BRITISH STANDARD

BS EN ISO 7899-1:1999
BS 6068-4.3:1999

Incorporating Technical Corrigendum No. 1

Water quality — Detection and enumeration of intestinal enterococci —

Part 1: Miniaturized method (Most Probable Number) for surface and waste water

The European Standard EN ISO 7899-1:1998 has the status of a British Standard

ICS 07.100.20

Confirmed
July 2008

National foreword

This British Standard is the English language version of EN ISO 7899-1:1998, including technical corrigendum May 2000. It is identical with ISO 7899-1:1998, including technical corrigendum May 2000. It supersedes BS 6068-4.3:1989 which is withdrawn.

The UK participation in its preparation was entrusted by Technical Committee EH/3, Water quality, to Subcommittee EH/3/4, Microbiological methods, which has the responsibility to:

— aid enquirers to understand the text;
— present to the responsible international/European committee any enquiries on the interpretation, or proposals for change, and keep the UK interests informed;
— monitor related international and European developments and promulgate them in the UK.

A list of organizations represented on this subcommittee can be obtained on request to its secretary.

BS EN ISO 7899-1 is one of a series of standards on water quality, others of which have been, or will be, published as sections of BS 6068. This standard has therefore been given the secondary identifier BS 6068-4.3. The various sections of BS 6068 are comprised within Parts 1 to 7, which, together with Part 0, are listed below.

— *Part 0: Introduction;*
— *Part 1: Glossary;*
— *Part 2: Physical, chemical and biochemical methods;*
— *Part 3: Radiological methods;*
— *Part 4: Microbiological methods;*
— *Part 5: Biological methods;*
— *Part 6: Sampling;*
— *Part 7: Precision and accuracy.*

NOTE The tests described in this British Standard should only be carried out by suitably qualified persons with an appropriate level of microbiological expertise. Standard microbiological procedures should be followed throughout.

Cross-references

The British Standards which implement international or European publications referred to in this document may be found in the BSI Standards Catalogue under the section entitled "International Standards Correspondence Index", or by using the "Find" facility of the BSI Standards Electronic Catalogue.

A British Standard does not purport to include all the necessary provisions of a contract. Users of British Standards are responsible for their correct application.

Compliance with a British Standard does not of itself confer immunity from legal obligations.

Summary of pages

This document comprises a front cover, an inside front cover, the EN ISO title page, the EN ISO foreword page, the ISO title page, pages ii to iv, pages 1 to 17 and a back cover.

The BSI copyright notice displayed in this document indicates when the document was last issued.

This British Standard, having been prepared under the direction of the Health and Environment Sector Committee, was published under the authority of the Standards Committee and comes into effect on 15 June 1999

Amendments issued since publication

Amd. No.	Date	Comments
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EUROPEAN STANDARD
NORME EUROPÉENNE
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EN ISO 7899-1

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English version

Water quality — Detection and enumeration of intestinal enterococci — Part 1: Miniaturized method (Most Probable Number) for surface and waste water

(ISO 7899-1:1998)

Qualité de l'eau — Recherche et dénombrement des entérocoques intestinaux —
Partie 1: Méthode miniaturisée (nombre le plus probable) pour les eaux de surface et résiduaires
(ISO 7899-1:1998)

Wasserbeschaffenheit — Nachweis und Zählung von intestinalen Enterokokken —
Teil 1: Miniaturisiertes Verfahren (MPN-Verfahren) für Oberflächenwasser und Abwasser
(ISO 7899-1:1998)

This European Standard was approved by CEN on 15 November 1998.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Central Secretariat or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and United Kingdom.

CEN

European Committee for Standardization
Comité Européen de Normalisation
Europäisches Komitee für Normung

Central Secretariat: rue de Stassart 36, B-1050 Brussels

Ref. No. EN ISO 7899-1:1998 E

Foreword

The text of the International Standard ISO 7899-1:1998 has been prepared by Technical Committee ISO/TC 147, *Water quality*, in collaboration with Technical Committee CEN/TC 230, *Water analysis*, the Secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 1999, and conflicting national standards shall be withdrawn at the latest by May 1999.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

Endorsement notice

The text of the International Standard ISO 7899-1:1998 was approved by CEN as a European Standard without any modification.

NOTE Normative references to International Standards are listed in Annex ZA (normative).

INTERNATIONAL STANDARD

ISO 7899-1

Second edition
1998-11-15

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2000-05-01

Water quality — Detection and enumeration of intestinal enterococci

Part 1:
Miniaturized method (Most Probable Number) for surface and waste water

Qualité de l'eau — Recherche et dénombrement des entérocoques intestinaux

Partie 1: Méthode miniaturisée (nombre le plus probable) pour les eaux de surface et résiduaires

Reference number
ISO 7899-1:1998(E)

Contents

Descriptors: water, surface water, sewage, quality, bacteriological quality, water pollution, tests, water tests, microbiological analysis, detection, counting, enterobacteriaceae.

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 7899-1 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 7899-1:1984), which has been technically revised.

ISO 7899 consists of the following parts, under the general title *Water quality — Detection and enumeration of intestinal enterococci in surface and waste water*.

— *Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium;*

— *Part 2: Method by membrane filtration.*

Annex E and Annex F form an integral part of this part of ISO 7899. Annex A, Annex B, Annex C, Annex D and Annex G are for information only.

