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Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer

Qualité de l'eau — Évaluation, en milieu aqueux, de la biodégradabilité aérobie ultime des composés organiques par détermination de la demande en oxygène dans un respiromètre fermé

Reference number
ISO 9408:1999(E)

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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 9408 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 9408:1991), which has been technically revised.

Annexes A to D of this International Standard are for information only.

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Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer

WARNING — Activated sludge and sewage contain potentially pathogenic organisms. Take appropriate precautions when handling them. Handle with care toxic test compounds and those whose properties are unknown.

1 Scope

This International Standard specifies a method, by determination of the oxygen demand in a closed respirometer, for the evaluation in aqueous medium of the ultimate biodegradability of organic compounds and waste waters at a given concentration by aerobic microorganisms.

The method applies to organic compounds which

a) are water-soluble under the conditions of the test;

b) are poorly water-soluble under the conditions of the test, in which case special measures may be necessary to achieve good dispersion of the compound (see for example, ISO 10634);

c) do not reach and react with the CO_2 absorbent;

d) are volatile, provided that a suitable respirometer or suitable conditions (e.g. a smaller ratio of volume head space to volume liquid medium) are used;

e) are not inhibitory to the test microorganisms at the concentration chosen for the test. The presence of inhibitory effects can be determined as specified in 7.3, or by using any other method for determining the inhibitory effect of a compound on bacteria (see, for example, ISO 8192).

NOTE The conditions described in this International Standard do not always correspond to the optimal conditions for allowing the maximum degree of biodegradation to occur. For alternative biodegradation methods, see ISO 15462.

2 Terms and definitions

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

2.1
ultimate aerobic biodegradation
breakdown of a chemical compound or organic matter by microorganisms in the presence of oxygen to carbon dioxide, water and mineral salts of any other elements present (mineralization) and the production of new biomass

2.2
primary biodegradation
structural change (transformation) of a chemical compound by microorganisms, resulting in the loss of a specific property

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2.3
activated sludge
biomass produced in the aerobic treatment of wastewater by the growth of bacteria and other microorganisms in the presence of dissolved oxygen

2.4
concentration of suspended solids of an activated sludge
amount of solids obtained by filtration or centrifugation of a known volume of activated sludge and drying at about 105 °C to constant mass

2.5
biochemical oxygen demand
BOD
mass concentration of dissolved oxygen consumed under specified conditions by the aerobic biological oxidation of a chemical compound or organic matter in water

NOTE It is expressed in this case as milligrams oxygen uptake per milligram (or gram) test compound.

2.6
chemical oxygen demand
COD
mass concentration of oxygen equivalent to the amount of a specified oxidant consumed by a chemical compound or organic matter when a water sample is treated with that oxidant under defined conditions

NOTE It is expressed in this case as milligrams oxygen consumed per milligram (or gram) test compound.

2.7
theoretical oxygen demand
ThOD
theoretical maximum amount of oxygen required to oxidize a chemical compound completely, calculated from the molecular formula

NOTE It is expressed in this case as milligrams oxygen required per milligram (or gram) test compound.

2.8
dissolved organic carbon
DOC
that part of the organic carbon in the water which cannot be removed by specified phase separation

NOTE Examples of specified phase separation are centrifugation at 40 000 $m \cdot s^{-2}$ for 15 min or by membrane filtration using membranes with pores of 0,2 µm to 0,45 µm diameter.

2.9
lag phase
time from the start of a test until adaptation and/or selection of the degrading microorganisms are achieved and the biodegradation degree of a chemical compound or organic matter has increased to about 10 % of the maximum level of biodegradation

NOTE It is expressed in days.

2.10
maximum level of biodegradation
maximum biodegradation degree of a chemical compound or organic matter in a test, above which no further biodegradation takes place during the test

NOTE It is expressed in percent.

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2.11
biodegradation phase
time from the end of the lag phase of a test until about 90 % of the maximum level of biodegradation has been reached

NOTE It is expressed in days.

2.12
plateau phase
time from the end of the biodegradation phase until the end of the test

NOTE It is expressed in days.

2.13
pre-exposure
pre-incubation of an inoculum in the presence of the test chemical compound or organic matter, with the aim of enhancing the ability of this inoculum to biodegrade the test material by adaptation and/or selection of the microorganisms

2.14
preconditioning
pre-incubation of an inoculum under the conditions of the subsequent test in the absence of the test chemical compound or organic matter, with the aim of improving the performance of the test by acclimatization of the microorganisms to the test conditions

3 Principle

Determination of the biodegradation of organic compounds by aerobic microorganisms is carried out using a static aqueous test system. Organic compounds in the context of this International Standard include waste waters. The test mixture contains an inorganic medium, the organic compound as the sole source of carbon and energy at a mass concentration of normally 100 mg/l organic carbon [but its theoretical oxygen demand (ThOD) shall be at least 100 mg/l], and a mixed inoculum obtained from a waste-water treatment plant or from another source in the environment.

