

British Standard

Water quality

Part 4. Microbiological methods

Section 4.2 Guide to the enumeration of micro-organisms by culture

[ISO title: Water quality — General guide to the enumeration of micro-organisms by culture]

Qualité de l'eau

Partie 4. Méthodes microbiologiques

Section 4.2 Guide pour le dénombrement des micro-organismes sur milieu de culture

Wasserqualität

Teil 4. Mikrobiologische Verfahren

Abschnitt 4.2 Anleitung zur Keimzahlbestimmung

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National foreword

This Section of BS 6068, which has been prepared under the direction of the Environment and Pollution Standards Policy Committee, is identical with ISO 8199 : 1988 'Water quality -- General guide to the enumeration of micro-organisms by culture'.

The international standard was prepared by subcommittee 4, Microbiological methods, of Technical Committee 147, Water quality, of the International Organization for Standardization (ISO) with the active approval and participation of the UK.

This British Standard is being published in a series of Parts subdivided into Sections that will generally correspond to particular international standards. Sections are being, or will be, published in Parts 1 to 6, 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

Techniques for the isolation and enumeration of micro-organisms, based on their ability to grow on specified culture media, are an important and widely used means of assessing the microbiological quality of water. The purpose of this guide is to gather in a single document the information common to the various enumeration techniques so as to avoid repetition of technical details in individual standards and to facilitate the choice of the technique most suitable for a particular problem.

NOTE. The tests described in this Section of BS 6068 should only be carried out in laboratories with suitable facilities and by suitably qualified persons with an appropriate level of microbiological expertise. Standard microbiological procedures should be followed throughout.

Terminology and conventions. The text of the international standard has been approved as suitable for publication as a British Standard without deviation. Some terminology and certain conventions are not identical with those used in British Standards; attention is drawn especially to the following.

The comma has been used as a decimal marker. In British Standards it is current practice to use a full point on the baseline as the decimal marker.

Wherever the words 'International Standard' appear, referring to this standard they should be read as 'Section of BS 6068'.

In British Standards it is current practice to use the symbol 'L' for litre (and in its submultiples) rather than 'l' and to use the spelling 'sulphur' etc. instead of 'sulfur', etc.

Cross-references

International standard	Corresponding British Standard
ISO 3534-1977	BS 5532 Statistical terminology Part 1 : 1978 Glossary of terms relating to probability and general terms relating to statistics (Identical)
ISO 3696-1987	BS 3978 : 1987 Specification for water for laboratory use (Identical)
ISO 5667/2-1982	BS 6068 Water quality Section 6.2 : 1983 Guidance on sampling techniques (Identical)
ISO 5667/3-1985	Section 6.3 : 1986 Guidance on the preservation and handling of samples (Identical)
ISO 6887-1983	BS 5763 Methods for microbiological examination of food and animal feeding stuffs Part 6 : 1983 Preparation of dilutions (Identical)

Textual errors. When adopting the text of the international standard, the following textual errors were discovered. They have been marked in the text and reported to ISO in a proposal to amend the text of the international standard.

In 5.2.4, the note should appear at the end of item (b).

In 10.2.1, final paragraph, '44 °C' should read '45 °C'.

On page 17 'Annexe A' should be read as 'Annex A' and 'Bibliographie' should be read as 'Bibliography'.

Additional information. (1) In the the UK *distilled* water has not generally hitherto been used as a diluent (see 5.2.5).

(2) In 8.2.5.2, final paragraph, the resulting absolute pressure will be about 30 kPa, i.e. a pressure of 70 kPa less than the usual atmospheric pressure.

1 Scope

This International Standard presents guidance for carrying out manipulations which are common to each technique for the microbiological examination of water, particularly the preparation of samples, culture media and apparatus. It also describes the various enumeration methods available and the criteria for the choice of a particular technique.

2 Normative references

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

ISO 3534: 1977, *Statistics — Vocabulary and symbols*.

ISO 3696: 1987, *Water for analytical laboratory use — Specification and test methods*.

ISO 5667-2: 1982, *Water quality — Sampling — Part 2: General guide to sampling techniques*.

ISO 5667-3: 1985, *Water quality — Sampling — Part 3: Recommendations for the preservation and handling of samples*.

ISO 6887: 1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*.

