INTERNATIONAL STANDARD

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Second edition
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Water quality — Determination of biochemical oxygen demand after *n* days (BOD_n) —

Part 1: Dilution and seeding method with allylthiourea addition

Qualité de l'eau — Détermination de la demande biochimique en oxygène après n jours (DBO_n) —

Partie 1: Méthode par dilution et ensemencement avec apport d'allylthiourée

Reference number
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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 5815-1:2003), which has been technically revised. The main changes compared to the previous edition are as follows:

— change of working range: 1 mg/l instead of 3 mg/l as lower limit;

— changes in test procedure;

— in 5.2, option to check seeding water suitability in advance with a CGA control analysis batch;

— in 5.3.2, phosphate buffer solution pH-value: requirement for preparation of a new solution if the pH value is out of the range pH 7 and pH 8;

— in 5.5, range for oxygen consumption of seeded dilution water 0,2 mg/l to 1,5 mg/l instead of upper limit 1,5 mg/l;

— in 5.9, allowable range BOD_5 of the CGA control solution changed to (198 ± 40) mg/l and BOD_7 (206 ± 40) mg/l;

— in 6.5, electrochemical probe option to measure the dissolved oxygen concentration added;

— in 8.4, interferences: subclause on presence of peroxides and peroxide compounds added;

— in 9.4, options to determinate the dilutions elaborated;

— in 9.7, control analysis: elaborated description of procedure;

— in 10.3, "approval of results/validity criteria" added;

— Annex A: title changed and "normative" instead of "informative"

— Annex C "Direct seeding of the analysis batches" added;

— new Annex D "Performance data" included.

A list of all parts in the ISO 5815 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The incubation time specified in this document is 5 d or 7 d. The latter corresponds to the practice in several Nordic countries. Annex A describes an incubation time of (2 + 5) d.

ISO 5815-1 specifies the determination of the biochemical oxygen demand (BOD) of waters with an expected BOD in the range 1 mg/l to 6 000 mg/l using the dilution method. A lower limit of working range may result from validation data in the laboratory. For samples with an expected low BOD in the range of 0,5 mg/l to 6 mg/l ISO 5815-2 provides the option of the determination of the (BOD) of waters using undiluted samples.

Water quality — Determination of biochemical oxygen demand after *n* days (BOD_n) —

Part 1:
Dilution and seeding method with allylthiourea addition

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This document specifies the determination of the biochemical oxygen demand of waters by dilution and seeding with suppression of nitrification after 5 d or 7 d incubation time.

It is applicable to all waters having biochemical oxygen demands usually between 1 mg/l and 6 000 mg/l. It applies particularly to waste waters but also suits for the analysis of natural waters. For biochemical oxygen demands greater than 6 000 mg/l of oxygen, the method is still applicable, but special care is needed taking into consideration the representativeness of subsampling for preparation of the dilution steps. The results obtained are the product of a combination of biochemical and chemical reactions in presence of living matter which behaves only with occasional reproducibility. The results do not have the rigorous and unambiguous character of those resulting from, for example, a single, well-defined, chemical process. Nevertheless, the results provide an indication from which the quality of waters can be estimated.

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.

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

ISO 5667-3, *Water quality — Preservation and handling of water samples*

ISO 5813, *Water quality — Determination of dissolved oxygen — Iodometric method*

ISO 5814, *Water quality — Determination of dissolved oxygen — Electrochemical probe method*

ISO 6060, *Water quality — Determination of the chemical oxygen demand*

ISO 8245, *Water quality — Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC)*

ISO 8467, *Water quality — Determination of permanganate index*

ISO 10523, *Water quality — Determination of pH*

ISO 15705, *Water quality — Determination of the chemical oxygen demand index (ST-COD) — Small-scale sealed-tube method*

ISO 17289, *Water quality — Determination of dissolved oxygen — Optical sensor method*

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:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
biochemical oxygen demand after *n* days
BOD_n
mass concentration of dissolved oxygen consumed under specified conditions by the biochemical oxidation of organic and/or inorganic matter in water where *n* is the incubation time equal to 5 d or 7 d

Note 1 to entry: For the purposes of this document "biochemical oxidation" is taken to mean "biological oxidation".

Note 2 to entry: *n* is either 5 or 7.

3.2
chemical oxygen demand
COD
mass concentration of oxygen equivalent to the amount of dichromate consumed by dissolved and suspended matter when a water sample is treated with that oxidant under defined conditions

[SOURCE: ISO 6060:1989, 3]

3.3
total organic carbon
TOC
sum of organically bound carbon present in water, bonded to dissolved or suspended matter, including cyanate, elemental carbon and thiocyanate

[SOURCE: ISO 8245:1999, 3.3]

3.4
permanganate index (of water)
mass concentration of oxygen equivalent to the amount of permanganate ion consumed when a water sample is treated with that oxidant under defined conditions

[SOURCE: ISO 8467:1993, 3.1]

3.5
seeding water
water with adapted (aerobic) microorganisms via which the oxidation of the water contents occurs

Note 1 to entry: The seeding water is used for producing the seeded dilution water.

3.6
dilution water
water added to the test sample to prepare a series of defined dilutions

[SOURCE: ISO 20079:2005, 3.7]

3.7
seeded dilution water
dilution water to which a definite amount of seeding water is added

3.8
free chlorine
chlorine present in the form of hypochlorous acid, hypochlorite ion or dissolved elemental chlorine

[SOURCE: ISO 7393-1:1985, 2.1]

3.9
combined chlorine
fraction of total chlorine present in the form of chloramines and organic chloramines

[SOURCE: ISO 7393-1:1985, 2.2]

3.10
nitrification
oxidation of ammonium salts by bacteria where usually the intermediate product is nitrite and the end product nitrate

[SOURCE: ISO 11733:2004, 3.9]

4 Principle

The BOD_n, with inhibition of nitrification is determined, using the dilution method. A batch series with different dilutions of a sample is prepared and examined. The dilution water is enriched with oxygen and seeded with adapted aerobic microorganisms.

