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Soil improvers and growing media —Enumeration of *Enterococcaceae*

Einführendes Element — Haupt-Element — Ergänzendes Element

Élément introductif — Élément central — Élément complémentaire

ICS:

CCMC will prepare and attach the official title page.

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European foreword

This document (prEN XXXX:XXXX) has been prepared by Technical Committee CEN/TC 223 "Soil improvers and growing media", the secretariat of which is held by NEN.

This document is a working document.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

This document shall be used in combination with EN 13040-2, *Soil improvers and growing media – Sample preparation – Part 2: Sample preparation for microbiological examination*

Introduction

This document describes a method for the enumeration of *Enterococcaceae* in soil improvers and growing media.

This methodology has been developed to enumerate *Enterococcaceae* in soil improvers and growing media in order to be able to control certain imposed hygienic requirements. The method is based on EN ISO 7899-2 [4].

Note: Validations are performed with specific materials that are present at the time of validation on the market. Materials/formulation can change over time regarding the composition.

Enterococcaceae in the sense of this document include several species of the genus *Enterococcus* (3.8, 3.9) with a faecal origin. Consequently, it can be used as an indicator of faecal contamination and as a parameter to evaluate the sanitation process during the manufacturing process of soil improvers or growing media. The presence or absence of *Enterococcaceae* does not reflect the presence or absence of other pathogens in the examined product.

Some components of soil improvers (e.g. bog peat, *Sphagnum*) have a very low pH value, others (e.g. compost) can be slightly alkaline and have a negative impact on the survivability of microorganisms during the preparation of the initial suspension. Therefore, it may be necessary to measure the pH of the initial suspension.

Some considerations have to be taken into account during sample preparation, for this see draft EN 13040-2 [2].

1 Scope

This document specifies a method for the enumeration of *Enterococcaceae* in soil improvers, soil improver blends, growing media and growing media blends. This document applies to material in solid form (including pre-shaped growing media) and liquid form.

Enterococcaceae as the entire family cannot be detected, and is not detected by this method. This document specifies a colony-count technique on a selective medium (Slanetz Bartley agar) with confirmation on Bile Esculin Azide agar, respectively (*enterococci*).

In the case a given limit value (e.g. [7]) is reached, the confirmation step shall be performed.

NOTE: This method has been validated in an interlaboratory study for the following soil improver and growing media materials:

— To be filled in after CEN/TC 223 decides on the materials to be used.

— Etc.

Validations have been performed with specific materials that were present on the market during the validation study. Materials/formulation can change over time regarding their composition.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 12579[1], *Soil improvers and growing media – Sampling*

EN 13040-2[2], *Soil improvers and growing media – Part 2: Sample preparation for microbiological examination*

WI 00223137[3]: –, *Soil improvers and growing media – Terminology*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in WI 00223137:–[3] and the following apply.

3.1

segment sample

combination of all incremental samples taken from one segment for microbiological testing and is to be used as a laboratory sample

Note 1 to entry: There will be five segment samples taken from each sampled portion.

[1] Under preparation. Stage at time of publication: prEN 12579:202X.

[2] Under preparation. Stage at time of publication: prEN 13040-2:202X.

[3] Under preparation. Stage at time of publication: prCEN/TS XXXX:202X.

3.2

laboratory sample

representative part of the final sample prepared for testing

3.3

test sample

sample prepared from the laboratory sample (3.2) and from which test portions will be taken

3.4

test portion

quantity of material drawn from the test sample (or from the laboratory sample, if both are the same) and on which the tests or observations are actually carried out

3.5

initial suspension

primary dilution obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed with, normally, a nine-fold quantity of diluent

Note 1 to entry: A closer ratio between the diluent and the quantity of product is often not recommended because of possible inhibiting influences of the matrix.

3.6

further dilutions

suspensions or solutions obtained by mixing a measured volume of the initial suspension (3.5) with an x-fold volume of diluent and by repeating this operation with further dilutions until a dilution series, suitable for the inoculation of culture media, is obtained

Note 1 to entry: Ten-fold dilutions are normally used to produce a decimal dilution series, but other ratios may be required for specific purposes.

