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Organic, organo-mineral and inorganic fertilisers — Enumeration of *Enterococcaceae*

Einführendes Element — Haupt-Element — Ergänzendes Element

Élément introductif — Élément central — Élément complémentaire

ICS:

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European foreword

This document (CEN/prEN 17804) has been prepared by Technical Committee CEN/TC 260 “Fertilisers and liming materials”, the secretariat of which is held by DIN.

This is a working document.

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

This document shall be used in combination with prEN 17803.

Introduction

This document describes a method for the enumeration of *Enterococcaceae* in fertilisers of the following Product Function Categories (PFCs) of EU fertilising products, as described in Regulation (EU) 2019/1009 [1]:

— PFC 1(A): Organic fertiliser;

— PFC 1(B): Organo-mineral fertiliser;

— PFC 1(C): Inorganic fertiliser, which contains more than 1 % by mass of organic carbon, other than organic carbon from chelating or complexing agents, nitrification inhibitors, denitrification inhibitors or urease inhibitors, coating agents, urea or calcium cyanamide.

This methodology has been developed to detect and enumerate *Enterococcaceae* in organic, organo-mineral and inorganic fertilisers in order to control certain hygienic requirements imposed by Regulation (EU) 2019/1009 [1]. The method described in this document is based on EN ISO 7899-2 [2].

Enterococcaceae includes several species of the genus *Enterococcus* and other genera. These bacteria occur ubiquitously in the environment (water, soil), in animals and in humans (in the normal intestinal flora). In the sense of this document, *Enterococcaceae* is referred to with the common name enterococci. Enterococci are considered indicator germs for faecal contamination (intestinal *enterococci*). Consequently, they can be used as a parameter to evaluate the sanitation process during the manufacturing process. The presence or absence of enterococci does not reflect the presence or absence of other pathogens in the material tested.

Because of the large variety of fertilisers, this method may not be appropriate in every detail for certain products. Different methods which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this method as far as possible.

Mineral components in fertilisers can have a negative impact on the survivability of microorganisms. In addition to an unfavourable shift in the pH value, these components can have a strong osmotic effect or be toxic to the microorganisms (e.g. copper). Therefore, it may be necessary to investigate the inhibitory effects of these components in a pre-test.

1 Scope

This document specifies a method for the enumeration of enterococci in organic fertilisers classified as PFC 1(A) in Regulation (EU) 2019/1009 [1], organo-mineral fertilisers classified as PFC 1(B), inorganic fertilisers classified as PFC 1 (C) which contain more than 1 % by mass of organic carbon, other than organic carbon from chelating or complexing agents, nitrification inhibitors, denitrification inhibitors or urease inhibitors, coating agents, urea or calcium cyanamide; and blends classified as PFC 7 in which the most abundant component by mass is PFC 1(A), PFC 1(B) or PFC 1(C) with the conditions stated above.

This document is also applicable to the blends of fertilising products where a blend is a mix of at least two of the following component EU fertilising products: fertilisers, liming materials, soil improvers, growing media, inhibitors or plant biostimulants, and where organic, organo-mineral or inorganic fertilisers as indicated above are the highest % in the blend by mass or volume, or in the case of liquid form by dry mass. If organic, organo-mineral or inorganic fertilisers as indicated above are not the highest % in the blend, the European Standard for the highest % of the blend applies. In case a blend of fertilising products is composed of components in equal quantity, or in case the component EU fertilising products used for the blend have identical formulations, the user decides which standard to apply.

Enterococci are part of the *Enterococcaceae* family. The entire *Enterococcaceae* family cannot be detected, and is not detected by this method.

NOTE This method has been validated in an interlaboratory study with specific products that were present on the market during the study (Annex C). Products can change over time regarding their microbial composition and other characteristics, and the formulation of the products may also change in the future.