The sample is incubated at (20 ± 1) °C for a specified period (n), 5 d or 7 d, in the dark, in a completely filled and stoppered bottle. The dissolved oxygen concentration is determined before and after incubation. The mass of consumed oxygen per litre sample is calculated.

5 Reagents

Use only reagents with the degree of purity "for analysis".

5.1 Water, at least grade 3 in accordance with ISO 3696.

The water shall not contain more than 0,01 mg/l of copper, nor chlorine or chloramines.

5.2 Seeding water, which can be obtained in one of the following ways:

a) municipal waste water, decanted or coarsely filtered;

b) surface water containing municipal waste water;

c) settled effluent from a waste water treatment plant;

d) water taken downstream from the discharge of the water to be analysed, or water containing microorganisms that are adapted to the water to be analysed;

e) commercially available seeding material.

Use seeding water with a COD of about 300 mg/l or a TOC of about 100 mg/l (see 5.5). If the COD or TOC are higher, adapt to these concentrations with dilution water (5.4) before preparing the seeded dilution water (5.5) or use a correspondingly changed volume of the seeding waters for seeding the dilution water (5.4).

If the sample comes from a process that has been subjected to disinfection treatment (chlorination, UV, ozone or other), use inoculum, even when there is no residual disinfectant present.

For commercially available seeding material consider respective application recommendations.

The selected seeding material can be checked in advance by running the procedure with a control analysis (9.7) batch only to prove its suitability for the analysis of samples.

5.3 Salt solutions

5.3.1 General

The following solutions can be kept for at least six months in glass bottles in the dark at (5 ± 3) °C. Discard the solutions at the first signs of precipitation or opaqueness.

5.3.2 Phosphate-buffer solution

Dissolve 8,50 g of potassium dihydrogen phosphate (KH_2PO_4), 21,75 g of dipotassium hydrogen phosphate (K_2HPO_4), 33,4 g of disodium hydrogen phosphate-heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) and 1,70 g of ammonium chloride (NH_4Cl), in about 500 ml of water (5.1). Dilute with water (5.1) to 1 000 ml and mix. Measure the pH value. If the pH value is outside the range pH 7 to pH 8, prepare a new solution.

5.3.3 Magnesium sulfate heptahydrate solution, ρ = 22,5 g/l.

Dissolve 22,5 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in water (5.1). Dilute with water (5.1) to 1 000 ml and mix.

5.3.4 Calcium chloride solution, ρ = 27,5 g/l.

Dissolve 27,5 g of anhydrous calcium chloride ($CaCl_2$) (or an equivalent amount, if the hydrate is used (for example 36,4 g $CaCl_2 \cdot 2H_2O$) in water (5.1), dilute with water (5.1) to 1 000 ml and mix.

5.3.5 Iron (III)-chloride-hexahydrate solution, ρ = 0,25 g/l.

Dissolve 0,25 g of iron (III)-chloride-hexahydrate ($FeCl_3 \cdot 6H_2O$), in water (5.1). Dilute with water (5.1) to 1 000 ml and mix.

5.4 Dilution water

Determine the total volume of dilution water required for the actual test. Pour about half the required volume of water (5.1) into the feed vessel (6.3) for the dilution water and add 1 ml of each of the salt solutions (5.3.2, 5.3.3, 5.3.4 and 5.3.5) for each litre of the total volume. Then fill to the required total volume with water (5.1) and mix by stirring, aeration or shaking. Bring the dilution water obtained in this way to a temperature of (20 ± 2) °C, keep at this temperature and aerate slightly by mixing. If, for example, specially adapted seeding water or seeding material is necessary, the procedure according to Annex C can be followed.

EXAMPLE If 20 l of dilution water are required, prepare 10 l of water (5.1). Stirring continuously, add 20 ml of each of the salt solutions individually and fill up with water (5.1) to 20 l.

5.5 Seeded dilution water

The preparation of a seeded dilution water is needed when the test solutions are prepared according to 9.3 The mass concentration of oxygen consumed over 5 d (or 7 d) at (20 ± 1) °C by the seeded dilution water with the addition of allylthiourea (ATU) solution to inhibit nitrification [blank value (see 9.5)], shall be between 0,2 mg/l and 1,5 mg/l.

The volume increase of the dilution water by seeding water should be as low as possible.

The amount of the seeding water (5.2) needed to attain a hypothetic COD of 0,6 mg/l to 3,0 mg/l, corresponding to the aimed oxygen consumption in the blank values (9.5), is calculated with Formula (1):

$$V_{\text{seeding water}} = \frac{\text{COD}_{\text{target}} \cdot V_{\text{dilution water}}}{\text{COD}_{\text{seeding water}}} \quad (1)$$

where

$V_{\text{seeding water}}$ is the volume of the seeding water (5.2) to be added to the dilution water (5.4) in litres, l;

$\text{COD}_{\text{target}}$ is the hypothetic COD (0,6 mg/l O_2 to 3 mg/l O_2) in the seeded dilution water (5.5) in milligrams per litre of oxygen, mg/l O_2;

$\text{COD}_{\text{seeding water}}$ is the COD of the seeding water (5.2) in milligrams per litre of oxygen, mg/l O_2;

$V_{\text{dilution water}}$ is the calculated amount of the dilution water to be seeded (5.4) in litres, l.

For direct seeding of test batches or automated systems which use a direct seeding, see instructions in Annex C.

Add the seeding water (5.2) to the dilution water (5.4) and mix by stirring or shaking. Determine the oxygen content as specified in ISO 5813, ISO 17289 or ISO 5814. Aerate the seeded dilution water up to an oxygen content of preferably a minimum 8 mg/l. The water shall not be supersaturated with oxygen by aeration: let it stand about 1 h in an unstopped container before use. Keep the seeded dilution water at (20 ± 2) °C. The so prepared seeded dilution water can be used immediately for the preparation of the analysis batches.