3.7

***Enterococcaceae* (in the sense of this document)**

Enterococcaceae in the sense of this document include several species of the genus *Enterococcus* (3.8, 3.9) with a faecal origin

[Comment from the WG 5-secretary: *Comments were received on definition 3.7 not being in line with other microbiology EN-ISO-standards, and the incorrectness of term 3.8. Also an alternative definition for 3.9 is given.****].***

3.8

genus *Enterococcus*

gram-positive, catalase-negative facultative-anaerobic cocci of the family *Enterococcaceae*. They often occur in pairs (diplococci) or short chains, unable of forming spores and tolerant of a wide range of environmental conditions: extreme temperature (10–45 °C), pH (4,5–10,0), and high sodium chloride concentrations. *Enterococci* occur ubiquitously in the environment (water, soil), in animals and in humans (in the normal intestinal flora). They are considered indicator germs for faecal contamination (intestinal *Enterococci*)

3.9

enterococci

gram-positive, catalase-negative cocci, able to reduce 2,3,5-triphenyl tetrazolium chloride to formazan on Slanetz Bartley agar **and** to hydrolyse esculin at 44 °C on Bile Esculin Azide agar

[Provided alternative:

Enterococci

bacteria which are able of reducing 2,3,5-triphenyltetrazolium chloride to formazan on the surface of a selective culture medium containing sodium azide (Slanetz-Bartley agar) and hydrolysis of aesculin at 44 °C on a medium containing bile salts (Bile aesculin azide agar) resulting in blackening of the agar medium

].

3.10

presumptive *enterococci*

gram-positive, catalase-negative cocci, able to reduce 2,3,5-triphenyl tetrazolium chloride to formazan on Slanetz Bartley agar

4 Principle

Duplicate plates of Slanetz Bartley agar are inoculated and incubated for 44 h to 48 h at 37 °C ± 1 °C The plates are thereafter examined to enumerate presumptive *Enterococci* colonies.

Confirmation of typical colonies from Slanetz Bartley agar by inoculation on pre-heated plates with Bile Esculin Azide agar at 44 °C ± 1 °C for 2 h or if no blackening occurs incubate again up to 20 h.,

5 Culture media and reagents

5.1 General

For current laboratory practice, standards comparable to EN ISO 7218 [5] and EN ISO 11133 [6] can be used.

Composition of culture media and reagents and their preparation are specified in Annex B.

For uniformity of results, in the preparation of media, either use a dehydrated complete medium or use constituents of uniform quality and chemicals of recognized analytical grade.

5.2 Diluents

5.2.1 General

Some materials can significantly change the pH value of the initial suspension, which can negatively affect the viability of the microorganisms under investigation. In general, a phosphate buffer is sufficient to prepare the initial suspension. When testing bog peat, *Sphagnum*, alkaline composts or blended products with a high content of minerals, the pH of the initial suspension shall be checked.

NOTE: If the samples are suspected to exhibit extreme pH values (pH lower than 4,5 and pH higher than 9, respectively), double buffered peptone water is recommended to be used as diluent for the initial suspension.

If the substrate is simultaneously tested for the presence of *Salmonella* (WI 00223121:–[4]), the initial suspension for enrichment can be used with buffered or double-buffered peptone water (with 10 g peptone). In this case, rapid processing of the initial suspension is necessary.

5.2.2 Phosphate buffer

See B.2.

5.2.3 Double-buffered phosphate buffer

See B.3.

5.3 Culture media

5.3.1 General

Slanetz Bartley agar and Bile Esculin Azide agar (commercially available from various suppliers) are used as selective media.

5.3.2 Slanetz Bartley agar

See B.4.

5.3.3 Bile Esculin Azide agar

See B.5.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (e.g. EN ISO 7218 [5]) and, in particular, the following.