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.

prEN 17803, *Detection of specific pathogens*

EN 1482 (all parts), *Fertilisers and liming materials — Sampling and sample preparation*

prEN ISO/DIS 7218, *Microbiology of food chain — General requirements and guidance for microbiological examinations*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at https://www.electropedia.org/
- ISO Online browsing platform: available at https://www.iso.org/obp

3.1
presumptive enterococci
Gram-positive, catalase-negative cocci, able to reduce 2,3,5-triphenyl tetrazolium chloride to formazan on Slanetz-Bartley agar under the conditions specified in this document

3.2
enterococci
bacteria which are able to reduce 2,3,5-triphenyltetrazolium chloride to formazan on the surface of a selective culture medium containing sodium azide (Slanetz-Bartley agar) and to hydrolyse esculin at

44 °C ± 1 °C on a medium containing bile salts (BEA agar), resulting in blackening of the medium under the conditions specified in this document

4 Principle

From the initial suspension, aliquots of appropriate dilutions are used to inoculate Slanetz-Bartley agar plates through spread plating, which are incubated at 37 °C ± 1 °C for 46 h ± 2 h. After incubation, presumptive enterococci colonies are enumerated. If the number of presumptive enterococci colonies isolated on Slanetz-Bartley agar exceeds the limit value in Regulation (EU) 2019/1009 [1], these are confirmed by inoculation on pre-heated plates of BEA agar, which are incubated at 44 °C ± 1 °C for 2 h to detect blackening of the agar medium. If no blackening occurs, the plates are incubated again at 44 °C ± 1 °C for up to 20 h.

5 Culture media and reagents

Current laboratory practices in accordance with standards comparable to prEN ISO/DIS 7218 shall be followed. The composition of culture media and reagents and their preparation are specified in Annex B. Use either dehydrated complete media or constituents of uniform quality and chemicals of recognized analytical grade for uniformity of results during the preparation of media. For performance testing of culture media, it is recommended to follow the procedures in accordance with standards comparable to EN ISO 11133:2014 [3] and Annex B.

6 Equipment and consumables

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

Usual microbiological laboratory equipment as described in prEN ISO/DIS 7218 shall be used and, in particular, the following.

6.1 Equipment for dry sterilization (oven) and wet sterilization (autoclave)

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

6.3 Homogenising equipment, as described in prEN 17803.

6.4 Scale of the required range and accuracy 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, capable of reading to the nearest 0,1 pH unit at 20 °C to 25 °C.

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

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

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

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

6.12 Sterile membrane filters or **sterile stamp pads**.

7 Sampling

Sampling is not included in this document. Sampling shall be performed carefully, following the principles described in EN 1482 (all parts) with appropriate adaptations to account for the specificities of organic, organo-mineral, mineral fertilisers and blends, and for the microbiological quality of the samples.

8 Preparation of test sample

The preparation of the test sample is not included in this document. The test sample shall be prepared following prEN 17803.

9 Procedure

9.1 General

The procedure as given in Annex A shall be followed.

The time elapsing between the start of the preparation of the initial suspension and the moment when the plates are inoculated shall not exceed 45 min.

9.2 Preparation of the initial suspension and decimal dilutions

Preparation of the initial suspension shall be performed according to prEN 17803.

To prepare a first decimal dilution, transfer 1 ml of the initial suspension using a sterile pipette (6.10) into a tube (6.9) containing 9 ml of sterile phosphate buffer solution (B.2 or B.3) brought to room temperature, and mix with a mechanical stirrer (6.3).

To prepare further decimal dilutions, using a sterile pipette (6.10) transfer 1 ml of the first dilution into a tube containing 9 ml of sterile phosphate buffer (B.2 or B.3) and mix with a mechanical stirrer (6.3).

Repeat this procedure until the dilution corresponds to an appropriate number of cells for enumeration.

9.3 Isolation on Slanetz-Bartley agar

Using a sterile pipette or a micropipette (6.10), transfer 0,1 ml of each appropriate dilution onto two Petri dishes (6.11) of Slanetz-Bartley agar (B.4).

If necessary, the inoculation procedure shall be repeated with further decimal dilutions, using a new sterile pipette for each dilution.

NOTE 1 A spread plating approach is necessary in order to be able to carry out a confirmation step that may be necessary later.

For enumeration, one plate per dilution shall be used with at least two successive dilutions. To improve the reliability of the results, two plates per dilution are recommended.

If only one dilution is used, then two plates of this dilution shall be used to improve reliability of the results.

For laboratories that do not operate under quality assurance principles, two plates per dilution shall be used to improve reliability of the results.