Throw away the residue of the dilution water at the end of the working day, unless the laboratory experience reveals via the complied-with control analysis (9.7) with the control solution (5.9), and the blank value determination (9.5) that the water is acceptable for a longer time.

5.6 Hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) solution, for example c(HCl) ≈ 0,5 mol/l or $c(H_2SO_4)$ ≈ 0,25 mol/l.

5.7 Sodium hydroxide (NaOH) solution, for example c(NaOH) = 0,5 mol/l, ρ ≈ 20 g/l.

5.8 Sodium sulfite (Na_2SO_3) solution, for example $\rho(Na_2SO_3)$ = 50 g/l.

5.9 Glucose-glutamic acid (GGA), control solution.

Dry about 200 mg to 300 mg of anhydrous D-glucose ($C_6H_{12}O_6$) and 200 mg to 300 mg of anhydrous L-glutamic acid ($C_5H_9NO_4$) at (105 ± 5) °C for 1 h. Weigh (150 ± 1) mg of each substance, dissolve in water (5.1), dilute with water to 1 000 ml, and mix. The theoretical oxygen demand of this solution is 307 mg/l of oxygen for BOD_5 (the empirical BOD_5 is (198 ± 40) mg/l of oxygen and the BOD_7 (based on conversion factor BOD_7/BOD_5 = 1,04 from Table D.3 and previous empirical BOD_5) is (206 ± 40) mg/l of oxygen).

Prepare the solution immediately before use and discard any remaining solution at the end of the working day. The solution may also be frozen in small amounts. The frozen solution can be kept for a maximum of three months. Use the thawed solution immediately after thawing.

5.10 Allylthiourea (ATU) solution, ρ = 1,0 g/l.

Dissolve 200 mg of allylthiourea ($C_4H_8N_2S$) in water (5.1), dilute with water (5.1) to 200 ml and mix. Store the solution at (5 ± 3) °C. The solution is stable for at least two weeks.

WARNING — The reagent is toxic and shall therefore be handled according to the safety data sheet.

The nitrification inhibition is not attained in all cases by addition of 2 ml of the ATU-solution (ρ = 1,0 g/l) per litre of analysis batch. The addition of a significantly higher volume than 2 ml of this ATU-solution can disturb the titration according to ISO 5813 (see 9.6.1).

6 Apparatus

Usual laboratory equipment, and in particular the following.

6.1 General

Plastic and glass vessels shall be carefully cleaned and, in particular, made free of absorbed toxic and biodegradable compounds and shall be protected from contamination.

6.2 Incubation bottles, BOD-bottles (Karlsruhe type) with a content between 100 ml and 300 ml or conical shoulder bottles with stoppers and a suitable funnel, or other suitable, bubble-free closing bottles. For the use of automatic systems, it is important to use incubation bottles with a definite volume, as the incubation bottles serve as dilution vessels.

6.3 Feed vessel for the seeded and non-seeded dilution water, made from glass or plastic.

Take measures to ensure that the vessel is kept clean and free from microorganism growths, and protected from light.

6.4 Tempering cabinet, room or incubator, capable of being maintained at (20 ± 1) °C and darkened.

6.5 Equipment for determining dissolved oxygen concentration, as specified in ISO 5813 (iodometric method), or ISO 5814 (electrochemical probe method) using an oxygen probe or ISO 17289 (optical sensor method) using optical oxygen measurement.

6.6 Cooling and freezing device, for transport and storage of the samples.

6.7 Dilution vessel, mixing vessel preferably made from glass, for example volumetric flask or graduated measuring cylinder, with sufficient volume capacity for the dilution batch and the possibility of a thorough mixing.

6.8 Aeration equipment, bottle of compressed air or a compressor. The air quality shall be such that the aeration does not lead to any contamination, especially by the addition of organic matter, oxidizing or reducing materials, or metals. If contamination is suspected, filter and wash the air.

6.9 pH-measuring equipment, which fulfils the requirements for the determination of pH, as specified in ISO 10523.

6.10 Stirrer, to ensure that the sample is homogeneous for the extraction of partial samples and no air is taken in.

6.11 Glass fibre filter GF 6.

7 Sampling and preservation

Closable vessels made from glass or plastic are suitable for sampling. The volume should be large enough to ensure that a proper dilution series can be derived. Fill the sampling vessels completely, close

them and cool to (5 ± 3) °C as soon as possible. Store the samples in the dark to prevent algal growth and cool at (5 ± 3) °C until processing at latest the day after the date of sampling.

If samples cannot be analysed in between the day after sampling freeze the samples as soon as possible after sampling and store samples at ≤−18 °C for up to one month, or up to six months if BOD is >50 mg/l, in a suitable vessel in the dark or in dark coloured bottles as specified in ISO 5667-3. Make sure that the bottles do not cause excessive blank values (9.5). Since completely filled sample containers are transported, the volume reduction up to a volume which allows expansion caused by freezing (prevention of breakage) takes place in the laboratory after homogenization of the sample.

Thaw the sample at maximum (20 ± 2) °C, but not for more than 16 h, as otherwise the onset of bacterial processes can falsify the results. The sample should therefore be frozen in portions, each of which does not exceed a volume of one litre. The thawing of the samples through any means of heating equipment, for example microwave or heating plate in which (a part of) the sample reaches a temperature of more than 22 °C is not allowed and leads to false results. The temperature of a heated water bath shall not exceed 22 °C. Complete thawing of a sample before use is essential, as the freezing process may lead to the concentration of certain components in the inner part of the sample which freezes last. A once thawed sample shall not be frozen again.

8 Interferences

8.1 General

Substances that are toxic to microorganisms, for example bactericides, free chlorine etc., inhibit the biochemical oxidation and lead to reduced findings. Increased results can occur due to the presence of nitrifying microorganisms. The presence of algae can lead to an overestimation of BOD and disturb the determination.