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Introduction

The aim of this part of ISO 7899 is to enumerate the major intestinal enterococci, namely *E. faecalis, E. faecium, E. durans* and *E. hirae*, which occur frequently in faeces of humans and homeothermic animals. Other faecal *Enterococcus* species, namely *E. avium, E. cecorum, E. columbae* and *E. gallinarum,* and *Streptococcus bovis/equinus* strains may occasionally be included, but they occur rarely in the environmental samples. Their recovery tends to be low. *Enterococcus casseliflavus* and *E. mundtii* are non-faecal species which, when present in water samples (e.g. because of influence of plant material and some industrial effluents), are enumerated as faecal enterococci. These species and other rare non-faecal species tend to produce yellow pigment on a non-selective medium. The possible interference of non-faecal *Enterococcus* species should therefore be considered in the interpretation of results.

1 Scope

This part of ISO 7899 specifies a miniaturized method for the detection and enumeration of major intestinal enterococci in surface and waste water by inoculation in a liquid medium. The method is applicable to all types of surface and waste waters, particularly those rich in suspended matter.

This method is not suitable for drinking water and any other type of water for which the guideline count is less than 15 per 100 ml.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 7899. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 7899 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3951:1989, *Sampling procedures and charts for inspection by variables for percent nonconforming.*

ISO 5667-1:1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

ISO 5667-3:1994, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples.*

ISO 8199:1988, *Water quality — General guide to the enumeration of microorganisms by culture.*

ISO/IEC Guide 2:1996, *Standardization and related activities — Vocabulary.*

3 Definitions

For the purposes of this part of ISO 7899, the definitions given in ISO/IEC Guide 2 and the following definition apply.

3.1
intestinal enterococci
microorganisms capable of aerobic growth at 44 °C and of hydrolysing the 4-methylumbelliferyl-β-D-glucoside (MUD), in the presence of thallium acetate, nalidixic acid and 2,3,5-triphenyltetrazolium chloride (TTC), in the liquid medium specified

4 Principle

The diluted sample is inoculated in a row of microtitre plate wells containing dehydrated culture medium.

The microtitre plates are examined under ultraviolet light at 366 nm in the dark after an incubation period of between 36 h and 72 h at 44 °C ± 0,5 °C. The presence of enterococci is indicated by fluorescence resulting from the hydrolysis of MUD. The results are given as Most Probable Number (MPN) per 100 ml.

5 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilized in accordance with the instructions given in ISO 8199.

Usual microbiological laboratory equipment, and in particular:

5.1 *Apparatus for sterilization* by dry heat (oven) or by steam (autoclave).

5.2 *Thermostatic incubator*, regulated at 44 °C ± 0,5 °C.

5.3 *Tunnel drier* or *vertical laminar air flow cabinet* (preferably class II).

5.4 *UV observation chamber* (Wood's Lamp 366 nm).

WARNING. UV light can cause irritation of skin and eyes. Use protective gloves and glasses.

5.5 *Portable refractometer* (optional).

5.6 *pH meter*, with an accuracy of ±0,1.

5.7 *Test tubes*, 16 mm × 160 mm and 20 mm × 200 mm, or *flasks* with similar capacity.

5.8 *Adjustable or pre-set 8-channel multipipette*, or any system suitable for measuring and distributing 200 µl per well.

5.9 *Sterile tips* for multipipette.

5.10 *Equipment for membrane filtration*, in accordance with ISO 8199, including membrane filters with a nominal pore size of 0,2 µm, for sterilization of liquid media.

5.11 *Sterile microtitre plates*, 96-well, 350 µl, flat-bottomed, nonfluorescent.

5.12 *Sterile adhesive cover strips* for sealing microtitre plates.

5.13 *Sterile Petri dishes*, 90 mm in diameter.

6 Sampling

Take the samples and deliver them to the laboratory in accordance with ISO 8199 and ISO 5667-1, ISO 5667-2 and ISO 5667-3.

7 Culture media and diluents

7.1 General instructions

To ensure reproducible results, prepare culture medium and diluents, using either constituents of uniform quality and chemicals of recognized analytical grade or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with distilled or demineralized water, free from substances capable of inhibiting or promoting growth under the test conditions. If the media are not used immediately, preserve them in the dark at (5 ± 3) °C, for up to one month in conditions avoiding any alterations to their composition.

NOTE The use of chemicals of other grades is permissible providing they are shown to be of equivalent performance in the test.

7.2 Diluent

7.2.1 ***Special Diluent (SD)***

Synthetic sea salt[a]	22,5 g
Bromophenol blue solution (optional)	10 ml
Demineralized or distilled water (**7.2.2**)	1 000 ml

[a] A typical analysis of a commercially available and suitable synthetic sea salt is given in Annex C. Pure NaCl solutions are not suitable, as they lead to marked inhibition.

Sterilize in the autoclave (**5.1**) at 121 °C ± 3 °C for 15 min to 20 min.

The bromophenol blue solution is prepared by adding 0,04 g in 100 ml of 50 % ethanol. It is used only to colour the SD blue and avoid confusing it with demineralized or distilled water.

7.2.2 ***Demineralized or distilled water***

Water used for dilution shall be demineralized or distilled water free from substances inhibiting growth under the test conditions.