NOTE 2 Where greater dilutions are made and low counts are expected, the number of inoculated plates for enumeration tests should be increased to ensure that an inoculation volume corresponding to at least 0,1 g of the test portion is distributed on the plates. 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 one Petri dish with a diameter of 140 mm or to use three Petri dishes with a diameter of 90 mm to make the counting result statistically more stable.

The inoculated Slanetz-Bartley agar plates shall be incubated upside down in an incubator (6.2) set at 37 °C ± 1 °C for 46 h ± 2 h.

9.4 Enumeration of colonies

After incubation, all plates (**Erreur ! Source du renvoi introuvable.**) showing less than 150 typical colonies and less than 300 total (typical and atypical) shall be used for the enumeration. Atypical colonies shall not be considered for the enumeration.

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

Typical colonies on Slanetz-Bartley agar are expressed as “presumptive enterococci” (3.1).

If there are no typical colonies on Slanetz-Bartley agar, the negative result should refer to “enterococci” (3.2) and not to “presumptive enterococci” (3.1).

9.5 Confirmation (optional)

9.5.1 General

If the calculated number of presumptive enterococci (3.1) colonies is above the limit values for *Enterococcaceae* in organic, organo-mineral and inorganic fertilisers specified in Regulation (EU) 2019/1009 [1], confirmation (9.5.2) shall be required.

Enterococci are confirmed by hydrolysis of esculin at 44 °C ± 1 °C on BEA agar (B.5), resulting in blackening of the agar medium.

NOTE Any other method capable of unambiguously identifying enterococci than the one described in this document [e.g. commercial available biochemical test kits, serological tests, polymerase chain reaction (PCR) analysis or Matrix-Assisted Laser Desorption Ionization - Time-of-Flight Mass Spectrometry (MALDI-TOF)] may be used for confirmation if giving the same results as the method specified in this document.

9.5.2 Confirmation on of selected typical colonies on BEA agar

At least five colonies of each colony type shall be transferred from each countable plate (**Erreur ! Source du renvoi introuvable.**) onto the BEA agar plates (B.5) preheated to 44 °C ± 1 °C, either by streaking (6.8) or as point inoculations.

If there are less than five colonies, all colonies shall be used 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 (6.12) (see Annex D). These methods are recommended when typical colonies are present in high numbers. In this case the subsequent calculation of the results shall be done according to Equation 1 (see Clause 10).

The BEA agar plates shall be incubated aerobically at 44 °C ± 1°C for 2 h (6.2). After incubation, typical colonies (brown to black) are counted as enterococci. If no blackening occurs after 2 h, the BEA agar plates can be incubated at 44 °C ± 1°C for up to 20 h (6.2).

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

9.5.3 Catalase test (optional)

A catalase test may be carried out on doubtful colonies to avoid false positives. Enterococci exhibit a negative reaction to the catalase test. If a positive reaction is detected, the colony shall be considered as non-enterococci.

10 Expression of results

The number of colony forming units (CFU) per gram or per ml of test portion (N) shall be calculated using Formula 1. In special cases (e.g. no colonies detected, more than 150 typical colonies, etc.) results shall be expressed according to the instructions stated in prEN ISO/DIS 7218:2022, Clause 11.2.6.4.

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

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 countable dilution;
- n_2 is the number of dishes retained at the second countable 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 confirmation is necessary, a given number A (generally 5 per plate) of presumptive colonies shall be confirmed from each of the dishes retained for counting.

After confirmation, the number of colonies (a) complying with identification criteria shall be calculated for each of the dishes using Formula 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 Formula 1 to account for the ratio of verified colonies.

NOTE 1 Σa is the total number of colonies complying with identification criteria from all of the dishes selected.

NOTE 2 If Σa is < 10, the final result is express as estimated.

11 Test report

The test report shall contain at least the following information:

— all information necessary for the complete identification of the sample;

— the sampling method used (prEN 12579[1]), with an indication of all deviations from the sampling method, if known;

— the size of the test portion and/or the nature of the object examined;

— the test method used, with reference to this document;

— any deviation in the media or the incubation conditions or confirmation used;

— any unusual features observed;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the result(s);

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

— if repeatability has been checked, the final result obtained; and

— the date(s) of start and end of the test.