8.2 Presence of free and/or combined chlorine

Remove free and/or combined chlorine in the sample by adding the required volume of sodium sulfite solution (5.8). Take care to avoid adding an excess: perform the determination of the free and combined chlorine concentration as specified in ISO 7393-1[3] or ISO 7393-2[4] and calculate the volume of the sodium sulfite solution according to Formula (2).

$$V_1 = \frac{1{,}78 \times V_p \times \rho_2}{\rho_3} \tag{2}$$

where

V_1 is the volume of the sodium sulfite solution (5.8) in millilitres, ml;

V_p is the sample volume to be treated in millilitres, ml;

ρ_2 is the measured free chlorine in the sample in grams per litre, g/l;

ρ_3 is the concentration of sodium sulfite solution in grams per litre, g/l.

8.3 Presence of algae

In the case of samples containing algae, consider filtration after sampling in the field or directly after arrival at the laboratory to avoid producing unusually high results. Use glass fibre filters (6.11). Filtering can change BOD results radically and it shall only be performed if deemed necessary in the evaluation of the quality of the water or if the analysis from an algae-free sample is required. Indicate any filtration in the test report (see Clause 11).

8.4 Presence of peroxides and peroxide compounds

NOTE Peroxides and peroxide compounds can be found in waste water of the chip industry or large laundries, etc.

Peroxides and peroxide compounds create reduced findings; hence peroxides shall be eliminated from samples to be examined.

The following methods can be applied for indication if peroxides are suspected:

— Proof with iodide starch paper. Iodide and filter paper containing starch is coloured blue in the presence of peroxide, but it reacts with chlorine as well. Peroxide-test strips are commercially available for this method.

— Proof by using oxygen content measurement (9.6.2). An oversaturation of the dissolved oxygen concentration in the sample can indicate peroxides and peroxide compounds.

If peroxides have been detected, various methods of destroying peroxides can be applied:

— Peroxides can be expelled by shaking or stirring the sample intensively in an open vessel. The presence of peroxides shall be tested using peroxide test strips or measurement of dissolved oxygen concentration from time to time, but not for more than a 2 h time period. The expulsion of the peroxides is finished when the dissolved oxygen concentration no longer decreases within a period of 30 min or the test strips no longer indicate any peroxides.

— Some peroxide compounds cannot be eliminated with these methods. The peroxide compounds can then be destroyed with a sodium sulfite solution (5.8). Use an aliquot of the sample to determine the volume of sodium sulfite solution (5.8) needed to destroy the peroxides. Avoid an excess and test the spontaneous oxygen consumption. The test for complete destruction can be carried out with peroxide test strips. Based on these results calculate the amount of sodium sulfite solution needed to remove peroxides and peroxide compounds in the sample volume required for the test. Add the equivalent to the sample aliquot that is used for the BOD determination.

It shall be noted that this treatment of the sample can change it, hence document the sample treatment for the elimination of peroxides in the test report.

9 Procedure

9.1 General

If the presence of substances that are toxic to microorganisms is suspected, Annex B with the testing of several different dilutions of the sample should be applied.

The number of bottles to be prepared depends on the technique to measure the dissolved oxygen content and the number of replicates desired.

The direct seeding of the analysis batches is described in Annex C.

9.2 Pretreatment

Pre-treatment is carried out for samples within at latest the day after sampling or for frozen samples after complete thawing of the sample.

9.2.1 Neutralization of the sample

Neutralize the sample or the diluted samples (for example in automatic systems) with hydrochloric acid (5.6) or sodium hydroxide solution (5.7) if the pH-value is not between 6 and 8. Choose the concentration of the hydrochloric acid (5.6) or the sodium hydroxide (5.7) solutions to restrict the

volume added to not more than 5 % of total volume. Indicate potential precipitation resulting hereby in the test report.

NOTE If the whole sample meets pH requirements, so will any dilution.

9.2.2 Homogenization

For sub-sampling the fresh or thawed sample shall be thoroughly mixed before distribution. An even distribution of all soluble and particulate components should be ensured (for example by gentle agitation or vigorous shaking). If the sample contains large particles, which complicate the withdrawal of partial samples of equal quality, homogenization by crushing the particles with for example a dispersing device is recommended.

9.3 Preparation of test solutions

Temper the sample to a temperature of (20 ± 2) °C. Assure homogeneity of the sample during preparation of the dilutions, for example by gentle agitation or vigorous shaking either manually or with a stirrer without the inclusion of air until all analysis batches are produced.

Carry out the test with preferably two replicates per dilution. Depending on the purpose of testing and quality assurance requirements for selected samples a testing of only one replicate per dilution is acceptable. Report the number of dilutions (see 9.4) and the number of replicates per dilution tested for the sample in the test report [Clause 11 d)].

The following description of the procedure takes into account at least two replicates per dilution and several dilutions.

Calculate the volumes of sample and seeded dilution water needed to prepare the test solutions for the selected number of dilutions and replicates considering 9.4 for the selection of dilutions. If the iodometric method (9.6.1) is used, a second series of bottles with replicates is needed to determine the concentration of dissolved oxygen at time zero.

Place a known volume of the sample (or pretreated sample) each in the dilution vessels (6.7), add 2 ml of allylthiourea solution (5.10) per litre of diluted sample and fill to the mark with seeded dilution water (5.5). If the dilution factor to be used is greater than 100, carry out serial dilutions in two or more steps. When automatic analysis systems are applied, add a definite volume of the sample (or the pre-treated sample) into the incubation bottle. The addition of 2 ml allylthiourea solution (5.10) per litre batch and the seeded dilution water (5.5) occur fully automatically through the system.