Sterilize in the autoclave (**5.1**) before use at 121 °C ± 3 °C for 15 min to 20 min.

7.3 Culture medium: MUD/SF medium

7.3.1 *Composition*

7.3.1.1 *Solution A*

Tryptose	40 g
KH_2PO_4	10 g
D(+)-galactose	2 g
Polyoxyethylenesorbitan monooleate (Tween® 80[a])	1,5 ml
Demineralized or distilled water (**7.2.2**)	900 ml

[a] Tween® 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 7899 and does not constitute an endorsement by ISO of this product.

Add tryptose, KH_2PO_4, galactose and Tween® 80 to 900 ml of water, whilst maintaining gentle heat and magnetic stirring, then bring to the boil until completely dissolved. Allow to cool.

7.3.1.2 *Solution B*

$NaHCO_3$	4 g
Nalidixic acid	250 mg
Demineralized or distilled water (**7.2.2**)	50 ml

Add both chemicals to 50 ml of water, whilst maintaining gentle heat and magnetic stirring. Allow to cool.

7.3.1.3 *Solution C*

Thallium(I) acetate	2 g
2,3,5-triphenyltetrazolium chloride	0,1 g
Demineralized or distilled water (**7.2.2**)	50 ml

Add both chemicals to 50 ml of water, whilst maintaining gentle heat and magnetic stirring. Allow to cool.

7.3.1.4 *Solution D*

MUD (4-methylumbelliferyl-β-D-glucoside)	150 mg
N,N-dimethylformamide	2 ml

WARNING. Thallium acetate and *N,N*-dimethylformamide are toxic. Use in a chemicals fume hood.

7.3.2 *Preparation*

Mix together solutions A+B+C+D.

Adjust the pH to 7,5 ± 0,2.

Sterilize by filtration through a membrane of average pore size 0,2 µm (**5.10**).

Distribute in 96-well microtitre plates (**5.11**) with a volume of 100 µl of media in each well (minimum capacity 350 µl) and dehydrate immediately in a tunnel drier or laminar air-flow cabinet (**5.3**).

The manufacturing of the medium shall meet the quality criteria given in Annex E.

8 Procedure

8.1 Choice of dilutions

The number of dilutions to inoculate varies according to the presumed level of contamination of the water to be tested. Table 1 gives some examples.

Table 1

Origin of sample	No. of dilutions	No. of wells/dilution	Measurement limits bacteria/100 ml
Bathing water	2	64 wells to 1/2 32 wells to 1/20	15 to $3,5 \times 10^4$
Other surface water	4	24 wells to 1/2 24 wells to 1/20 24 wells to 1/200 24 wells to 1/2 000	40 to $3,2 \times 10^6$
Waste water and treatment plants	6	16 wells to 1/2 Up to 16 wells to 1/200 000	60 to $6,7 \times 10^8$

8.2 Preparation of dilutions

NOTE These procedures should be performed in a biological safety cabinet, as aerosols may be created by diluting and pipetting.

8.2.1 ***Fresh and brackish (waste) water*** [salinity < 30 g/kg, measured with refractometer (**5.5**) or equivalent method]

Prepare the relevant number of sterile tubes (**5.7**) in a rack, according to the number of selected dilutions; add 9 ml of the special diluent (**7.2.1**) to each tube.

Vigorously stir the sample (see clause **6**) in order to obtain a homogeneous distribution of the microorganisms and, using a sterile pipette, immediately transfer 9 ml of this homogenized sample to the first tube containing 9 ml of diluent (**7.2.1**) (1/2 dilution).

Using a fresh pipette, transfer 1 ml of this dilution (homogenized) to the second tube (1/20 dilution).

From the second tube (dilution 1/20 carefully homogenized) proceed, if necessary, to another 1/10 dilution giving the dilution 1/200.

Continue as above until all the dilutions have been prepared.

8.2.2 ***Sea water*** (salinity ≥ 30 g/kg)

Prepare the relevant number of sterile tubes (**5.7**) in a rack, according to the number of selected dilutions, add 9 ml of demineralized or distilled water (**7.2.2**) to the first tube and 9 ml of the special diluent (**7.2.1**) to the other tubes.

Vigorously stir the sample (see clause **6**) in order to obtain a homogeneous distribution of the microorganisms and, using a sterile pipette, immediately transfer 9 ml of this homogenized sample to the first tube containing 9 ml water (**7.2.2**) (1/2 dilution).

Using a fresh sterile pipette, transfer 1 ml of this dilution (homogenized) to the second tube (1/20 dilution).

From the second tube (dilution 1/20 carefully homogenized) proceed, if necessary, to another 1/10 dilution giving the following dilution (1/200).

Continue as above until all the dilutions have been prepared.

8.3 Inoculation and incubation of microtitre plates

8.3.1 ***Inoculation***

Transfer the contents of the first tube of dilution to an empty, sterile Petri dish, of minimum diameter 90 mm.

Using a multichannel pipette (**5.8**) with 8 sterile tips (**5.9**), distribute 200 µl into each well of a microtitre plate (**5.11**) corresponding to this first dilution.

For subsequent dilutions (1/20, 1/200, etc.) operate in an identical manner, changing the Petri dish and the row of 8 sterile tips between each dilution.

Alternatively, any other suitable system (**5.8**) may be used to distribute 200 µl of each dilution per well in accordance with Table 1.