The mixture is agitated in a closed test vessel and the consumption of oxygen is determined either by measuring the amount of oxygen required to maintain a constant gas volume in the respirometer vessel, or by measuring the change in volume or pressure (or a combination of the two) in the apparatus. The evolved carbon dioxide is absorbed in a suitable substance in the test vessel.

The degradation is followed over a period of 28 d, or longer if necessary, by determining the consumption of oxygen either automatically or manually. The amount of oxygen consumed by the organic compound (after correction by comparison with blank control) is expressed as a percentage of either the theoretical oxygen demand (ThOD), calculated from the formula of the compound, or the chemical oxygen demand (COD).

For sufficiently water-soluble compounds, removal of dissolved organic carbon (DOC) may be determined (optionally) by measuring the concentration of DOC at the beginning and the end of incubation to obtain additional information on the ultimate biodegradability. If a substance-specific analytical method is available, information on the primary degradability may be obtained.

4 Test environment

Incubation shall take place in the dark or in diffused light, at a temperature within the range 20 °C to 25 °C which shall not vary by more than ± 1 °C during the test.

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5 Reagents

Use only reagents of recognized analytical grade.

5.1 Water

Distilled or deionized water containing less than 1 mg/l DOC.

5.2 Test medium

5.2.1 Composition

5.2.1.1 Solution a)

Dissolve

anhydrous potassium dihydrogenphosphate (KH_2PO_4)	8,5 g
anhydrous dipotassium hydrogenphosphate (K_2HPO_4)	21,75 g
disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	33,4 g
ammonium chloride (NH_4Cl)	0,5 g
water (5.1), in quantity necessary to make up to	1000 ml

In order to check this buffer solution, it is recommended to measure the pH, which should be at about 7,4. If this is not the case prepare a new solution.

5.2.1.2 Solution b)

Dissolve 22,5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot H_2O$) in water (5.1), quantity necessary to make up to 1000 ml.

5.2.1.3 Solution c)

Dissolve 36,4 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in water (5.1), quantity necessary to make up to 1000 ml.

5.2.1.4 Solution d)

Dissolve 0,25 g iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in water (5.1), quantity necessary to make up to 1000 ml. Prepare this solution freshly before use, or add a drop of concentrated hydrochloric acid (HCl) to avoid precipitation

5.2.2 Preparation of the test medium

For 1000 ml of test medium, add to about 800 ml of water (5.1):

— 10 ml of solution a);

— 1 ml of each of the solutions b) to d).

Make up to 1000 ml with the water (5.1). Prepare the test medium freshly before use. The solutions a) to c) may be stored up to 6 months in the dark at room temperature.

5.3 Carbon dioxide absorber

Potassium hydroxide solution (about 10 mol/l), soda lime pellets or other suitable absorbent.

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5.4 Mercury chloride solution

Dissolve 1 g of mercury(II) chloride ($HgCl_2$) in 100 ml of the water (5.1).

5.5 Sodium hydroxide solution

Dissolve sodium hydroxide (NaOH) in the water (5.1) to obtain a solution of concentration 0,1 mol/l to 0,5 mol/l.

5.6 Hydrochloric acid solution

Dilute concentrated hydrochloric acid (HCl) in the water (5.1) to obtain a solution of concentration 0,1 mol/l to 0,5 mol/l.

6 Apparatus

Ensure that all glassware is thoroughly cleaned and free from organic or toxic matter.

6.1 Closed respirometer

The principle of a closed respirometer is given in annex D. The respirometer contains test vessels allowing oxygen supply and stirring, including tubing nonpermeable to oxygen and carbon dioxide. The respirometer vessels are located in a constant temperature room or in a thermostatically controlled water-bath. When testing volatile compounds, the apparatus used shall be appropriate or adapted to this particular purpose. Care shall be taken that there is no loss of compound due to the apparatus.

6.2 Water-bath or constant temperature room (to comply with clause 4)

6.3 Equipment for measurement of dissolved organic carbon

Instrument of sufficient sensitivity for the measurement of dissolved organic carbon (DOC) (optional).

6.4 Device for determining chemical oxygen demand (COD) (optional)

6.5 Centrifuge or device for filtration

The centrifuge shall be capable of producing an acceleration of 4 000 g.

The filtration apparatus shall be equipped with membrane filters (nominal aperture diameter of 0,2 μm to 0,45 μm pore size) which do not adsorb or release organic carbon.