9.4 Calculation of dilutions

9.4.1 Empirical determination of the dilutions

As the correct dilution degree of the sample which gives a measurable BOD_n in at least one of the dilutions cannot be precisely attained, several different dilutions varying according to the dilution factor (as reciprocal value of sample volume to total volume of test batch) and including the dilution corresponding to the expected BOD_n (see Table 1 and Annex B) are prepared. Various options for the determination of dilutions factors and dilution series are described in 9.4.2 to 9.4.3. If sufficient information on the oxygen consumption of the sample is available (for example known, reproducible, stable composition of the sample type under investigation), the testing of only one dilution is acceptable, as far as the test results for this dilution comply with the validity criteria (10.3). If the presence of substances that are toxic is suspected (for example strong chemical odour), dilutions are indispensable and the preparation of dilutions over a broader range of concentrations is recommended.

Table 1 — Examples of typical dilution series (3 dilution stages each) for determination of BOD_5

Expected BOD_n n = 5 d mg/l oxygen	Possible sample volume in ml/l (for example as respective dilution series) ml/l	Dilution factor[a]
1 to 6	250, 500, 750[b] or 200, 400, 600	4 to 1,33
4 to 12	200, 400, 600[b] or 200, 300, 400	5 to 1,67
10 to 30	200, 400, 600 or 50, 100, 150	20 to 1,67
20 to 60	100, 200, 300 or 40, 60, 80	25 to 3,33
40 to 120	40, 80, 120 or 20, 30, 40	50 to 8,33
100 to 300	30, 40, 50 or 5, 10, 15	200 to 20
200 to 600	10, 20, 30 or 3, 6, 9	333 to 33,3
400 to 1 200	4, 8, 12 or 1, 2, 3	1 000 to 83,3
1 000 to 3 000	2, 4, 6 or 0,5; 1,0; 1,5[c]	2 000 to 167
2 000 to 6 000	1, 2, 3 or 0,3; 0,6; 0,9	3 333 to 333

[a] Applied sample volume in relation to the total volume of the analysis batch.

[b] Recommendation for samples from sewage treatment processes with good biological treatment.

[c] For this preferably prepare dilutions of for example 1:10. (Data including preliminary dilution).

9.4.2 Determination of dilutions via the factors *R* of the TOC, the permanganate index or the COD

The determination of the total organic carbon (TOC) (according to ISO 8245), the permanganate index (according to ISO 8467) or the chemical oxygen demand (COD) (according to ISO 6060 or ISO 15705) in a representative aliquot of the sample can provide valuable decision aids for a dilution series in this respect.

Table 2 shows typical intervals for *R*, the ratio of BOD_n to TOC, permanganate index or COD, for some sample types.

Table 2 — Typical values for ratio *R*

	Total organic carbon BOD_n/TOC	Permanganate-index BOD_n/Permanganate-index	Chemical oxygen demand BOD_n/COD
Untreated waste water	1,2 to 2,8	1,2 to 1,5	0,35 to 0,65
Biologically treated waste water	0,3 to 1,0	0,5 to 1,2	0,10 to 0,35

Select from experience appropriate R-values from Table 2 and calculate the expected BOD*n* values according to Formula (3):

$$BOD_{n(\text{expected})} = R \cdot y \tag{3}$$

where

y is the measured TOC-value or permanganate-index or COD-value in milligrams per litre, mg/l;

R is the respective dimensionless ratio from Table 2

Use the lower and upper values of the ratios *R* from Table 2 respectively to calculate the expected BOD_n. Based on these calculated expected BOD_n concentrations, seek out possible volumes of sample per litre test batch and the respective dilution factors from Table 1. Select the third dilution in relation to the two calculated dilutions.

EXAMPLE Untreated waste water, *R* is from 1,2 to 2,8 and the measured TOC (y) is 100 mg/l.

Expected BOD_n = 1,2 × 100 mg/l = 120 mg/l.

Expected BOD_n = 2,8 × 100 mg/l = 280 mg/l.

As the expected BOD_n is between 120 mg/l and 280 mg/l, a dilution series with 30 ml/l, 40 ml/l and 50 ml/l is appropriate.

9.4.3 Calculation of dilution stages via the COD

For samples with a COD >60 mg/l preferably use following Formula (4). Calculate the lowest dilution stage F (i.e. the stage with the highest sample volume content) via the COD according to Formula (4):

$$F = \frac{10\,000}{\mathrm{COD}} \tag{4}$$

where

F is the sample volume in millilitres per litre, ml/l, of total batch volume;

COD is the numerical value of the chemical oxygen demand in milligrams per litre, mg/l.

Half F to determine the highest dilution stage with the lowest sample volume (lowest sample volume = $F/2$). The third dilution stage is calculated as mean value of both calculated dilution stages.

EXAMPLE The COD is 250 mg/l O_2.

$$F = \frac{10\,000}{250} = 40$$

The lowest dilution stage is 40 ml sample volume per litre total batch volume. If F = 40 ml/l is divided in two, the highest dilution stage with 20 ml/l sample volume is determined. The mean value of both sample volumes is 30 ml/l sample volume and 30 ml/l is the average dilution stage. The dilution series to be aimed for should include these concentrations.

9.5 Blank value determination

Carry out a blank test in parallel with the determination, using the seeded dilution water (5.5) including 2 ml of ATU solution (5.10) per litre of analysis batch.

If the BOD of the blank solutions exceeds 1,5 mg/l O_2, possible causes, for example sources of contamination shall be sought.

If the analysis batches are directly seeded, use the procedure described in Annex C.

9.6 Determination of dissolved oxygen

9.6.1 Measurement of dissolved oxygen using iodometric method (in accordance with ISO 5813)

Fill two series of incubation bottles (6.2) with one or more replicates for each dilution stage (9.4) with the diluted test solutions (9.3), and at least two bottles with the blank solution (9.5) allowing them to overflow slightly.

During filling, precautions shall be taken to prevent changing the oxygen content of the solutions.

Allow any air bubbles adhering to the walls to escape. Stopper the bottles, taking care to avoid trapping air bubbles.

Divide the bottles into two series, each containing at least two bottles of each dilution and at least two bottles of blank solution (see 9.5).