6.1 Equipment for dry sterilization (oven) and wet sterilization (autoclave), as specified in e.g. EN ISO 7218 [5].

6.2 Incubator, capable of maintaining a temperature of operating at 44 °C ± 1 °C, and optionally capable of maintaining a temperature of 37 °C ± 1 °C and/ and/or 44 °C to 47 °C.

6.3 Homogenization devices as described in EN 13040-2 [2] shall be used

6.4 Scales with the required range and accuracy as described in standards comparable to EN ISO 7218 [5] for the different products to be weighed.

6.5 Water bath, capable of maintaining temperatures of 44 °C to 47 °C.

6.6 Cooling unit, adjustable at 5 °C ± 3 °C.

6.7 pH meter, with an accuracy of ± 0,1 pH unit at 20 °C to 25 °C.

6.8 Sterile loops of appropriate diameter, 3 mm (10 µl volume????).

6.9 Spreaders (hockey-stick type), sterile (see EN ISO 7218).

[4] Under preparation. Stage at time of publication: prENXXXX:202X.

6.10 Sterile straight wire, **sterile membrane filters** or **sterile stamp pads**, for transferring colonies for confirmation.

6.11 Sterile tubes, bottles, or flasks with caps of appropriate capacity.

6.12 Pipettes or pipettor and sterile tips of nominal capacities of 10 ml and 1 ml.

6.31 Sterile Petri dishes with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

7 Sampling

Sampling shall have been conducted following EN 12579 [1].

8 Preparation of test sample

The test sample shall have been prepared following EN 13040-2 [2].

9 Procedure (see diagram in Annex A)

9.1 Preparation of the initial suspension and decimal dilutions

Preparation of the initial suspension and further decimal dilutions shall be performed according to EN 13040-2 [2].

If the initial suspension is also used for the detection of *Salmonella*, the amount of sample has to be adapted, for example weigh 26 g into a sterile contained and add 234 ml buffered peptone water or double-buffered peptone water.

Note: In the case materials containing mineral components are brought into solution, pH should be corrected to a pH between 6 and 8.

[Comment from WG 5-secretary: *Please note that the consequence of the pH should be tested in trials****].***

9.2 Inoculation and incubation

9.2.1 General

Some materials can significantly change the pH value of the initial suspension, which can negatively affect the viability of the microorganisms under investigation. In general, a phosphate buffer is sufficient to prepare the initial suspension. When testing bog peat, Sphagnum, alkaline composts or blended products with a high content of minerals, the pH of the initial suspension shall be checked.

NOTE: If the samples are suspected to exhibit extreme pH values (pH lower than 4,5 and pH higher than 9, respectively), double buffered peptone water is recommended to be used as diluent for the initial suspension.

If the substrate is simultaneously tested for the presence of *Salmonella* (WI 00223121:–[5]), the initial suspension for enrichment can be used with buffered or double-buffered peptone water (with 10 g peptone). In this case, rapid processing of the initial suspension is necessary.

[5] Under preparation. Stage at time of publication: prENXXXX:202X.

9.2.2 Procedure

Using a sterile pipette or a micropipette (6.11), transfer 0,1 ml of each prepared solution and dilution onto two Slanetz Bartley plates.

Repeat the procedure with further decimal dilutions, if necessary, using a new sterile pipette for each dilution.

NOTE: Where greater dilutions are made, the number of inoculated plates for enumeration tests should be increased to ensure a minimum 0,1 g of the test portion is distributed between all plates when low counts are expected. If the colony counts expected from the first dilution are ≤ 10, it is recommended to add 1 ml of the first dilution (10^{-2}) to two Petri dishes with a diameter of 140 mm or to use 2 × 3 Petri dishes with a diameter of 90 mm to make the counting result statistically more stable.

Invert the inoculated dishes so that the bottom is uppermost and place them in an incubator (6.2) set at 37 °C ± 1 °C for 44 h to 48 h.

NOTE: A spread plating approach is necessary in order to be able to carry out a confirmation step that may be necessary later.