3 Principle

The general principle of these techniques consists of inoculating a known volume of a water sample into a culture medium (solid or liquid). It is assumed that on incubation each micro-organism present multiplies giving either a colony visible directly on the solid medium, or changes in appearance in the liquid medium. The choice of a particular method depends not only on the nature of the micro-organisms sought, but also on the nature of the water and the reasons for the examination.

4 Safety and hygiene in the laboratory

As well as general and statutory considerations for safety in the laboratory, particular care and a high standard of personal hygiene are essential in microbiological work because pathogenic organisms may always be encountered. In addition, media not necessarily designed for pathogens may support their growth so that great care is needed in the handling and disposal of all cultures. Sound technique is the basis of safe microbiological procedures and it is important that all individuals concerned with microbiological work should have received adequate training. It is also important that the necessary laboratory equipment and facilities should conform to accepted codes of safety and good laboratory practice.

Certain precautions are essential in the laboratory not only to prevent contamination of samples and culture media but also to avoid infection hazards to personnel, namely:

- strictly observe personal hygienic precautions, keep nails short, and use hair and beard protection if necessary;
- wash hands with soap and warm water both before and after microbiological work as well as after use of the toilet;
- always wear protective clothing in the laboratory;
- sterilize all cultures by autoclaving, and sterilize or disinfect associated apparatus and materials before disposal or re-use;
- do not eat, drink or smoke in the laboratory;
- report any accident, spillage or unusual occurrence to senior personnel;
- if in doubt, ask for advice.

5 Diluents and culture media

5.1 General

Constituents of uniform quality and analytical grade chemicals should be used for the preparation of media. Alternatively, dehydrated complete media may be used. The manufacturer's instructions should be strictly followed.

The water used should be glass-distilled or demineralized and free from substances that might inhibit growth of micro-organisms under the test conditions. It should comply with the requirements of ISO 3696, Grade 3.

5.1.1 Sterilization

Diluents and culture media should be dispensed in containers suitable for sterilization by autoclaving. For most purposes, a temperature of $121\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 15 min is adequate. However, a different time and temperature may sometimes be required and details are given in each individual standard.

Alternatively, with thermolabile substances sterilization may be effected by filtration.

5.2 Diluents

One of the following diluents should be used.

5.2.1 Peptone diluent

Composition

Peptone	1,0 g
Water	to 1 000 ml

Preparation

The peptone should be dissolved in approximately 950 ml of water. The pH should be adjusted by adding sodium hydroxide solution [$c(\text{NaOH}) = 1\text{ mol/l}$] or hydrochloric acid [$c(\text{HCl}) = 1\text{ mol/l}$] so that after sterilization the pH will be $7,0 \pm 0,1$. The solution should be made up to 1 000 ml with water, distributed and sterilized.

5.2.2 Peptone saline solution

Composition

Peptone	1,0 g
Sodium chloride	8,5 g
Water	to 1 000 ml

Preparation

The constituents should be dissolved in the water and the pH adjusted by adding sodium hydroxide solution [$c(\text{NaOH}) = 1\text{ mol/l}$] or hydrochloric acid [$c(\text{HCl}) = 1\text{ mol/l}$] so that after sterilization it will be $7,0 \pm 0,1$. The solution should be made up to 1 000 ml with water, distributed and sterilized.

5.2.3 Ringer's solution — quarter-strength

Composition

Sodium chloride	2,25 g
Potassium chloride	0,105 g
Calcium chloride (anhydrous)	0,12 g
Sodium hydrogen carbonate	0,05 g
Water	to 1 000 ml

Preparation

The constituents should be dissolved in the water and the solution made up to 1 000 ml.

5.2.4 Phosphate buffer solution

Composition

Potassium dihydrogenorthophosphate (KH_2PO_4)	42,5 mg
Magnesium chloride (MgCl_2) (see note)	190 mg
Water	1 000 ml

Preparation

a) Phosphate solution

To prepare the solution 34 g of potassium dihydrogen-orthophosphate should be dissolved in 500 ml of distilled water. The pH should be adjusted to a value of $7,2 \pm 0,5$ with 1 mol/l sodium hydroxide solution and the solution made up to 1 000 ml with distilled water.

NOTE — Alternatively, an equivalent amount of magnesium sulfate* ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) may be used.

b) Magnesium chloride solution

To prepare the solution 38 g of magnesium chloride should be dissolved in 1 000 ml of distilled water.

Final solution

For use, 1,25 ml of phosphate solution a) and 5,0 ml of magnesium chloride solution b) should be added to 1000 ml of distilled water. The solution should be dispensed in convenient volumes and sterilized by autoclaving at $121\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 15 min.