Put the first series of bottles with diluted test solutions and the blank solution in the incubator (6.4) and leave in darkness for *n* days ±4 h at (20 ± 1) °C.

In the second series of bottles, measure the concentration of dissolved oxygen at time zero. Use the method specified in ISO 5813.

After the incubation, determine the dissolved oxygen concentration in each of the bottles, using the method specified in ISO 5813.

9.6.2 Measurement of dissolved oxygen using probes (in accordance with ISO 5814 or ISO 17289)

Fill one or more replicate incubation bottles (6.2) each with the diluted test solutions (9.3) and two bottles each with the blank solution (9.5) allowing them to overflow slightly. Take precautions to prevent changing the oxygen content of the medium.

Allow any air bubbles adhering to the walls to escape.

Measure the dissolved oxygen concentration in each of the bottles at time zero, using the method specified in ISO 5814 or ISO 17289.

If necessary, add some dilution water to substitute the overflowed water and stopper the bottles, taking care to avoid trapping air bubbles.

Put the bottles with diluted test solutions (9.3) and the blank solution (9.5) in the incubator (6.4) and leave in darkness for *n* days ±4 h at (20 ± 1) °C.

After the incubation, determine the dissolved oxygen concentration in each of the bottles, using the method specified in ISO 5814 or ISO 17289.

9.7 Control analysis

Check the method for each sample series by using the glucose-glutamic acid control solution (5.9). For this, pour 20 ml/l each of the glucose-glutamic acid control solution (5.9) into the dilution vessel or incubation bottle. Add the ATU solution (5.10) in such way that a dilution of 2 ml ATU solution (5.10) per 1 l of the analysis batch is attained and fill with water (5.1)

For practical reasons, the number of control measurements may be reduced, for example in labs where seeded dilution water is stored in large vessels for several days a control can be tested in the first and the last series with this dilution water preparation. This approach is acceptable, if sufficient practical experience regarding the trend of the results for testing the blank value and the control solution (5.9) is available and the other validity criteria (10.3) are met for each series. Perform the measurement according to 9.6.

EXAMPLE Prepare a control with a 20 ml/l glucose-glutamic acid control solution (5.9) at the beginning and end of the batch using a prepared filling vessel (6.3).

If a higher precision is requested, a dilution series with 10 ml/l, 20 ml/l and 30 ml/l of solution 5.9 can be included.

The attained BOD_n values shall be within the range (198 ± 40) mg/l of oxygen during the BOD_5 determination and within the range (206 ± 40) mg/l of oxygen for the BOD_7 determination.

NOTE The large GGA range reflects the accepted level of sample and analytical variability.

The precise control limits for each laboratory may be lower (for example (198 ± 20) mg/l) and shall be derived by the elaboration of a control chart based on a minimum of 25 determinations over a period of at least several weeks. The mean value calculated from results for all dilutions and replicates and the standard deviations can then be used to calculate control limits for quality control checks.

If the attained BOD_n cannot be achieved, check if the procedure was carried out in a proper way.

10 Calculation and indication of the results

10.1 Examination of test solutions for valid oxygen consumption during test

Test solutions are valid and can be used for the calculation of the BOD_n test result if at least 2,5 mg/l are depleted as oxygen consumption during the incubation and at least 1 mg/l dissolved oxygen remains at the end of the 5-day or 7-day period in the dilution taken.

The minimum oxygen consumption of 2,5 mg/l does not apply in case the lowest possible dilution factor is used.

The lower limit of working range results from validation data in the laboratory and can be calculated by three times the reproducibility standard deviation ($3 \times s_R$) of a test solution near the limit of quantification (LOQ) with the lowest possible dilution factor.

10.2 Calculation of biochemical oxygen demand after *n* days (BOD_n)

Calculate the biochemical oxygen demand (BOD_n), expressed in milligrams per litre of oxygen for each bottle, using the Formula (5):

$$BOD_n = \left[(\rho_1 - \rho_2) - \frac{V_t - V_{sam}}{V_t} \times (\rho_3 - \rho_4) \right] \times \frac{V_t}{V_{sam}} \quad (5)$$

where

ρ_1 is the dissolved oxygen concentration of one of the test solutions at time zero, in milligrams per litre, mg/l;

ρ_2 is the dissolved oxygen concentration of this same test solution after *n* days, in milligrams per litre, mg/l;

ρ_3 is the dissolved oxygen concentration of the blank solution at time zero, in milligrams per litre, mg/l;

ρ_4 is the dissolved oxygen concentration of the blank solution after *n* days, in milligrams per litre, mg/l;

V_{sam} is the volume of sample used for the preparation of the test solution concerned, in millilitres, ml;

V_t is the total volume, in millilitres, ml, of this test solution.

If the method according to Annex C is used (see Clause 9), apply the calculation with Formula (C.1) in Annex C.

If at least two test dilutions are valid according to 10.1, calculate the average of the results obtained for these solutions as BOD_n test result.

Use the result of a single dilution for the final calculation of BOD_n result

a) only if this dilution complies with all approval criteria (10.1), or

b) select one out of at least three dilutions as BOD_n test result if the calculation using all valid test solutions (10.1) and (10.3) according to Formula (B.4) reveals $r \geq 0{,}995$.

Express results in milligrams of oxygen per litre. Report results less than 10 mg/l of oxygen to the nearest mg/l. Results above 10 mg/l of oxygen can be reported to two significant figures, for example 12 mg/l of oxygen or 380 mg/l of oxygen.

10.3 Validity criteria

The results obtained in the test of each set of analysis are reliable and therefore deemed to be approved, if they meet the following acceptance criteria:

— the oxygen consumption of the blank solution (9.5) in the test is 0,2 mg/l to 1,5 mg/l O_2;

— the BOD_n of the glutamic-glucose acid solution (9.7) in the set is within the allowable range (198 ± 40) mg/l for BOD_5 and (206 ± 40) mg/l for $BOD_{7;}$

— at least 2,5 mg/l shall be depleted as oxygen consumption during the incubation and at least 1 mg/l dissolved oxygen remains at the end of the 5-day (or 7-day period) in the dilutions taken for the calculation of the BOD_n test result

The results that do not meet the preassigned criteria cannot be considered valid.