6.6 pH meter (usual laboratory equipment)

7 Procedure

7.1 Preparation of the test solutions

7.1.1 Test compound

Prepare a stock solution of a sufficiently water-soluble test compound in the test medium (5.2) and add a suitable amount of this solution to the test vessels to obtain a final mass concentration of 100 mg/l test compound, but equivalent to at least 100 mg/l ThOD. Depending on the properties of the test compound (e.g. toxicity) and the purpose of the test, other concentrations may be used. Add compounds of low water-solubility directly into the test vessels. Determine the added amount exactly. Determine, if required, the COD of the test compound using e.g. ISO 6060.

NOTE For more details on handling poorly water-soluble compounds, see ISO 10634.

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7.1.2 Solution of the reference compound

Use as reference compound an organic compound of known biodegradability, such as aniline or sodium benzoate which have degradation degrees >60 %. Prepare a stock solution of the reference compound in the test medium (5.2) in the same way as with a water-soluble test compound (7.1.1), in order to obtain a final mass concentration of 100 mg reference compound per litre test medium.

7.1.3 Solution to check inhibition

If required (e.g. when no information on the toxicity of test compound is available), prepare a solution containing, in the test medium (5.2), both the test compound (7.1.1) and the reference compound (7.1.2) preferably at mass concentrations of 100 mg/l for each.

7.2 Preparation of the inoculum

7.2.1 General

Prepare the inoculum, using preferably activated sludge or the following sources (7.2.2 to 7.2.4) or a mixture of these sources, to obtain a microbial population that offers sufficient biodegradative activity. Check the activity of the inoculum by means of the reference compound (7.1.2 and clause 9). The BOD of the blank shall fulfil the validity criteria (see clause 9). To reduce the influence of the blank, it may be helpful to precondition the inoculum, e.g. by aerating it, up to one week before use. Use a suitable volume for inoculation.

NOTE Normally the inoculum should not be pre-exposed to the test compound, to allow a general prediction of the degradation behaviour in the environment. In certain circumstances, depending on the purpose of the test, pre-exposed inocula may be used, provided that this is clearly stated in the test report (e.g. percent biodegradation = x %, using pre-exposed inocula) and the method of pre-exposure is detailed in the test report. Pre-exposed inocula can be obtained from laboratory biodegradation tests conducted under a variety of conditions [e.g. Zahn-Wellens test (ISO 9888) and SCAS test (ISO 9887)] or from samples collected from locations where relevant environmental conditions exist (e.g. treatment plants dealing with similar compounds or contaminated areas).

Based on experience, suitable volume means:

— sufficient to give a population which offers enough biodegradation activity;

— degrades the reference compound by the stipulated percentage (see clause 9);

— gives between 10^3 to 10^6 colony-forming units per millilitre in the final mixture;

— gives not greater than the equivalent of 30 mg/l suspended solids of activated sludge in the final mixture;

— the quantity of dissolved organic carbon provided by the inoculum should be less than 10 % of the initial concentration of organic carbon introduced by the test compound;

— generally 1 ml to 10 ml of inoculum are sufficient for 1000 ml of test solution.

7.2.2 Inoculum from an activated sludge plant

Take a sample of activated sludge collected from the aeration tank of a full-scale or a laboratory waste water treatment plant dealing with predominantly domestic sewage. Mix well and determine the concentration of suspended solids of the activated sludge (use e.g. ISO 11923). If necessary, concentrate the sludge by settling so that the volume of sludge added to the test assay is minimal but nevertheless fulfils the criteria of 7.2.1. If it is suspected that the sludge contains inhibiting matter, centrifuge, wash with medium (5.2), recentrifuge and resuspend in the medium. Keep the sample under aerobic conditions and use preferably on the day of collection. Use a suitable volume (see 7.2.1) to obtain 30 mg/l of suspended solids in the final mixture.

7.2.3 Inoculum from waste water

Take a sample from the influent or from the effluent of a full-scale or a laboratory waste-water treatment plant dealing with predominantly domestic sewage. If necessary, concentrate the sample by filtration or centrifugation.

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Mix well, keep the sample under aerobic conditions and use preferably on the day of collection. Before use, let the sample settle for 1 h and take a suitable volume of the supernatant for inoculation.

7.2.4 Inoculum from a surface water

Take a sample of an appropriate surface water. If necessary concentrate the sample by filtration or centrifugation. Keep the sample under aerobic conditions and use preferably on the day of collection. Use a suitable volume as inoculum.