5.2.5 Distilled water

Distilled water of suitable grade should be distributed and sterilized (see 5.1).

5.3 Culture media

In general most media after sterilization in sealed containers may be stored satisfactorily for several months at room temperature provided they are kept in the dark and remain sealed. Media dispensed aseptically may be stored at between $4\text{ }^{\circ}\text{C}$ and $10\text{ }^{\circ}\text{C}$ for up to 1 month; before use, they should be inspected carefully for contamination, excessive evaporation, or other evidence of deterioration. Most reagents are best kept at $4\text{ }^{\circ}\text{C}$. Culture media supplied pre-poured should be used in accordance with the manufacturer's instructions.

*See national foreword for details of textual error.

6 Sterilization of apparatus and glassware

Apparatus and glassware not supplied sterile should be sterilized by one of the following methods:

- a) in an oven, at $160\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for 1 h (or $170\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for 40 min);
- b) in an autoclave, at $121\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for a minimum of 20 min.

If membrane filters are not obtained sterile, they should be sterilized before use according to the manufacturer's instructions or, in their absence, by autoclaving at $115\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 10 min or, for vegetative organisms, disinfected by boiling gently in distilled water for 10 min.

7 Samples

7.1 Sampling

For guidance on sampling techniques, refer to ISO 5667-2.

The prime objective is to obtain a sample which is representative, as far as possible, of the water to be examined. To achieve this, certain precautions are necessary which are common to all sampling procedures for bacteriological examination:

- a) for re-use, sampling bottles should be able to withstand repeated sterilization without producing or releasing substances likely to inhibit or promote bacterial growth;
- b) sampling bottles should contain an agent to neutralize any residual disinfectant in the water and should be sterile. The neutralizing agent should not affect the viability or growth of the organisms sought. For chlorine, 0,1 ml of a 1,8 % (m/m) sterile solution of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) should be added before sterilization to each bottle for each 100 ml of capacity;
- c) scrupulous care should be taken to avoid accidental contamination of the sample during collection and subsequent handling;
- d) the sample should be examined as soon as possible after collection, preferably within 6 h;
- e) every sample bottle should be clearly identifiable, and should be accompanied by sufficient information concerning the nature of the sample and the reasons for the examination requested.

7.2 Preservation and handling

For guidance on preservation and handling of samples, refer to ISO 5667-3.

In general, samples should be transported to the laboratory for examination as soon as possible. If there is likely to be a delay, the samples should be placed in a cool insulated container.

7.3 Preparation of test sample

Before examination, the sample should be mixed thoroughly by vigorous agitation to achieve uniform distribution of micro-organisms and, depending on the nature of the water and the bacterial content anticipated, any dilutions necessary made at this stage.

For ten-fold dilutions, 90 ml or 9 ml of the diluent should be measured out into sterile dilution bottles or tubes. Alternatively, volumes of diluent, pre-sterilized in screwcapped bottles, should be used. One or more ten-fold dilutions should be made by transferring one volume of water sample to nine volumes of diluent. With a fresh pipette or by mechanical means, the solution should be mixed thoroughly and one volume of this dilution transferred to another nine volumes of diluent. These steps should be repeated as often as required. Sufficient of each dilution for all the tests to be carried out on the sample should be prepared. For dilutions other than ten-fold, the volume of diluent to volume of sample should be adjusted accordingly.

For general guidance on the preparation of ten-fold dilutions, refer to ISO 6887; this guidance can also be applied to making dilutions with a dilution factor other than 10.

8 Enumeration after inoculation of test portions of the sample in (or on) solid media

8.1 Principle

A test portion of the water sample is inoculated by mixing with or spreading on the surface of a specified solid culture medium so that on incubation, micro-organisms form colonies either on or in the medium.

For practical purposes each colony is considered to have originated from a single micro-organism or a clump of micro-organisms present in the sample at the moment of inoculation. Taking into account the volume of the test portion and the number of colonies formed, the result can therefore be expressed as a number of colony-forming units in a given volume of the sample, e.g. 1 ml or 100 ml.

8.2 Procedures

8.2.1 General

Three main procedures may be used for the inoculation of solid media.