If the failure is due to some of the quality controls, the entire test shall be rejected.

11 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO/FDIS 5815-1:2019;

b) specification, that the test was carried out with suppression of nitrification;

c) the number of days of incubation (n);

d) number of dilutions and respective replicates per dilution tested;

e) the BOD_n , in milligrams of oxygen per litre (reported as described in 10.2) for the dilutions tested and the calculated or selected BOD_n as final test result;

f) for results below the working range, a documentation for an adequate detection limit;

g) any special details which may have been noted during the test (for example precipitation);

h) details of any operations not specified in this document, or which were regarded as optional, such as filtration (see 8.3), freezing (see Clause 7), homogenization (see 9.2.2), elimination of peroxides (see 8.4), alternative incubation periods (BOD_{2+5}) (see Annex A) and multitesting (see Annex B).

Annex A
(normative)

Influence of incubation periods and temperatures

The rate of oxidation of carbon during the first stage of the BOD test is expressed by Phelps' law [Formula (A.1)]:

$$\log_{10} \frac{L}{L-x} = k \cdot t \qquad \text{(A.1)}$$

where

L is the ultimate BOD at infinite time, in milligrams per litre of oxygen, mg/l O_2;

x is the BOD at time t, in milligrams per litre of oxygen, mg/l O_2;

t is the time, in days, d;

k is the rate constant, expressed as the reciprocal day.

For a given type of organic matter and microbial seed, the effect of temperature on the rate constant k and on the value of L can be predicted to a first approximation. This may be useful when considering the use of the BOD test in warm climates, or in studies of long rivers which traverse a number of climatic regions. It is essential that such relationships, however, are used with caution.

The standard BOD result is obtained after 5-day or 7-day incubation at (20 ± 1) °C.

Due to practical reasons the BOD_5-determination can be substituted by BOD_{2+5}-determination. By incubating for 2 d at (0 to 4) °C followed by 5 d at (20 ± 1) °C, a BOD_{2+5} result is obtained. It has been observed (Reference [1] and Table D.2) that there is no significant difference between BOD_5 and BOD_{2+5} after sample dilution.

When determining BOD_{2+5}, keep the bottles with the diluted test solutions (see 9.3) and the blank solution (9.5) in darkness at (0 to 4) °C for 2 d ± 2 h, put them afterwards in the incubator (6.4) and leave in darkness with the temperature of the solutions equilibrated at (20 ± 1) °C for 5 d ± 2 h. The temperature rise of the samples takes place at (20 ± 1) °C, in the dark and during the time interval of 2 h required to the maximum.

NOTE If the same incubator is used for the samples at both temperatures, it can be necessary to use an incubator with a ventilator in order to enable the temperature change in the required time span of up to 2 h.

When BOD_5 determinations are substituted by BOD_{2+5} determinations, the laboratory has to verify that its procedure for BOD_{2+5} determination gives equivalent results to BOD_5 determination.

The results differ from BOD_7-determination, where the incubation temperature is constant during the whole incubation period. If BOD_7 is measured, a conversion factor (BOD_5 / BOD_7) should be used in order to calculate BOD_5 (see Annex D).

Annex B
(informative)

Multitesting

Multitesting is the analysis of a sample in two or more different dilutions and replicates and the evaluation using all dilutions and replicates. This procedure may be used if enhanced precision is desired, or the presence of substances toxic to microorganisms is suspected.

The sample is analysed as described in 9.3. Oxygen consumption during incubation is determined for each bottle of the dilution batches of the samples and the dilution water batches of the blank solution are calculated according to Formula (B.1):

$$S\rho_{ij} = \rho_1 - \rho_2 \tag{B.1}$$

where

ρ_1 is the dissolved oxygen concentration of one of the test solutions or blank solution at time zero, in milligrams per litre, mg/l;

ρ_2 is the dissolved oxygen concentration of this same test solution or blank solution after n days, in milligrams per litre, mg/l;

$S\rho_{ij}$ is the oxygen consumption of the j-th replicate sample of the i-th dilution or blank solution in milligrams per litre, mg/l.

The sample parts of the dilutions that are part of the further evaluation are calculated according to Formula (B.2):

$$\Phi_i = \frac{V_{\text{sam}}}{V_{\text{t}}} \tag{B.2}$$

where

Φ_i is the volume fraction of the water sample in the i-th dilution (sample content) in millilitres per millilitre, ml/ml;

V_{sam} is the volume of sample used for the preparation of the test solution concerned, in millilitres, ml;

V_{t} is the total volume, in millilitres, ml, of this test solution.

The validity of the oxygen consumption of the test batches is proven with Formula (5) in 10.2. The numerical evaluation presupposes the validity of the mixing rule, which can be checked in practice graphically simplified. The oxygen consumption during incubation $S\rho_{ij}$ for each bottle with a dilution is plotted against the volume of sample in each dilution Φ_i.

The oxygen consumption of the blank bottles is plotted as the zero-volume value.

An example with a prepared evaluation table of the test results (Table B.1) and the plot of these data is given in Figure B.1. If the relationship between the oxygen consumption and the sample volume in the plot is linear, the BOD_n does not reveal components in the sample that inhibit microorganisms. If the plot shows a linear relationship between the oxygen consumption and the sample volume only for low sample concentrations, only sample dilutions within the linear range are used for determining BOD_n. The BOD_n is calculated as described in Clause 10 and is the average value of all determinations within the linear range.

If reliably appearing values for the oxygen consumption are present for the dilution water and for at least two dilution stages, the collated results can in addition be evaluated statistically. The coefficient of correlation (r) is calculated for these results additionally to the BOD_n.

If the points are located along a straight line, the measuring series can be evaluated without restrictions.