7.3 Test

Set up the closed respirometer (see 6.1 and the example described in annex D). Assemble a sufficient number of test vessels in order to have

- at least 2 test vessels (symbol F_T) for the test compound (7.1.1);
- at least 2 blank vessels (symbol F_B) containing test medium and inoculum;
- at least 1 vessel, for checking the procedure (symbol F_C) containing the reference compound (7.1.2);
- if needed, 1 vessel for checking a possible inhibitory effect of the test compound (symbol F_I) containing solution (7.1.3).
- if needed 1 vessel for checking a possible abiotic elimination (symbol F_S) containing the test compound (7.1.1) but no inoculum, sterilized by addition of a suitable inorganic toxic compound to prevent microbial activity. Use, for example, 1 ml/l of the mercury(II) chloride solution (5.4). Add the same amount of the toxic substance two weeks after the test is begun, if required.

Add appropriate amounts of the test medium (5.2), the inoculum (7.2), the test (7.1.1) and the reference compounds (7.1.2) at the desired concentrations to the respective vessels in accordance with Table 1 to obtain the desired final test volume. Add absorbent (5.3) to the CO_2-absorber compartments of the vessels. Measure the pH value of the vessel contents and adjust if necessary to 7,4 with solutions 5.5 or 5.6.

Place all test vessels in the water-bath or constant temperature room (6.2), allow them to reach the desired temperature (see clause 4), seal the vessels, and in the case of an automatic respirometer make any necessary connections, and start the stirrer. Take the readings of biochemical oxygen demand (oxygen consumption) on the manometers (if manual) or verify that the recorder of an automatic respirometer is functioning properly. Use the method given by the manufacturer for the appropriate type of respirometer.

If a nearly constant level of oxygen consumption is attained (plateau phase) and no further biodegradation is expected, the test is considered to be completed. Usually the maximum test period shall not exceed 28 d. Extend the test by one week to two weeks, if degradation has obviously started but has not reached a plateau.

On the last day of the test, measure the pH.

When DOC is being monitored, withdraw appropriately sized samples from the test vessels at the beginning (time 0) and end (time t) of the test period. Alternatively, determine the initial value (time 0) in a separately prepared vessel or calculate it from the added test compound. Either filter these samples through a membrane filter or centrifuge at 4 000 g for 15 min (see 6.5). When DOC measurements are not conducted the same day, keep the samples up to 48 h at 4 °C in the dark and in full tightly stoppered glass vessels.

NOTE DOC removal may be due to biodegradation but also to abiotic processes such as adsorption on the inoculum or the vessel wall or, in the case of volatile test compounds, stripping and adsorption on the tubing. When dealing with mixtures, selective adsorption of different components may occur.

When primary degradation is being monitored, determine the concentration of the test compound using specific analysis in vessels F_T and F_S at the end of the test (time t).

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Table 1 — Final distribution of test and reference compounds in the test vessels

Vessel	Test medium (5.2)	Test compound (7.1.1)	Reference compound (7.1.2)	Inoculum (7.2)
F_T Test compound	+	+	–	+
F_T Test compound	+	+	–	+
F_B Blank	+	–	–	+
F_B Blank	+	–	–	+
F_C Inoculum check	+	–	+	+
F_I Inhibition control (optional)	+	+	+	+
F_S Abiotic elimination check (optional)	+	+	–	–

If the test compound contains nitrogen, determine the final concentration of nitrate and nitrite immediately at the end of the test, or on suitably preserved samples. Alternatively, use a qualitative spot test procedure for nitrite and nitrate on a small volume of reaction mixture taken from each vessel and apply a quantitative method only if positive results are obtained. If nitrification has taken place, correct the oxygen consumption (see annex B).

8 Calculation and expression of results

8.1 Calculation

8.1.1 Specific biochemical oxygen demand

Express the oxygen consumption values for each vessel obtained from the reading of the respirometer as biochemical oxygen demand. Calculate the specific biochemical oxygen demand B_s according to equation (1). Correct the oxygen consumption in the case of nitrification (see 7.3 and annex B).

$$B_S = \frac{B_t - B_{Bt}}{\rho_{TC}} \qquad (1)$$

where

B_S is the specific biochemical oxygen demand, in milligrams of oxygen per gram of the test compound;

B_t is the measured biochemical oxygen demand of the test compound F_T at time t, in milligrams per litre;

B_{Bt} is the measured biochemical oxygen demand of the blank control F_B at time t, in milligrams per litre;

ρ_{TC} is the mass concentration of the test compound, in grams per litre.

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8.1.2 Percentage biodegradation

The biodegradation is defined as the ratio of the specific biochemical oxygen demand to either the theoretical oxygen demand (ThOD) (for an example of the calculation see annex A) or the chemical oxygen demand (COD). Determine the percentage degradation for each test vessel, using equations (2) and/or (3):

$$D_{\mathrm{ThOD}} = \frac{B_{\mathrm{S}}}{\mathrm{ThOD}} \times 100 \qquad (2)$$

$$D_{\mathrm{COD}} = \frac{B_{\mathrm{S}}}{\mathrm{COD}} \times 100 \qquad (3)$$

where

D_{ThOD} is the percentage biodegradation of ThOD at time t;

D_{COD} is the percentage biodegradation of COD at time t;

B_{S} is the specific oxygen demand of the test compound, expressed in milligrams per gram of test compound;

ThOD is the theoretical oxygen demand, expressed in milligrams per gram of test compound;

COD is the chemical oxygen demand determined experimentally, expressed in milligrams per gram of test compound.