- a) The sample is mixed with the medium which has previously been melted and cooled to a temperature close to that of solidification; after incubation the colonies which develop within and on the surface of the medium are counted.
- b) The sample is spread over the dry surface of an agar medium and after incubation, colonies which develop on its surface are counted.

c) The sample is passed through a membrane filter, which retains the micro-organisms sought; the membrane is then placed on an agar medium or on an absorbent pad impregnated with broth. On incubation, colonies form on the surface of the membrane. Alternatively, for certain organisms, such as anaerobes, the membrane may be placed face downwards in a Petri dish and overlaid with molten agar medium.

8.2.2 Choice of technique

The choice of technique depends on several factors including the physical and chemical characteristics of the water (see 10.2.2) as well as the nature of the micro-organisms sought (see 10.2.1), their probable concentration, and the test precision required. Indications are given in 8.2.3.1, 8.2.4.1, and 8.2.5.1 of the volumes of water samples which can be used for each method and the limits of detection are discussed in 10.1.3. In addition the precision of the methods is discussed in 10.1.2. The requirements of regulations may also influence the choice of technique to be used by indicating, for example, the precision desired or whether the presence or absence of an organism in a specified test volume will be sufficient.

8.2.3 Pour plate method

8.2.3.1 Test portion

The volume of the test portion of the sample, or of a dilution of the sample, can vary between 0,1 ml and 5 ml depending on the size of the Petri dish and the volume of culture medium used. The dilution should be chosen so that the expected number of colonies formed on plates of diameter 90/100 mm is between about 25 and 300.

8.2.3.2 Inoculation

The medium required should be melted in boiling water or steam, placed in a water bath at $45\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and with sterile precautions any additional ingredients necessary added at this stage.

The Petri dishes required should be prepared and marked, any dilutions necessary made in accordance with 7.3 and, after thorough mixing, the test portions distributed into the dishes.

Each tube of melted medium should be removed in turn from the water bath, the outside of the tube dried and the neck flamed. The medium should be added to the test portion in each Petri dish without delay and mixed carefully so as to obtain a uniform distribution of micro-organisms. The plates should be left to cool on a horizontal surface and then they should be incubated in accordance with 8.2.6. Generally 15 ml of medium is used for a test portion of 1 ml or 2 ml; for larger test portions, the composition of the medium should be adjusted accordingly.

8.2.4 Spread plate method

8.2.4.1 Test portion

For a Petri dish of between 90 mm and 100 mm diameter the volume of the test portion of the sample, or of a dilution of the

sample, should be 0,1 ml to 0,5 ml. The dilution should be chosen so that the expected number of colonies formed lies between about 25 and 300.

8.2.4.2 Inoculation

Plates, each containing about 15 ml of culture medium, should be prepared and marked; the surface of the medium should be dried before use. The test portion should be pipetted on to the surface of the medium and spread over the surface with a sterile glass rod or mechanical device. After absorption of the inoculum, incubation should be carried out in accordance with 8.2.6.

8.2.5 Membrane filtration method

8.2.5.1 Test portion

The maximum volume of the test portion depends on the filterability of the water sample and on the membranes used. This technique is suitable for waters which contain little particulate matter in suspension, for example drinking water. With membranes of mean pore diameter 0,45 μm , it may be possible to filter several litres of such water through a single membrane, and so achieve a high level of test sensitivity; for some organisms, however, filtration through a membrane with a mean pore diameter of 0,22 μm may be necessary. A test volume of the sample or a dilution of it should be chosen to yield less than about 100 colonies on a membrane of 47 mm or 50 mm in diameter.

8.2.5.2 Filtration

The sterile filtration apparatus should be connected to a source of vacuum. A sterile membrane should be placed, grid-side upwards, on the porous disc of the filter base, only the outer part of the membrane filter being grasped with flat-ended sterile forceps. The sterile funnel should be positioned securely on the filter base. With the vacuum stopcock turned off, one of the following should be poured or pipetted into the funnel:

- a known volume of the sample, or dilution of it, carefully mixed;
- the contents of a flask or bottle containing the test portion and sufficient diluent to bring the total volume to at least 20 ml to 30 ml;
- at least 20 ml of diluent, to which the test portion, measured with a pipette, is added directly and mixed with the pipette.

The stopcock should be opened and a vacuum of about 70 kPa applied to filter the water slowly through the membrane. The stopcock should be closed as soon as the sample has been filtered.

8.2.5.3 Transfer of membrane

The funnel should then be removed and the membrane transferred with sterile forceps to one of the following, ensuring that no air bubbles are trapped between the membrane and the medium:

- an agar medium in a Petri dish;