CAUTION. Beware of contamination via overflow from one well to another.

8.3.2 ***Incubation***

Once the microtitre plate is inoculated, cover with the disposable sterile adhesive tape (**5.12**) provided for this purpose.

Incubate the microtitre plate (**5.2**) at 44 °C ± 0,5 °C for a minimum of 36 h and a maximum of 72 h.

NOTE The microtitre plates should be handled with care, without tilting.

8.4 Reading of results

Place each microtitre plate, including adhesive, in the UV observation chamber (**5.4**).

Consider all wells in which a blue fluorescence is observed as being positive.

NOTE The reading may be carried out any time after 36 h, as the fluorescence does not alter with time.

9 Expression of results

9.1 Determination of characteristic number

For each chosen dilution, note the number of positive (+) wells.

EXAMPLE 1:	Bathing water	
	1/2	32 + out of 64
	1/20	5 + out of 32
	Record 32/5 as characteristic number	
EXAMPLE 2:	Other surface water	
	1/2	24 + out of 24
	1/20	18 + out of 24
	1/200	5 + out of 24
	1/2 000	1 + out of 24
	Record 18/5/1 as characteristic number	
EXAMPLE 3:	Waste water	
	1/2	16 + out of 16
	1/20	16 + out of 16
	1/200	12 + out of 16
	1/2 000	5 + out of 16
	1/20 000	0 + out of 16
	1/200 000	0 + out of 16
	Record 12/5/0 as characteristic number	

Where three or more dilutions have been inoculated, a characteristic number of three digits, ending in 0 where possible, shall be recorded, in accordance with ISO 8199.

9.2 Calculation of the MPN and its confidence interval

The MPN is a statistical estimation of the density of microorganisms, assumed to correspond to a Poisson distribution in the volumes inoculated. Confidence intervals are attached to this MPN.

Software shown in Annex A or Annex B enable the calculation of the MPN of intestinal enterococci per millilitre of water for each configuration of inoculations and the confidence interval at 95 %.

EXAMPLE 1: Assuming CN is the Characteristic Number, LO the Lower Limit and UP the Upper Limit:

If CN = 32/5, the software in Annex A gives 7,56 enterococci per millilitre,
[LO = 5,42 – UP = 10,54],
i.e. 756/100 ml (542 to 1 054)

EXAMPLE 2:

If CN = 18/5/1, the software in Annex A gives 159,08/ml,
[LO = 101,99 – UP = 248,11]

EXAMPLE 3:

If CN = 12/5/0, the software in Annex A gives 1 724,61/ml
[LO = 1 003,98 – UP = 2 962,50]

If none of the wells is positive, express the result in the following form:

< *n*/100 ml

where *n* is the MPN for 1 positive well under the dilution conditions employed.

10 Test report

The test report shall include all details necessary for the complete identification of the sample, referring to the method used and the results.

The test report shall also mention any special phenomena observed during the test and any non-specified or optional operations used in the method which may have altered the results.

11 Performance data

Information concerning the repeatability and reproducibility of the procedure, obtained from interlaboratory tests, is given in Annex D.