NOTE Since the COD of a chemical is rarely as large as the ThOD, the percentage degradation of the COD is usually higher than the percentage degradation of the ThOD. The latter value is more accurate and should be used preferably.

8.1.3 Calculation of DOC removal

When DOC removal of a water-soluble test compound is determined, calculate for each test vessel F_T the percentage elimination of dissolved organic carbon D_C using equation (4):

$$D_C = \left(1 - \frac{\rho_{\mathrm{cT}t} - \rho_{\mathrm{cB}t}}{\rho_{\mathrm{cT0}} - \rho_{\mathrm{cB0}}}\right) \times 100 \qquad (4)$$

where

ρ_{cT0} is the DOC mass concentration, in milligrams per litre, at time 0, in the test vessel F_T;

ρ_{cB0} is the DOC mass concentration, in milligrams per litre, at time 0 in the blank vessel F_B;

$\rho_{\mathrm{cT}t}$ is the DOC mass concentration, in milligrams per litre, at time t in the test vessel F_T;

$\rho_{\mathrm{cB}t}$ is the DOC mass concentration, in milligrams per litre, at time t in the blank vessel F_B.

If ρ_{cT0} is calculated from the added test compound, neglect ρ_{cB0}.

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8.1.4 Calculation of primary biodegradation

When specific analyses of the test compound are performed, calculate the percentage of primary biodegradation D_S of the test compound compared to the amount of test compound in vessel F_S at the end of the test using equation (5).

$$D_S = \frac{\rho_S - \rho_T}{\rho_S} \times 100 \quad (5)$$

where

ρ_T is the mass concentration of the test compound, in milligrams per litre, in vessel F_T at time *t*;

ρ_S is the mass concentration of the test compound, in milligrams per litre, in vessel F_s at time *t*.

8.1.5 Reference compound, abiotic elimination and inhibition control

Calculate in the same way the biodegradation degree and the DOC removal of the reference compound F_C and, if included, of the abiotic elimination check F_S and the inhibition control F_I.

8.2 Expression of results

Compile a table of measured BOD values and the percentages of biodegradation D_{ThOD} and/or D_{COD} for each test vessel and for each measuring interval. In the case of automatic respirometers, appropriate timed points may be chosen from the automatically drawn oxygen-uptake curve. Plot a biodegradation curve, in percent, as a function of time, and indicate lag phase and degradation phase. If comparable results are obtained for the duplicate test vessels F_T (<20 % difference), plot a mean curve, otherwise plot curves for each vessel (see example in annex C). Plot in the same way a biodegradation curve of the reference compound F_C and, if included, of the abiotic elimination check F_S and the inhibition control F_I.

Determine the mean value of percent biodegradation in the plateau phase or use the highest value, e.g. when the curve decreases in the plateau phase, and indicate this maximum level of biodegradation as "degree of biodegradation of the test compound" in the test report.

Information on the toxicity of the test compound may be useful in the interpretation of test results showing a low biodegradation. If in vessel F_I the degradation percentage is <25 % and insufficient degradation of the test compound is observed in vessels F_T, it can be assumed that the test compound is inhibitory. In this case the test shall be repeated using a lower test concentration or another inoculum. If in vessel F_S (abiotic elimination check if included), a significant amount (>10 %) of BOD is observed, abiotic degradation processes may have taken place.

If DOC removal, primary degradation and/or nitrite/nitrate have been determined, indicate the measured and calculated values. Indicate the measured pH values.

9 Validity of results

9.1 Validity criteria

The test is considered as valid if

a) the percentage degradation in vessel F_C (inoculum check) is greater than 60 % on the 14th day;

b) the amount of BOD in the blank F_B at the end of the test, which is usually at about 20 mg/l to 30 mg/l, does not exceed 60 mg/l after 28 d.

If either a) or b) is not met, the test should be repeated using another or a better preconditioned inoculum.

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9.2 Inhibition

If the vessel F_I (inhibition control) was included, the test compound is assumed to be inhibiting if the degradation percentage of the reference compound in vessel F_I is lower than 40 % at the end of the test. In this case, it is advisable to repeat the test with a lower concentration of the test compound.

9.3 pH value

If the pH value at the end of the test is outside the range 6 to 8,5 (e.g. by nitrification of nitrogen-containing test compound) and if the percentage degradation of the test compound is less than 60 %, it is advisable to repeat the test with a lower concentration of the test compound, using a non-nitrifying activated sludge as inoculum or increasing the buffer capacity of the inorganic medium. This shall be indicated in the test report.