9.3 Enumeration of colonies

After incubation under the conditions above specified, all plates showing less than 150 typical colonies are used for the enumeration.

Typical formazan positive colonies on Slanetz Bartley agar are dark red, maroon or pink colour, either in the center surroundete by a narrow colourless zone, or throughout the colony with a variation in diameter from 0,3 mm to 2 mm.

9.4 Confirmation

9.4.1 General

Confirmation is only required if the calculated result will be above the given (e.g. [7]) limit value.

If there are typical colonies that are not confirmed by the laboratory, the result can be expressed as "presumptive *Enterococci*". If there are no typical colonies, then the result should refer to *Enterococci* and not the presumptive *Enterococci*.

Enterococci are confirmed by hydrolysis of aesculinat 44 °C on Bile aesculin azide agar resulting in blackening of the agar medium.

Note: Any other method capable of unambiguously identifying enterococci than the one described below may be used on typical colonies (e.g. commercial available biochemical test kits, serological confirmation (Lancefield group D), polymerase chain reaction (PCR)-analysis or Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF)).

9.4.2 Confirmation of selected typical colonies

Transfer five colonies of each colony type from each countable plate (9.3) either by streaking (6.9) or as point inoculation onto the Bile Aesculin azide agar plates preheated to 44 °C.

If there are less than 5 colonies, use all colonies for confirmation.

NOTE: It is also possible to transfer all colonies of a plate to the respective confirmation medium by using a sterile stamp pad or a membrane filter. In this case the subsequent calculation of the results is done according to Equation 1 (see Clause 10).

The plates are incubated aerobically for 2 h at 44 °C ± 1 °C or if no blackening occurs incubate up to 20 h at 44 °C ± 1 °C (6.2). After incubation typical colonies (brown to black) are counted as enterococci.

These results form the basis for calculating the number of colony-forming units (CFU) per gram or per milliliter of product.

9.4.3 Catalase test

On selected colonies a catalase test can be carried out to avoid false positives. Enterococci exhibit a negative reaction to the catalase test. If positive, consider the colony as non-Enterococci.

10 Expression of results

Results shall be checked according to general instructions stated in ISO 7218 [5].

The number of colony forming units (CFU) per gram or per millilitre of the test portion is calculated comparable to EN ISO 7218 [5] using Equation 1:

$$N = \frac{\Sigma C}{V(n_1+0{,}1n_2)d} \quad (1)$$

Where:

ΣC is the sum of the colonies counted on all the dishes retained from one or two successive dilutions;

V is the volume of inoculum applied to each Petri dish, in millilitres (ml);

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution (n_2= 0 if it is not performed);

d is the dilution factor corresponding to the more concentrated dilution retained (d= 1 for liquid product undiluted).

When the method requires confirmation, a given number A (generally 5) of presumptive colonies are confirmed from each of the dishes retained for counting.

After confirmation calculate, for each of the dishes, the number of colonies (a) complying with identification criteria using Equation 2:

$$a = \frac{b}{A} \times C \quad (2)$$

Where:

A is the number of presumptive colonies selected for confirmation from each Petri dish;

b is the number of colonies confirmed from A;

C is the total number of colonies in the Petri dish.

And replace ΣC by Σa in Equation 1.

NOTE: If Σa is < 10, the final result is express as estimated.

11 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used (EN 12579 [1]);

c) the size pf the test portion and/or the nature of the object examined;

d) the test method used, with reference to this document;

e) all operating details not specified in this part, or regarded as optional, together with details of any incidents which could have influenced the test result(s);

f) the result(s) obtained, indicating clearly the method of expression used; and

g) if the repeatability has been checked, the final result obtained.