Annex A
(normative)

Diagram of the procedure

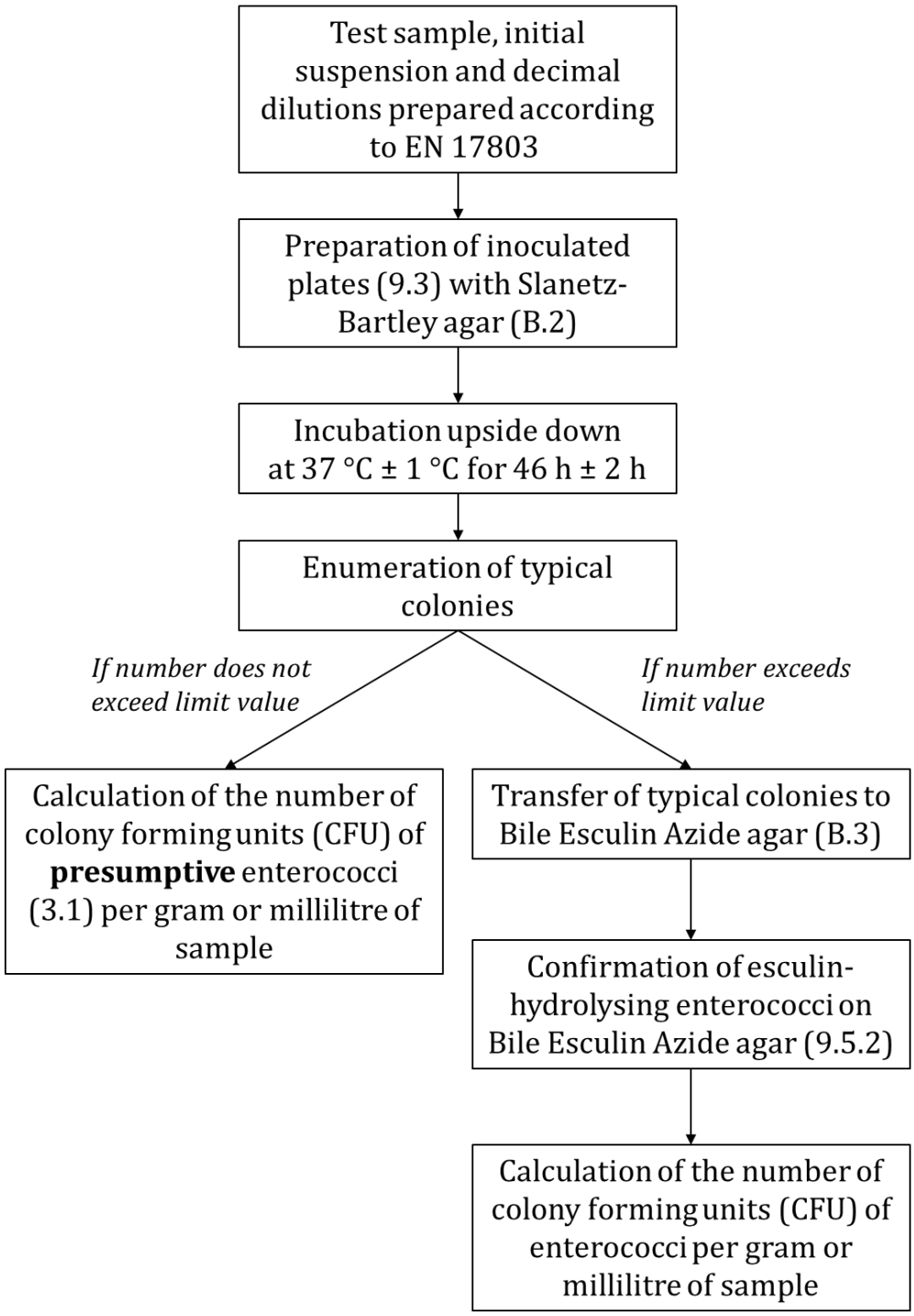

Figure A.1 — Flow diagram of the method for the detection and enumeration of *Enterococcaceae* in organic, organo-mineral and inorganic fertilisers

Annex B
(normative)

Composition and preparation of diluents, 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. Is is recommended that the users verify this under their own storage conditions according to EN ISO 11133 [3].