10 Test report

The test report shall contain at least the following information:

a) a reference to this International Standard;

b) all necessary information for the identification of the test compound ;

c) all the measured and calculated data (for example in tabular form) obtained, as well as the degradation curve;

d) the concentration, ThOD and/or COD of the test and reference compounds used;

e) the name of the reference compound used and the degradation obtained with this compound;

f) the source, the characteristics, the concentration or the volume of the inoculum used and information on any pretreatment;

g) the main characteristics of the respirometer used ;

h) the incubation temperature of the test ;

i) if included, the percentage of DOC removal or primary biodegradation;

j) if included, the percentage of degradation obtained in vessel F_S (abiotic elimination);

k) if included, the percentage of degradation in vessel F_I (inhibition check) and a statement on the toxicity of the test compound;

l) the reasons, in the event of rejection of the test;

m) any alteration of the standard procedure or any other circumstance that may have affected the results.

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Annex A
(informative)

Example of calculation of theoretical oxygen demand

A.1 General

The theoretical oxygen demand (ThOD) of the hypothetical substance $C_c\ H_h\ Cl_{cl}\ N_n\ Na_{na}\ O_o\ P_p\ S_s$, of relative molecular mass M_r, can be calculated according to:

$$\mathrm{ThOD}_{\mathrm{NH_3}} = \frac{16\left[2c + \frac{1}{2}(h - cl - 3n) + 3s + \frac{5}{2}p + \frac{1}{2}na - o\right]}{M_r}$$

This calculation implies that C is mineralized to CO_2, H to H_2O, P to P_2O_5 and Na to Na_2O. The halogen is eliminated as hydrogen halide. Nitrogen is eliminated as ammonia and not oxidized to nitrite or nitrate. Sulfur is assumed to be oxidized to the state of +VI.

In the case of a nitrogen-containing compound, the nitrogen may be eliminated after nitrification as nitrite or nitrate, with theoretical oxygen demands respectively equal to:

$$\mathrm{ThOD}_{\mathrm{NO_2}} = \frac{16\left[2c + \frac{1}{2}(h - cl) + 3s + \frac{3}{2}n + \frac{5}{2}p + \frac{1}{2}na - o\right]}{M_r}$$

$$\mathrm{ThOD}_{\mathrm{NO_3}} = \frac{16\left[2c + \frac{1}{2}(h - cl) + 3s + \frac{5}{2}n + \frac{5}{2}p + \frac{1}{2}na - o\right]}{M_r}$$

A.2 Example: glucose

Molecular formula $C_6H_{12}O_6$ and molecular mass M_r = 180.

$$\mathrm{ThOD} = \frac{16\left[2 \times 6 + \frac{1}{2} \times 12 - 6\right]}{180} = 1{,}07 \text{ mg/mg compound}$$

A.3 Example: sodium *n*-dodecylbenzenesulfonate

Molecular formula $C_{18}H_{29}SO_3Na$ and molecular mass M_r = 348.

$$\mathrm{ThOD} = \frac{16\left[2 \times 18 + \frac{1}{2} \times 29 + 3 + \frac{1}{2} - 3\right]}{348} = 2{,}34 \text{ mg/mg compound}$$

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A.4 Example: di-*n*-dodecylamine

Molecular formula $(C_{12}H_{25})_2NH$ and molecular mass M_r = 353. Assume full nitrate formation observed by analysis.

$$\mathrm{ThOD}_{\mathrm{NO_3}} = \frac{16\left[2 \times 24 + \frac{1}{2} \times 51 + \frac{5}{2}\right]}{353} = 3{,}44 \text{ mg/mg compound}$$

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Annex B
(informative)

Correction of oxygen uptake for interference by nitrification

If nitrification has occurred but is not complete, the observed oxygen uptake by the reaction mixture may be corrected for the amount of oxygen used in oxidizing ammonium to nitrite, and nitrite to nitrate, if the changes in concentration during incubation of nitrite and nitrate are determined, by considering the following equations:

$$2NH_4Cl + 3O_2 = 2HNO_2 + 2HCl + 2H_2O \qquad (B.1)$$

$$2HNO_2 + O_2 = 2HNO_3 \qquad (B.2)$$

Overall:

$$2NH_4Cl + 4O_2 = 2HNO_3 + 2HCl + 2H_2O \qquad (B.3)$$

From equation (B.1), the oxygen uptake when 28 g of nitrogen contained in ammonium chloride (NH_4Cl) is oxidized to nitrite is 96 g, i.e. a factor of 3,43 (96/28). In the same way, from equation (B.3), the oxygen uptake when 28 g of nitrogen is oxidized to nitrate is 128 g, i.e. a factor of 4,57 (128/28).