Annex A (informative)
Example of software for statistical analysis of MPNs

```
10      REM ********************************************************************************************
20      REM GENERAL PURPOSE PROGRAM FOR MPN, ITS S.E., C.I.
30      REM AND HOMOGENEITY TEST STATISTICS
40      REM ********************************************************************************************
50      REM
60      DIM A(10,6),X2(3,9)
70      REM SET PROGRAM LIMITS
80      D9=10
90      U9=50
100     L9=0
110     A1=.0005
120     E1=85
130     REM SET CHI-SQUARED SIGNIFICANCE LEVELS
140     GOSUB 1000
150     REM READ IN RESULTS OF A DILUTION SERIES
160     GOSUB 2000
170     REM CALC AND PRINT THE MPN
180     GOSUB 3000
190     REM CALC AND PRINT S.E. OF LOG10(MPN)
200     GOSUB 4000
210     REM CALC AND PRINT 95 PERCENT C.I. FOR MPN
220     GOSUB 5000
230     REM CALC AND PRINT DEVIANCE
240     GOSUB 6000
250     STOP
1000    REM SET CHI-SQUARED SIGNIFICANCE LEVELS
1010    FOR I=1 TO 3
1020    FOR J=1 TO 9
1030    READ X2(I,J)
1040    NEXT J
1050    NEXT I
1060    REM 5 PERCENT LEVELS DF=1...9
1070    DATA 3.84, 5.99, 7.81, 9.49, 11.07
1080    DATA 12.59, 14.07, 15.51, 16.92
1090    REM 1 PERCENT LEVELS
1100    DATA 6.63, 9.21, 11.34, 13.28, 15.09
1110    DATA 16.81, 18.48, 20.09, 21.67
1120    REM .1 PERCENT LEVELS
1130    DATA 10.83, 13.81, 16.27, 18.47, 20.52
1140    DATA 22.46, 24.32, 26.12, 27.88
1150    RETURN
2000    REM READ IN RESULTS OF A DILUTION SERIES
2010    PRINT "MPN GENERAL PURPOSE PROGRAM"
2020    PRINT "******************************************"
2030    PRINT " "
2040    PRINT "M.A. HURLEY AND M.E. ROSCOE"
```

```
2050    PRINT " "
2060    PRINT "NUMBER OF DILUTION LEVELS......K=";
2070    INPUT N
2080    IF N<1 THEN GOTO 2060
2090    IF N<=D9 THEN GOTO 2120
2100    PRINT "ERROR *** LEVELS EXCEED MAXIMUM"
2110    STOP
2120    S1=0
2130    FOR I=1 TO N
2140    PRINT " "
2150    PRINT "LEVEL NUMBER  .......................................I=";I
2160    PRINT "DILUTION FACTOR.................................D=";
2170    INPUT A(I,2)
2180    PRINT "SUBSAMPLE VOLUME...........................V=";
2190    INPUT A(I,1)
2200    PRINT "NUMBER OF SUBSAMPLES....................N=";
2210    INPUT A(I,3)
2220    PRINT "NUMBER OF POSITIVE SUBSAMPLES..P=";
2230    INPUT A(I,4)
2240    PRINT "IS THE DATA CORRECT FOR LEVEL ";I;"(Y OR N)";
2250    INPUT R$
2260    IF R$="Y" THEN 2280
2270    GOTO 2140
2280    A(I,5)=A(I,1)*A(I,2)
2290    A(I,6)=A(I,5)*A(I,4)
2300    S1=S1+A(I,5)*A(I,3)
2310    NEXT I
2320    RETURN
3000    REM CALCULATES AND PRINTS MPN
3010    B1=0
3020    S3=0
3030    FOR J=1 TO N
3040    E2=A(J,5)*U9
3050    IF E2<E1 GOTO 3080
3060    E2=0
3070    GOTO 3090
3080    E2=EXP(–E2)
3090    S3=S3+A(J,6)/(1–E2)
3100    NEXT J
3110    IF S3–S1>=0 THEN 3130
3120    GOTO 3200
3130    FOR I=1 TO N
3140    A(I,5)=A(I,5)*2
3150    A(I,6)=A(I,6)*2
3160    NEXT I
3170    S1=S1*2
3180    B1=B1+1
3190    GOTO 3020
3200    X3=L9
```



```
3210  X4=U9
3220  X=(X3+X4)/2
3230  S=0
3240  FOR I=1 TO N
3250  E2=A(I,5)*X
3260  IF E2<E1 GOTO 3290
3270  E2=0
3280  GOTO 3300
3290  E2=EXP(–E2)
3300  S=S+A(I,6)/(1–E2)
3310  NEXT I
3320  IF ABS(S–S1)<A1 THEN 3380
3330  IF S–S1>0 THEN 3360
3340  X4=X
3350  GOTO 3220
3360  X3=X
3370  GOTO 3220
3380  X5=X*(2^B1)
3390  PRINT " "
3400  PRINT "MPN=";X5
3410  PRINT "FOR A SAMPLE WITH DILUTION FACTOR 1"
3420  PRINT "                              AND VOLUME 1"
3430  RETURN
4000  REM CALCS AND PRINTS S.E. OF LOG10 (MPN)
4010  S2=0
4020  FOR I=1 TO N
4030  X3=A(I,5)
4040  E2=X3*X
4050  IF E2<E1 GOTO 4080
4060  X4=0
4070  GOTO 4090
4080  X4=EXP(–E2)
4090  S3=X3*X3*A(I,3)*X4
4100  S3=S3/(1–X4)
4110  S2=S2+S3
4120  NEXT I
4130  V=I/(X*X*S2)
4140  S1=SQR(V)/LOG(10)
4150  PRINT " "
4160  PRINT "S.E. OF LOG10 (MPN)=";S1
4170  RETURN
5000  REM CALCS 95 PERCENT C.I. FOR MPN
5010  X3=LOG(X)+B1*LOG(2)
5020  S2=SQR(V)
5030  U=EXP(X3+1.96*S2)
5040  L=EXP(X3–1.96*S2)
5050  PRINT " "
5060  PRINT "95 PERCENT C.I. =";L;"TO";U
5070  RETURN
```

```
6000 REM CALCS. AND PRINTS DEVIANCE
6010 S3=0
6020 FOR I=1 TO N
6030 S4=0
6040 IF A(I,4)<=0 GOTO 6110
6050 E2=A(I,5)*X
6060 IF E2<E1 GOTO 6090
6070 E2=0
6080 GOTO 6100
6090 E2=EXP(–E2)
6100 S4=A(I,4)*LOG(A(I,4)/(A(I,3)*(1–E2)))
6110 S3=S3+S4
6120 S4=0
6130 IF A(I,4)>=A(I,3) GOTO 6160
6140 S4=A(I,3)–A(I,4)
6150 S4=S4*(LOG(S4/A(I,3))+A(I,5)*X)
6160 S3=S3+S4
6170 NEXT I
6180 D=2*S3
6190 REM CHI-SQUARED TEST OF DEVIANCE
6200 V=N–1
6210 PRINT “ ”
6220 PRINT “DEFIANCE =”;D;“ON”;V;“ D.F.”
6230 PRINT “ ”
6240 PRINT “CHI-SQUARED SIGNIFICANCE LEVELS FOR”;V;“ D.F.”
6250 PRINT “     5 PERCENT      ”;X2(1,V)
6260 PRINT “     1 PERCENT      ”;X2(2,V)
6270 PRINT “     .1 PERCENT     ” ;X2(3,V)
6280 RETURN
7000 END
```