Annex A
(normative)

Flow diagram of the procedure

[Comment from the WG 5-secretary: *Please note that this flow diagram needs to be updated..****].***

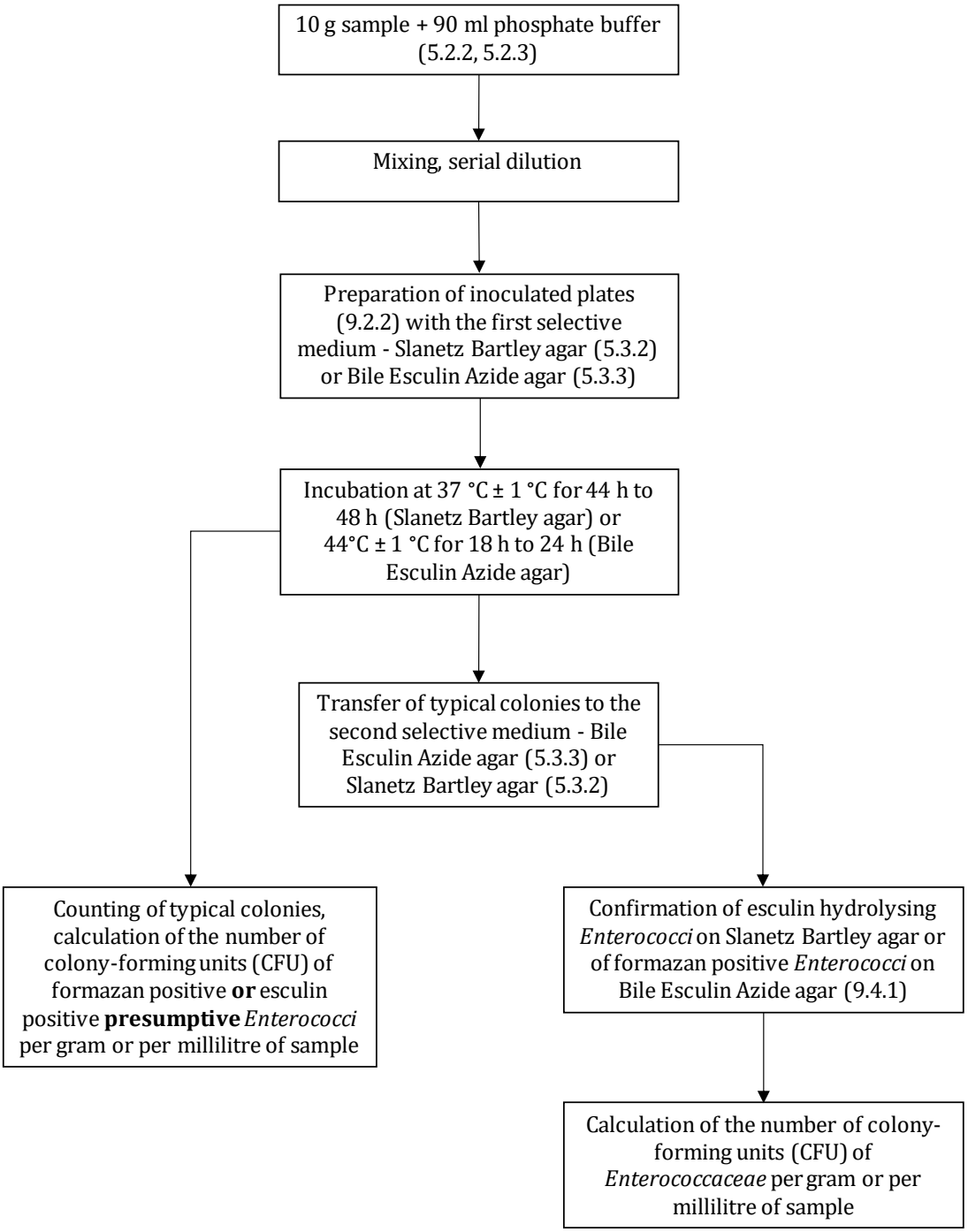

Figure A.1 – Flow diagram of the method for the detection and enumeration of *Enterococcaceae* in soil improvers and growing media

Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 General

If media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf lives of the media indicated in this annex have been shown in some studies. The user should verify this under their own storage conditions (see e.g. EN ISO 11133 [6]).