Since the reactions are sequential, being carried out by distinct and different bacterial species, it is possible for the concentration of nitrite to increase or decrease; in the latter case an equivalent concentration of nitrate would be formed. Thus, the oxygen consumed in the formation of nitrate is 4,57 times the increase in concentration of nitrate-N whereas the oxygen associated with the formation of nitrite is 3,43 times the increase in the concentration of nitrite-N. With the decrease in its concentration, the oxygen loss is 3,43 times the decrease in concentration.

That is:

$$O_1 = 4{,}57 \times \Delta_{NO_3\text{–}N} \qquad (B.4)$$

$$O_2 = 3{,}43 \times \Delta_{NO_2\text{–}N} \qquad (B.5)$$

$$O_3 = -\,[3{,}43 \times \Delta_{NO_2\text{–}N}] \qquad (B.6)$$

where

O_1 is the oxygen consumed in nitrate formation;

O_2 is the oxygen consumed in nitrite formation;

O_3 is the oxygen lost in nitrite disappearance;

$\Delta_{NO_3\text{–}N}$ is the increase in nitrate-N concentration;

$\Delta_{NO_2\text{–}N}$ is the change in nitrite-N concentration;

So that , using equations (B.4) and (B.5) or (B.6):

$$O_4 = [4{,}57 \times \Delta_{NO_3\text{–}N}] \pm [3{,}43 \times \Delta_{NO_2\text{–}N}] \qquad (B.7)$$

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and thus

$$O_5 = O_6 - O_4 \quad \text{(B.8)}$$

where

O_4 is the oxygen uptake due to nitrification;

O_5 is the oxygen uptake due to carbon oxidation;

O_6 is the total observed oxygen uptake.

Alternatively, if only total oxidized nitrogen is determined, the oxygen uptake due to nitrification may be taken to be, as a first approximation, 4,57 times the increase in the concentration of oxidized nitrogen.

The corrected value for oxygen consumption due to oxidation of carbon is then compared with $ThOD_{NH_3}$, as calculated in annex A.

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Annex C
(informative)