Annex B (informative)
Example of software for computation of MPNs

```
10   DIM T (20)
20   DIM M (20)
30   DIM P (20)
40   L   = 0
45   CLS : L = L + 1
50   PRINT “CALCULATION OF MPN N° ”;L
60   PRINT “ .........................................”
70   A   = 1
80   PRINT
90   INPUT “NB OF DILUTIONS”;DI : PRINT : PRINT
110  PRINT
120  P   = 0 : S = : U = 0
130  FOR I = 1 TO DI
140  PRINT “DILUTION” ; I
150  INPUT “NB OF POSITIVE WELLS ..” ; P(I)
155  INPUT “NB OF WELLS ....................”;T(I)
160  INPUT “WATER VOLUME/WELL (ML)...” ;M(I) : PRINT
170  P   =   P(I) + P
180  S   =   ((T(I) – P(I))*M(I)) + S
190  U   =   (M(I) * TI)) + U
200  NEXT I
280  K   = 1
290  NP = (P/U) * 2 ^ (K + K/2 + K/4 + K/8 + K/16 + K/32 + K/64 + K/128 + K/256 + K/512)
300  PM = 0
310  FOR I= 1 TO DI
320  PM = PM + ((P(I) * M (I) * EXP ( – M(I) * NP))/(1 – EXP (– M(I) * NP )))
330  NEXT I
340  IF PM < S THEN 370
350  K   = K + A
360  GOTO 290
370  DT = S – PM
380  IF ABS (DT) < = .000005 GOTO 702
390  K   = K – A
400  A   = A/10
410  GOTO 290
702  FOR I = 1 TO DI:ES = ES + P(I):NEXT I
710  FOR I = 1 TO DI
720  K (I)= (T(I) * (M(I)^2))
730  NEXT I
740  FOR I= 1 TO DI
745  IF (NP * M(I) > 88 THEN E(I) = 1.65E38:GOTO 760
750  E(I) = (EXP (M(I) * NP) – 1)
760  NEXT I
770  LL = 0
780  FOR I = 1 TO DI
790  LL = LL + (K (I)/E (I))
```

```
800   NEXT I
810   CL = (LOG (NP) – (1.96 * (1/(NP * SQR (LL)))))
817   CU = (LOG (NP) + (1.96 * (1/(NP * SQR (LL)))))
840   NP = INT (NP * 100 + .5)/100
850   PRINT : PRINT " NPP = ";NP;"/ML"
860   PRINT : PRINT
870   PRINT "LIMITS INF=";INT ( EXP (CL)* 100 + .5)/100;"/ML SUP=" ;
      INT (EXP (CU) * 100 + .5)/ 100;"/ML"
880   PRINT : PRINT: INPUT "DO YOUWANT ANOTHER MPN (Y/N)?";RE$
890   IF RE$ = "N" THEN END
900   GOTO 45
```

Comment

After display of the run number (L, line 50),

Input the number of dilutions (line 90).

For each dilution:

- — display the dilution rank (line 140),
- — input the number of positive wells or tubes (line 150),
- — input the number of wells inoculated with this dilution (line 155),
- — input the volume of water inoculated per well, in millilitres (line 160).

Calculation of the MPN according to De Man [2] (lines 280–410).

Calculation of the lower and upper limits of confidence interval according to De Man [2] (lines 770–817).

Display

- — of MPN (per ml) (line 850),
- — the lower limit (per ml) (line 870),
- — the upper limit (per ml) (line 870),

Question: "Another run ?"

If no: END.

Annex C (informative)
Synthetic sea salt

C.1 Major ion composition of a convenient ocean synthetic sea salt

Major iron		% Total weight	Ionic concentration at 34 g/kg salinity (mg/l)
Chloride	(Cl^-)	47,470	18 740
Sodium	(Na^+)	26,280	10 454
Sulfate	(SO_4-^2)	6,602	2 631
Magnesium	(Mg^{+2})	3,230	1 256
Calcium	(Ca^{+2})	1,013	400
Potassium	(K^+)	1,015	401
Bicarbonate	(HCO_3-)	0,491	194
Borate	(B)	0,015	6,0
Strontium	(Sr^{+2})	0,001	7,5
	SOLIDS TOTAL	86,11	34 089,50
Water	(H_2O)	13,88	
	TOTAL	99,99	

C.2 Example for preparation from defined substances

Three basic solutions are to be made as follows:

Solution A	
$CaCl_2 \cdot 2H_2O$	83,6 g
KCl	43,5 g
$SrCl_2 \cdot 6H_2O$	0,07 g
Distilled water	to 1 000 ml
Solution B	
$NaHCO_3$	15,15 g
$Na_2B_4O_7$	3,0 g
Distilled water	to 1 000 ml
Solution C	
$MgSO_4 \cdot 7H_2O$	190,0 g
$MgCl_2 \cdot 6H_2O$	147,0 g
Distilled water	to 1 000 ml

The diluent is made by adding to 960 ml distilled water 10 ml of solution A, 10 ml of solution B, 20 ml of solution C, and then 14,9 g sodium chloride, mixing until completely dissolved and setting the pH to 7,5 ± 0,2. The diluent is distributed to containers of desired volumes, and sterilized by autoclaving at (121 ± 3) °C for 15 min.