WARNING — The selective media described in this standard contain sodium azide. As this substance is highly toxic and mutagenic, precautions shall be taken to avoid contact with, especially by the inhalation of fine dust during the preparation of commercially available dehydrated complete media. Azide-containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide (HN_3) may be produced. Solutions containing azide can also form explosive compounds when in contact with metal pipework, for example from sinks.

B.2 Phosphate buffer

B.2.1 Composition

Peptone [a]	1,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate dodecahydrate ‡ ($Na_2HPO_4 \cdot 12H_2O$)	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4) ‡	1,5 g
Water	1 000 ml

[a] For example, enzymatic digest of casein.

NOTE: When using buffered peptone water (BPW), the peptone content increases to 10 g/l. Commercially available, ready-to-use BPW medium is suitable.

B.2.2 Preparation

Dissolve the components in the water in flasks, bottles or test tubes (6.10), by heating if necessary, Adjust the pH (6.8), if necessary, so that after sterilization, it is 7,0 ± 0,2 at 25 °C.

B.3 Double buffered phosphate buffer

This buffer contains twice the amount of the two buffer components (marked ‡ in B.2.1).

NOTE: When using buffered peptone water (BPW), the peptone content increases to 10 g/l. Commercially available, ready-to-use BPW medium is suitable.

B.4 Slanetz Bartley agar

[Comment from the WG 5-secretary: *This part must be revised. Comment received that in other documents, the TTC is not part of the basic medium, but is added after autoclaving. And if it is included, the culture medium must only be heated to 100 °C. Need to mention basic medium, production, TTC solution, complete medium. And noting that the complete medium shall not be autoclaved.]*.

B.4.1 Composition

Tryptose	20,0 g
Yeast extract	5,0 g
Glucose	2,0 g
Dipotassium dihydrogen phosphate (K_2HPO_4)	4,0 g
Sodium Azide	0,4 g
Triphenyltetrazolium chloride	0,1 g
Agar	10,0 g
Water	1 000 ml

B.4.2 Preparation

Dissolve the components in the water in flasks, bottles or test tubes (6.10), by heating if necessary. Adjust the pH (6.8), if necessary, so that after sterilization, it is 7,0 ± 0,2 at 25 °C.

Commercially available preparations can be used if they respect the composition.

B.5 Bile Esculin Azide agar

B.5.1 Composition

Tryptone	17,0 g
Peptic digest of meat	3,0 g
Yeast extract	5,0 g
Bacteriological ox bile	10,0 g
Sodium chloride	5,0 g
Aesculin	1,0 g
Ferric ammonium citrate	0,5 g
Sodium Azide	0,15 g
Agar	13,0 g
Water	1 000 ml

B.5.2 Preparation

Dissolve the components in the water in flasks, bottles or test tubes (6.10), by heating if necessary. Adjust the pH (6.8), if necessary, so that after sterilization, it is 7,0 ± 0,2 at 25 °C. Sterilize by autoclave for 15 min at 121 °C ± 3 °C.

Cool to 50 °C to 60 °C and pour into Petri dishes to a depth of 3 mm to 5 mm and allow to set on a cool, horizontal surface.

Commercially available preparations can be used if they respect the composition.

Annex C
(informative)

Method validation studies and performance characteristics

This annex, now intentionally left blank, will be filled with data about method validation studies and performance characteristics, as soon as results on this will be available at the next stage of this document.

Bibliography

[1] EN 12579, *Soil improvers and growing media – Sampling*

[2] EN 13040-2, *Soil improvers and growing media – Sample preparation – Part 2: Sample preparation for microbiological examination*

[3] WI 00223137: –, *Soil improvers and growing media – Terminology*

[4] EN ISO 7899-2, *Water quality — Detection and enumeration of intestinal enterococci — Part 2: Membrane filtration method*

[5] EN ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

[6] EN ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

[7] REGULATION (EU) 2019/1009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 5 June 2019 laying down rules on the making available on the market of EU fertilizing products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003