The preparation and performance of culture media is a fundamental step to ensure the integrity of microbiological examination. When ready-to-use media are used, the manufacturers of this available media should have a quality program that ensures the quality of the media they supply, comparable to EN ISO 11133 [3]. Under these conditions, the user/laboratory does not need to run additional testing on such media, but shall ensure the storage condition according to the ones recommended by the manufactures.

The performance for diluents and media prepared by the user/laboratory directly from commercially available dehydrated formulations and/or from basic individual components should be evaluated comparable to EN ISO 11133 [3].

B.2 Basic phosphate buffer

B.2.1 Composition

Peptone[a]	1,0 g
Sodium chloride (NaCl)	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. ‡ See B.3.	

Commercially available, ready-to-use diluent is suitable.

B.2.2 Preparation

Dissolve the components in water in flasks, bottles or test tubes (6.9), by heating if necessary.

If necessary, adjust the pH (6.7) to 7,0 ± 0,2 at 25 °C after sterilization.

B.3 Double-buffered phosphate buffer

This buffer contains twice the amount of the two buffer components (marked ‡ in B.2.1) with respect to the basic phosphate buffer.

Commercially available, ready-to-use diluent is suitable.

B.4 Slanetz-Bartley agar

B.4.1 Basal medium

B.4.1.1 Composition

Tryptose	20,0 g
Yeast extract	5,0 g
Glucose	2,0 g
Dipotassium hydrogenphosphate (K_2HPO_4)	4,0 g
Sodium azide (NaN_3)	0,4 g
Agar	8,0 g to 18,0 g [a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

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

B.4.1.2 Preparation

Dissolve the ingredients in boiling water.

Once dissolution is complete, sterilize at 121 °C for 15 min (6.1).

Cool to 44 °C - 47 °C in the water bath (6.5).

B.4.2 TTC solution

B.4.2.1 Composition

2,3,5-triphenyltetrazolium chloride	1,0 g
Water	100 ml

B.2.2.2. Preparation

Dissolve the indicator in water by stirring.

Sterilize by filtration with 0,2 µm filters (6.12).

Protect the solution against the action of light and discard it if a pink tinge develops.

B.4.3 Complete medium

B.4.3.1 Composition

Basal medium (B.2.1)	1000 ml
TTC solution (B.2.2)	10 ml

B.4.3.2 Preparation

Once the basal medium is complete, sterilize at 121 °C for 15 min (6.1). Cool to 44 °C - 47 °C in the water bath (6.5). Add the TTC-solution so the final concentration in the medium is 1%.

If necessary, adjust the pH (6.7) to 7,2 ± 0,1 at 25 °C with a solution of sodium carbonate (100 g/l), sodium hydroxide (40 g/l) or hydrochloric acid (36,5 g/l).

Pour 20 ml of medium into Petri dishes of a diameter of 90 mm, or an equivalent amount in a dish of another size (6.11), and allow to set on a cool, horizontal surface.

Poured plates can be stored in the dark for up to 2 weeks at 5 °C ± 3 °C (6.6).

B.5 Bile Esculin Azide (BEA) 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
Esculin	1,0 g
Ferric ammonium citrate	0,5 g
Sodium azide	0,15 g
Agar	13,0 g
Water	1 000 ml

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

B.5.2 Preparation

Dissolve the components in water in flasks, bottles or test tubes (6.9), by heating if necessary. Sterilize by autoclave for 15 min at 121 °C ± 3 °C (6.1).

If necessary, adjust the pH (6.7) to 7,0 ± 0,2 at 25 °C after sterilization.

Cool to 44 °C - 47 °C in the water bath (6.5) and pour into Petri dishes (6.11) 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.

Bibliography

[1] 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 fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003

[2] EN ISO 7899-2:2000, *Water quality - Detection and enumeration of intestinal enterococci - Part 2: Membrane filtration method (ISO 7899-2:2000)*

[3] EN ISO 11133:2014[1], *Microbiology of food, animal feed and water - Preparation, production, storage and performance testing of culture media (ISO 11133)*

[4] Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA), 2017: Method 28.3.4, Determination of enterococci and intestinal enterococci. In: VDLUFA Methods Book, Vol. III Feedstuff Analysis, VDLUFA-Verlag, Darmstadt

[1] As impacted by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020.