Annex D (informative) Performance characteristics of the method

The performances of repeatability (*r*) and reproducibility (*R*) calculated according to ISO 5725-2 as part of interlaboratory tests have shown:

On bathing waters

(spiked samples, with 100 French laboratories, on three occasions in 1995 and 1996) (one sea water and two fresh waters, without significant difference):

i) At the level 100 intestinal enterococci/100 ml	*r*	≈ 3,6
	R	≈ 5,2
ii) At the level 400 intestinal enterococci/100 ml	*r*	≈ 2,1
	R	≈ 3,7

and, for information only, during tests (in 1993 and 1994) limited to nine laboratories and four samples (naturally contaminated):

b) On river waters containing

between $2,2 \times 10^3$ and $1,5 \times 10^4$ intestinal enterococci/100 ml:	*r*	≈ 1,5
	R	≈ 2,7

c) On sewage waters containing

between $1,9 \times 10^4$ and $5,1 \times 10^6$ intestinal enterococci/100 ml:	*r*	≈ 2,6
	R	≈ 3,9

Annex E (normative) Quality criteria for manufacturing of the medium in microtitre plates

E.1 General

For each of the criteria which follow, a quality control shall be made on each batch of manufactured microtitre plates. The microtitre plates to be tested are taken at random or in a systematic way to constitute a sample in accordance with ISO 3951, respecting the general control level No. II of the normal control.

The threshold of positivity of a microtitre plate is defined as being the fluorescence level leading to a positive reading without ambiguity, to the eye, under a Wood light (366 nm). It shall be measured in application of the protocol of Annex F.

The quality criteria to be respected are given in **E.2** to **E.5**. The batch is rejected if any of the criteria is not respected.

E.2 Background noise

Absence of positive well in each microtitre plate of the sample, after inoculation with sterile special diluent and incubation of 48 h at (44 ± 0,5) °C. The medium background noise of the sample shall be inferior to 25 % of the positivity threshold defined above and the variation coefficient shall be inferior to 10 %.

E.3 Average level of fluorescence

This is the geometric mean of the fluorescence signal obtained from the 96 wells of a microtitre plate inoculated uniformly with 200 µl per well of a suspension of *E. faecalis* CCM 2541 containing 500 microorganisms per ml of Special Diluent (**7.2.1**), and incubated for 48 h at (44 ± 0,5) °C. The average signal so obtained shall be at least twice the threshold of positivity and the variation coefficient shall be inferior to 10 %.

E.4 Fertility

Fertility is calculated as the ratio of the number of germs observed with the batch of microtitre plates under test to the number of microorganisms expected with a stable reference material (target value). The level of concentration should be brought up to around the maximum of the precision of the method, that is to say about one microorganism per well (500/100 ml). The stability and the homogeneity (target value and confidence intervals) of the reference material should have been determined with one (or several) batch(es) of microtitre plates already accepted. The threshold of acceptance of the microtitre plates tested is 0,66 to 1,5 of the target value. The variation coefficient should be inferior to 10 %).

The strains to be tested are:

- *E. faecium* WR63 — Obtainable from RIVM, Bilthoven, The Netherlands
- *E. faecalis* CCM 2541
- *E. hirae* CCM 2423

} Obtainable from the Czech Collection of Microorganisms, Brno, Czech Republic.

The incubation period is 48 h at (44 ± 0,5) °C.

E.5 Interferences

The selective power of the microtitre plates is tested by inoculating microorganisms similar to the target species:

- *Aerococcus viridans* CIP 54145T
- *Lactococcus lactis* CIP 7056T
- *Staphylococcus epidermidis* CIP 8155T

} Obtainable from Institut Pasteur, Paris, France

Suspensions containing from 10^4 to 10^5 of these microorganisms per 100 ml are inoculated in each microtitre plate tested on the basis of 32 wells per strain. After an incubation of 48 h at (44 ± 5) °C, no well shall show fluorescence superior to the criteria defined for the background noise (25 % of the positivity threshold), or a formazan precipitate.

Annex F (normative) Preparation of calibration microtitre plates

F.1 Material and reagents

Current laboratory apparatus (precision balance, pH meter, glassware) and also:

F.1.1 4-methylumbelliferone.

F.1.2 Absolute ethanol.

F.1.3 0,8 % sodium chloride solution.

F.1.4 Glycine.

F.1.5 Sodium hydroxide.

F.1.6 Microtitre plates with 96 wells.

F.2 Preparation of the stock solution

Prepare a stock solution of 4-methylumbelliferone in ethanol by mixing the following:

4-Methylumbelliferone	0,044 g
Ethanol	10 ml

F.3 Preparation of the buffer solution

F.3.1 Prepare the following solutions:

Glycine solution: Dissolve 7,507 g of glycine and 5,84 g of sodium chloride in 1 000 ml distilled water.

Sodium hydroxide solution: Dissolve 4 g of sodium hydroxide in 1 000 ml distilled water.

F.3.2 In a beaker with agitator, add 56,5 ml of the glycine solution [**F.3.1**a)] to 43,5 ml of sodium hydroxide solution [**F.3.1**b)]. Check with the pH meter that the pH value is 10,3.

F.4 Preparation of the calibration plate

All the following operations shall take place at room temperature.