Outliers will remain unconsidered during the further evaluation. The causes of divergent measuring values should nevertheless be investigated. Large variations in dissolved oxygen readings between bottles containing the same concentration of sample are indicative of either contamination or use of a non-homogenous sample.

If the validity of the mixing rule is successfully checked graphically, r, the coefficient of correlation, can be calculated according to Formula (B.3):

$$r = \frac{N \cdot \Sigma \phi_i \cdot S\rho_{ij} - \Sigma \phi_i \cdot \Sigma S\rho_{ij}}{\sqrt{\left[N \cdot \Sigma \phi_i^2 - \left(\Sigma \phi_i\right)^2\right] \cdot \left[N \cdot \Sigma S\rho_{ij}^2 - \left(\Sigma S\rho_{ij}\right)^2\right]}} \tag{B.3}$$

The size of the coefficient of correlation and the deviation between the oxygen consumption of the dilution water as found in the test and as averaged by regression calculation provide together significant evidence for the validity of the mixing rule. If the coefficient of correlation is greater than 0,995 and at the same time the difference between the oxygen consumption found and the oxygen consumption averaged by regression calculation is not greater than 0,1 mg/l, it can be assumed that the mixing rule is fulfilled. In all other cases, the graphic control method can be used to decide whether the statistical evaluation based on the collated results can be carried out with a correspondingly restricted data set.

EXAMPLE As an example, a table (see Table B.1) can be created in an evaluation program.

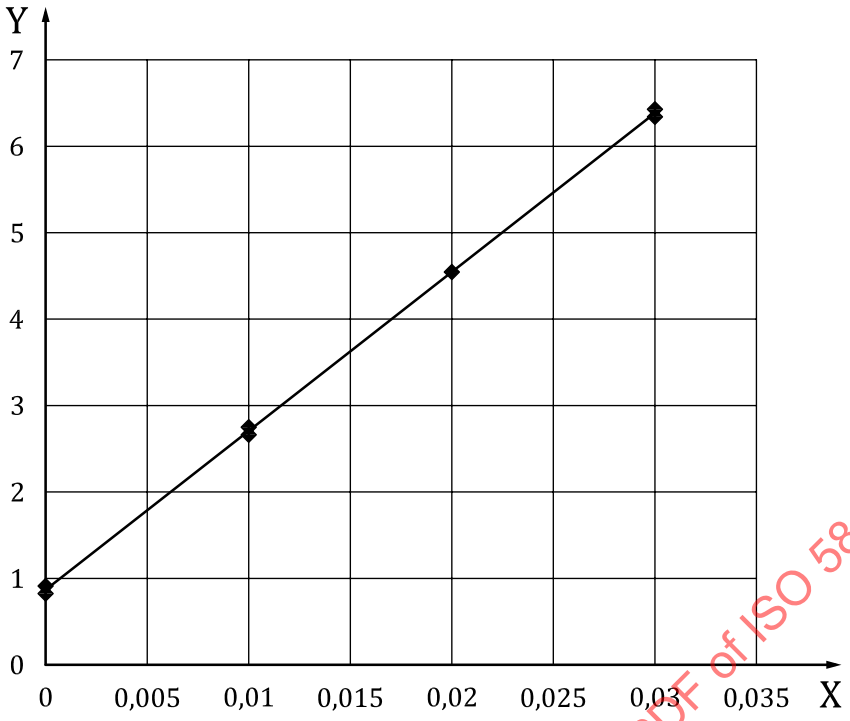
Table B.1 — Evaluation table

Batch	N	Sample content ml/ml	Square sample content	Initial oxygen concentration mg/l	Final oxygen concentration after n days mg/l	Oxygen consumption mg/l	Square oxygen consumption	Product of sample content and oxygen consumption
		Φ_i	Φ_i^2	ρ_1	ρ_2	$\rho_1 - \rho_2 = S\rho_{ij}$	$S\rho_{ij}^2$	$\Phi_i \cdot S\rho_{ij}$
Dilution water	1	0	0	8,5	7,7	0,8	0,64	0
Dilution water	2	0	0	8,6	7,7	0,9	0,81	0
1. Dilution	3	0,01	0,000 1	8,5	5,8	2,7	7,29	0,027
1. Dilution	4	0,01	0,000 1	8,6	6,0	2,6	6,76	0,026
2. Dilution	5	0,02	0,000 4	8,5	4,0	4,5	20,25	0,09
2. Dilution	6	0,02	0,000 4	8,6	4,1	4,5	20,25	0,09
3. Dilution	7	0,03	0,000 9	8,6	2,2	6,4	40,96	0,192
3. Dilution	8	0,03	0,000 9	8,5	2,2	6,3	39,69	0,189
Sums	N*	$\Sigma\Phi_i$	$\Sigma\Phi_i^2$			$\Sigma S\rho_{ij}$	$\Sigma S\rho_{ij}^2$	$\Sigma\left(\Phi_i \cdot S\rho_{ij}\right)$
Intermediate results:	8	0,12	0,002 8			28,7	136,65	0,614

N Bottle number.

N* Quantity of results.

From Table B.1, the oxygen consumption of the batches ($S\rho_{ij}$) can be transferred in a Cartesian coordinate system (Figure B.1) over the associated volume fraction of the sample (Φ_i) in the dilution batch.



Key

X dilution in ml/l

Y oxygen consumption in mg/l O_2

Figure B.1 — Graphic test in respect to validity of the mixing rule in the case of an evaluation example with plotted compensation line

From Table B.1, the sums of the last line can be put into Formula (B.3) to calculate the coefficient of correlation *r*:

$$r = \frac{8 \times 0{,}614 - 0{,}12 \times 28{,}7}{\sqrt{\left[8 \times 0{,}002\,8 - 0{,}12^2\right] \times \left[8 \times 136{,}65 - 28{,}7^2\right]}} = 0{,}999\,8 \qquad \text{(B.4)}$$