Example of a biodegradation curve

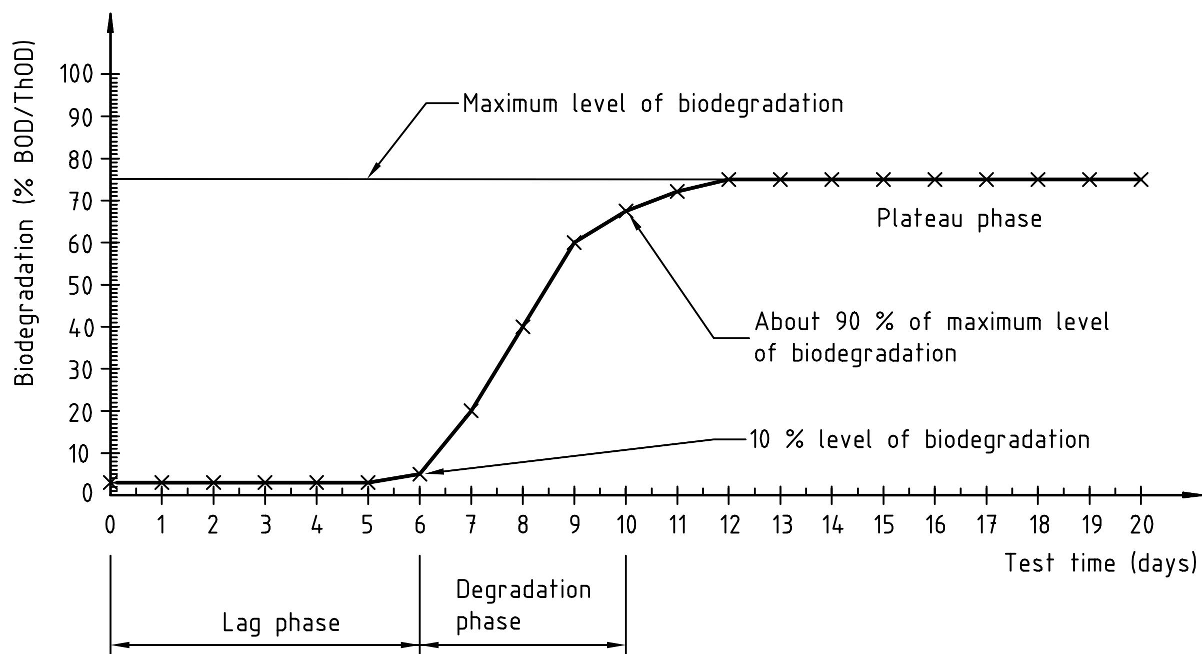

Figure C.1 — Biodegradation of aniline in the respirometric test

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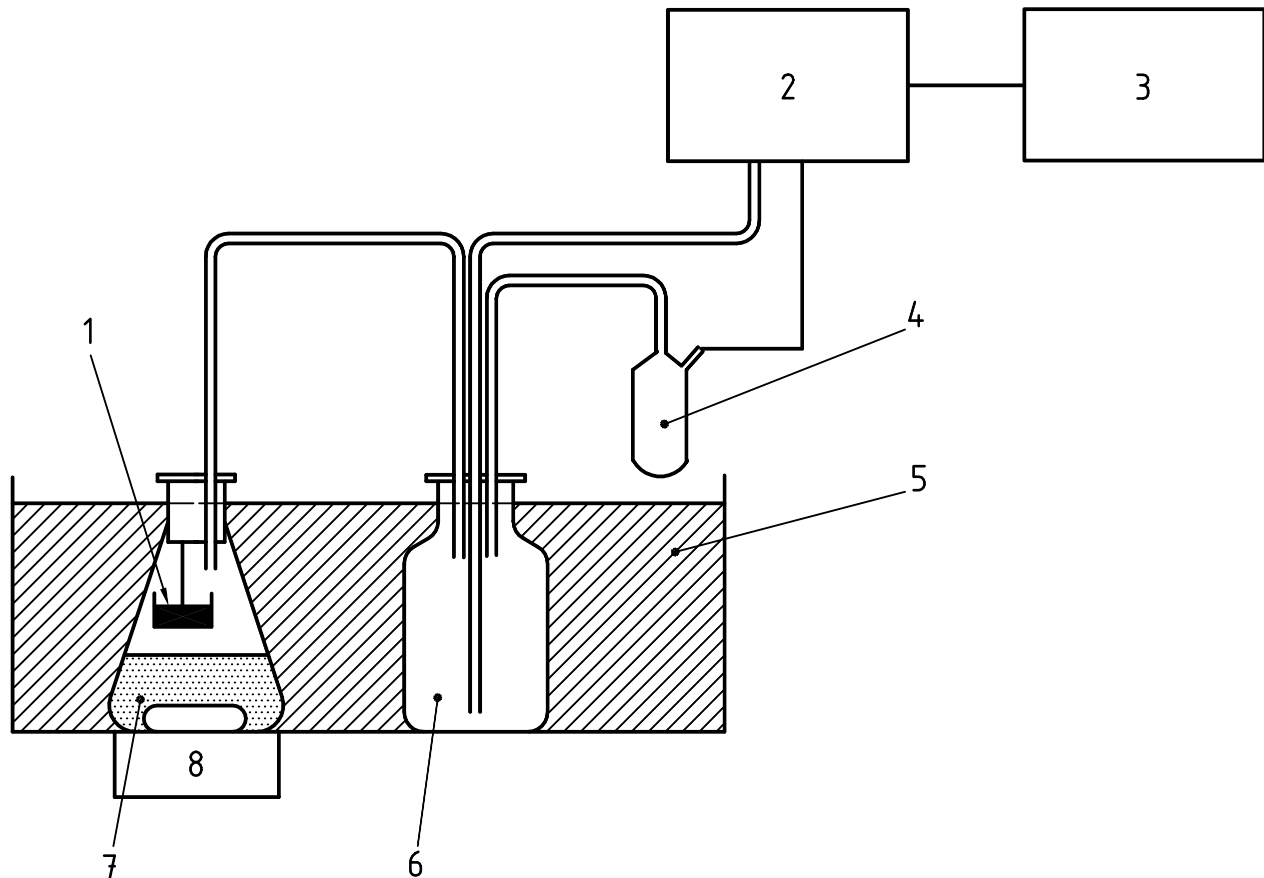
Annex D
(informative)

Closed respirometer



Key

1 CO_2 absorber

2 Monitor

3 Printer, plotter or computer

4 Manometer

5 Water bath

6 Oxygen-producing unit

7 Test flask

8 Stirrer

Figure D.1 — Principle of a closed respirometer

The test mixture is stirred by a magnetic stirrer in the test vessel, which is about one-third filled with liquid. If biodegradation takes place, the microorganisms consume oxygen and produce carbon dioxide. Oxygen in the gaseous phase of the vessel is then dissolved in the liquid. The carbon dioxide in the upper part of the vessel is absorbed, and the total pressure in the vessel then decreases.

This pressure drop is detected by a manometer, which is used to initiate the electrolytic generation of oxygen. When the original pressure is re-established, the electrolysis is stopped and the quantity of electricity used is measured in the monitor. The amount of electricity used is proportional to the consumed oxygen. This is indicated on a plotter, a printer, or directly on a computer.

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