All the dilutions shall be made using a solution of 0,8 % sodium chloride (**F.1.3**).

Prepare an intermediate solution at 1/100 of the stock solution (**F.2**). From the obtained solution, prepare daily the following solutions.

ST 1:	100 µl of the intermediate solution	+ 0,9 ml of 0,8 % NaCl
ST 2:	0,5 ml of ST1	+ 0,5 ml of 0,8 % NaCl
ST 3:	0,5 ml of ST2	+ 0,5 ml of 0,8 % NaCl
ST 4:	0,5 ml of ST3	+ 0,5 ml of 0,8 % NaCl
ST 5:	0,5 ml of ST4	+ 0,5 ml of 0,8 % NaCl

Display the different solutions in the uneven columns of the microtitre plate (**F.1.6**), following the instructions in Figure F.1 (the even columns are not used).

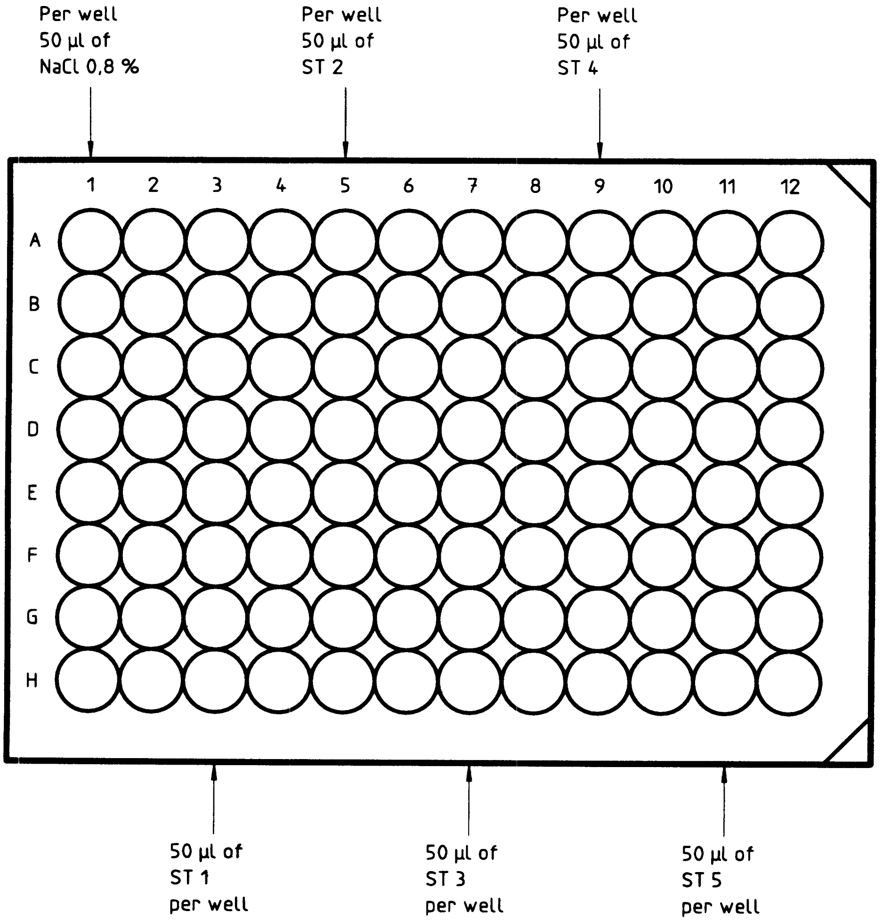

Figure F.1

Add in each well 100 µl of the pH 10,3 buffer solution (**F.3.2**). A range from 0 pg to about 1 500 pg of 4-methylumbelliferone per microlitre is thus obtained.

NOTE Experimentally it has been observed that the positivity threshold with 4-methylumbelliferone SIGMA (batch No. M-1381) and transparent microtitre plates with flat bottom NUNC (ref. 1352) is 367 pg/µl when the microtitre plate is filled as described in this annex with the solution ST3.

Annex G (informative) Bibliography

[1] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[2] DE MAN J.C. The probability of most probable numbers. *Eur. J. Appl. Microbiol.*, **1**, 1975, pp. 67–78

[3] DEVRIESE L.A., COLLINS M.D. and WIRTH R. The Genus Enterococcus. In: A. Balows *et al.* (Ed.) *The Prokaryotes*. 2nd edn., Vol. ii, 1992, pp. 1485–1481, Springer, NY

[4] HURLEY M. A. and ROSCOE M. E. Automated statistical analysis of microbial enumeration by dilution series. *J. Appl. Bacteriol.*, **55**, 1983, pp. 159–164

[5] KLEE A.J. A computer program for the determination of most probable numbers and its confidence limits. *Microbiol. Methods*, **18**, 1993, pp. 91–98

Annex ZA (normative)
Normative references to international publications with their relevant European publications

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the 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 European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

Publication	Year	Title	EN	Year
ISO 5667-1	1980	*Water quality — Sampling — Part 1: Guidance on the design of sampling programmes*	EN 25667-1	1993
ISO 5667-2	1991	*Water quality — Sampling — Part 2: Guidance on sampling techniques*	EN 25667-2	1993
ISO 5667-3	1994	*Water quality — Sampling — Part 3: Guidance on the preservation and handling of sample*s	EN ISO 5667-3	1995

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