ml. Calibrated automatic pipettes with disposable sterile tips may be used.

7.10 Refrigerator capable of being controlled at 2 °C to 8 °C.

7.11 Tweezers

7.12 Scalpel

7.13 Forceps

7.14 Funnel

7.15 Silk gauze for the preparation of TCS 3

7.16 Gauze for the preparation of *Ascaris* ova suspension

8 Procedure

8.1 Preparation of the parasite ova suspension

Eggs of *Ascaris suum* are required for the preparation of the parasite ova suspension.

Principle:

- Open the uterus of the female parasite in longitudinal direction using a scalpel (see Fig. 1 in Annex A)
- Remove the uterus with forceps into a sterile petri dish with approximately 20 ml tap water
- Scratch the eggs out of the uterus using a scalpel
- Transfer the preliminary egg suspension carefully from the petri dish using a pipette and remove remaining debris from the adult worms by filtering the suspension through a gauze into a glass flask with 30 ml tap water. The ova will pass through the filter into the glass flask
- This is the *Ascaris suum* ova test-suspension

In order to determine the number of developed *Ascaris* ova in 1 ml of the suspension by microscopic examination, transfer 3 x 10 µl of the test- suspension on the microscope slide and note the total number of eggs. Calculate the mean and multiply with 100. The calculated number is the number of *Ascaris* ova in 1 ml of prepared suspension.

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The number of the Ascaris egg in the 1 ml of the suspension shall not be less than 1 000 000/ml

NOTE 1: Origin of the *Ascaris suum* worms: slaughter houses from the gut of slaughtered pigs. Only female *Ascaris* worms must be used (> 15 cm in length). In order to obtain approximately 1 000 000 eggs/ml in the 1 ml of ova suspension, 10 uteri muss be prepared as described.

NOTE 2: Suspension of eggs can be stored at +4 °C for 3-6 months. In order to keep the eggs viable during storage, they require oxygene, therefore the flask in which the eggs are stored shall not be closed tightly, in order to allow the gas exchange. The eggs settle down in the flask and can be found as a layer on the bottom. A part of the water (approx. 6 ml) above the egg-layer shall be exchanged regularly, at least once a week by fresh tap water. During this time approximately 70 – 75 % of the *Ascaris* eggs will developed (embryonation).

8.2 Preparation of test containment system type 3 (TCS 3)

Principle: The procedure for the preparation of TCS 3 is shown in Fig. 2 (Annex A).

- Prepare a piece of silk gauze 12 cm x 12 cm (pt. 1)
- Fold the gauze piece along the middle line (pt. 2)
- Join the edges at two ends by heating the silk gauze (e.g. with a sealing wand or heat gun as used for shrink-wrapping), leaving one side open (pt. 3)
- Make two more welding seams in the middle to obtain three pockets (pt. 4)
- Pipette into each bag 1 ml egg-test suspension with approx. 1 million eggs
- tie the bags firmly with cable straps
- If necessary the prepared bags with ova suspension can be placed into raschel bags or TCS 1 (see Part 1) for additional mechanical protection during exposure

NOTE 1: the prepared TCS 3 can be stored in a glass flask with tap water at +4 °C for 3-6 months (see 8.2. Note 2)

8.2 Exposure of the TCS 3 to the process

The exposure of the TCS 3 to the treatment process is carried out depending on the situation in the treatment process. The TCS 3 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.

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.

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8.3 Collecting of TCS 3 and determination of residual *Ascaris* ova count

At the end of the treatment process, the TCS 3 must be removed, placed into sterile Petri dishes and immediately transported to the laboratory. In order to prevent drying of the TCS 3 during transport, the petri dishes shall be kept humid by placing the TCS 3 on a layer of wet paper towel in the Petri dish.

Procedure in the laboratory:

- Carefully open the TCS 3 using scissors
- Remove the *Ascaris* eggs into a sterile petri dish with approximately 20 ml tap water by forceps and
- Incubate at 29 °C for 4 weeks. The eggs require oxygene for development so that the petri dishes should not be completely closed during incubation

NOTE 1: Transfer 20 ml of the prepared *Ascaris* eggs test-suspension stored in the laboratory at 4 °C (8.1) into a sterile Petri dish and incubate in parallel with the egg suspension from TCS 3. This solution is used as a positive *Ascaris* ova control.

In order to determine the number of developed *Ascaris* ova in the suspension by microscopic examination, count exactly 100 eggs. Classify the counted ova as either unembryonated or embryonated to the second larval stage. If no development or just simple embryogenesis is observed, the egg is considered unembryonated. The inactivation rate of *Ascaris* eggs is calculated as the percentage of unembryonated eggs.

8.4 Determination of the inactivation rate.

The validation of chemical processes must demonstrate that the process achieves the following overall reduction:

- reduction of resistant parasites such as eggs of *Ascaris* sp. by at least 99,9 % (3 log10) of viable stages.

If the reduction of *Ascaris* sp. 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)

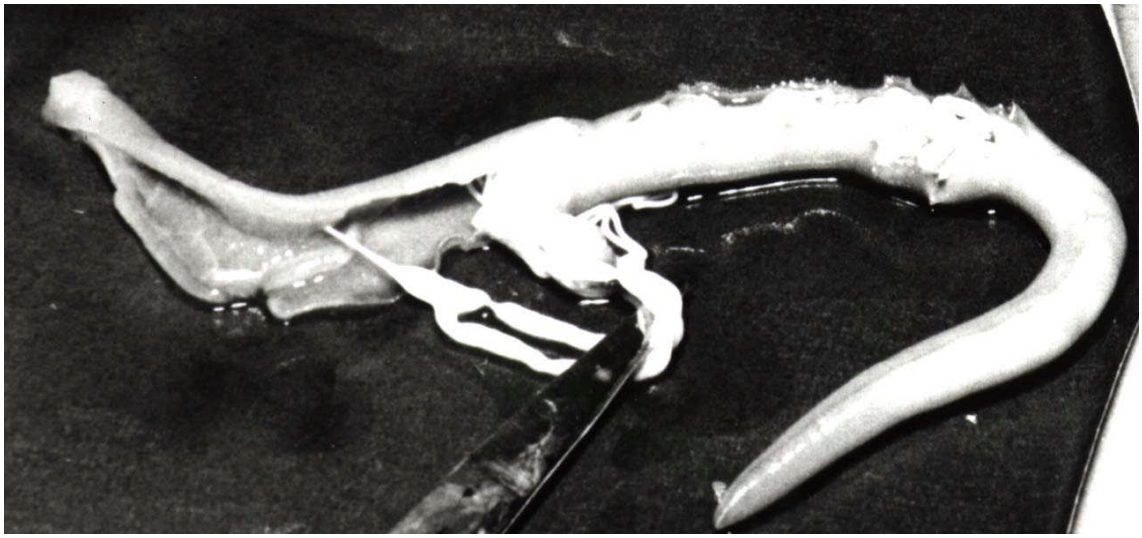

Fig. 1: Preparation of the *Ascaris* ova suspension

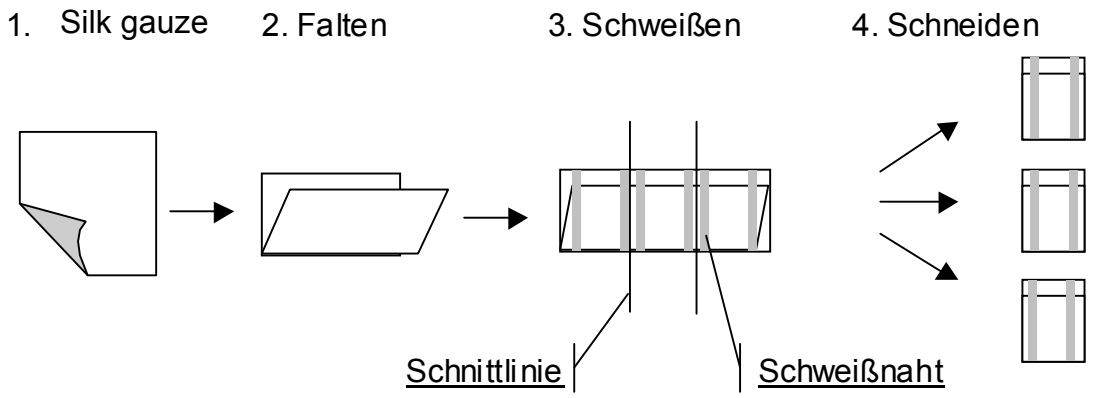

Fig. 2: Test containment system type 3

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

[2] Regulation (EC) No 208/2006 of Feburary 2006: Amending Annexes VI and VIII to Regulations (EC) No 1774/2002 of the European Parliement and of the Council as regards processing standards for biogas and composting plants and requirements for manure