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Water quality — Evaluation in an aqueous medium of the ultimate aerobic biodegradability of organic compounds — Determination of biochemical oxygen demand in a two-phase closed bottle test

Qualité de l'eau — Évaluation en milieu aqueux de la biodégradabilité aérobie ultime des composés organiques — Détermination de la demande biochimique en oxygène en fiole fermée à deux phases

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Contents

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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.

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 10708 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

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

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Water quality — Evaluation in an aqueous medium of the ultimate aerobic biodegradability of organic compounds — Determination of biochemical oxygen demand in a two-phase closed bottle test

WARNING – SAFETY PRECAUTIONS – Activated sludge and sewage may contain potentially pathogenic organisms. Therefore appropriate precautions should be taken when handling them. Toxic test compounds and those whose properties are unknown should be handled with care.

1 Scope

This International Standard specifies a method for the evaluation of the ultimate biodegradability by aerobic microorganisms of organic compounds, in particular poorly water-soluble compounds, at a given concentration.

NOTE 1 The conditions described in this International Standard do not necessarily always correspond to the optimal conditions allowing the maximum degree of biodegradation to occur.

The method applies to organic compounds which

– are water soluble or poorly water soluble at the concentration used under the test conditions;

– do not adsorb onto or have any effect on the oxygen electrode (see 8.1.2 and 8.3.4);

– are not inhibitory to the test microorganisms at the concentration chosen for the test.

NOTE 2 For special measures to introduce poorly water-soluble test compounds into the test vessels, see ISO 10634.

NOTE 3 Inhibitory effects can be determined as described in 8.1.4 and 8.3.1, or by using any other method for determining the inhibitory effect on bacteria of a substance (e.g. ISO 8192).

2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was 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 edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 10634:1995, *Water quality – Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium.*

3 Definitions

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

3.1 ultimate biodegradation
breakdown of an organic chemical compound by microorganisms in the presence of oxygen to yield carbon dioxide, water and mineral salts of any other elements present (mineralization) and new biomass

3.2 primary biodegradation
structural change (transformation) of an organic chemical compound by microorganisms resulting in the loss of a specific property

3.3 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, expressed in this case as milligrams of oxygen uptake per milligram or gram of test compound

3.4 theoretical oxygen demand (ThOD)
theoretical maximum amount of oxygen required to oxidize a chemical compound completely, calculated from the molecular formula, expressed in this case as milligrams of oxygen required per milligram or gram of test compound

3.5 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, expressed in this case as milligrams of oxygen consumed per milligram or gram of test compound

3.6 dissolved organic carbon (DOC)
that part of the organic carbon in water which cannot be removed by specified phase separation, for example by 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

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

3.8 lag phase
time from the start of a test until adaptation and 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 theoretical maximum biodegradation; it is expressed in days

3.9 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; it is expressed as a percentage

3.10 degradation phase
time from the end of the lag phase of a test until about 90 % of the maximum level of biodegradation has been reached; it is expressed in days

3.11 plateau phase
time from the end of the biodegradation phase when the maximum level of biodegradation is reached until the end of the test

3.12 pre-exposure
pre-incubation of an inoculum in the presence of the test compound, with the aim of enhancing the ability of an inoculum to biodegrade the test compound by adaptation and selection of the microorganisms

3.13 pre-conditioning
pre-incubation of an inoculum under the conditions of the test in the absence of the test compound, with the aim of improving the performance of the test by acclimatization of the microorganisms to the test conditions

4 Principle

Biodegradation of organic compounds by aerobic microorganisms is determined in an aquatic medium. The organic compound is the sole source of carbon and energy in the medium. The inoculated medium is shaken or stirred in closed bottles, containing known volumes of medium and air, to assure steady-state oxygen partitioning between liquid and gas phases. The degradation is followed by regular measurements of the dissolved oxygen concentration in the aqueous phase for up to 28 days. The total oxygen uptake in the test flasks is calculated from the difference in the measured dissolved oxygen concentrations in the blank and test flasks divided by the oxygen saturation value under normal conditions and multiplied by the total oxygen content originally present in the liquid and gaseous phases. Biodegradability is calculated as the total oxygen uptake divided by the theoretical oxygen demand (ThOD) or chemical oxygen demand (COD), expressed as a percentage.

5 Test environment

Incubation shall take place in the dark or in diffused light in an enclosure which is maintained at a constant temperature (± 0,5 °C) in a range between 20 °C and 25 °C.

6 Reagents

Use only reagents of recognized analytical grade.

6.1 Water

Distilled or deionized water containing less than 2 mg/l of DOC and/or less than 10 % of the initial organic carbon content introduced by the test compound.

6.2 Test medium

6.2.1 Composition

6.2.1.1 Solution A

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 (6.1) in a quantity necessary to make up to	1 litre

NOTE – The correct composition of the medium can be checked by measuring the pH value, which should be 7,4.

6.2.1.2 Solution B

Dissolve 22,5 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in 1 000 ml of water (6.1).

6.2.1.3 Solution C

Dissolve 27,5 g of anhydrous calcium chloride ($CaCl_2$) in 1 000 ml of water (6.1).

6.2.1.4 Solution D

Dissolve 0,25 g of iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in 1 000 ml of water (6.1). Prepare this solution freshly before use.

NOTE – It is not necessary to prepare this solution just before use if a drop of concentrated hydrochloric acid (HCl) or 0,4 g/l ethylenediaminetetraacetic acid (EDTA) is added.

6.2.2 Preparation

For 1 litre of test medium, add to about 500 ml of water (6.1)

- 10 ml of solution A;
- 1 ml of each of the solutions B to D.

Make up to 1 000 ml with water (6.1).

6.3 Sodium hydroxide solution

Dissolve sodium hydroxide (NaOH) in water (6.1) to obtain a solution of 0,1 mol/l to 0,5 mol/l.

6.4 Hydrochloric acid solution

Dissolve hydrochloric acid (HCl) in water (6.1) to obtain a solution of 0,1 mol/l to 0,5 mol/l.

7 Apparatus

Ensure that all glassware is thoroughly cleaned and, in particular, is free from organic or toxic matter. Use usual laboratory equipment and the following items.

7.1 Incubation bottles, gas-tight, e.g. narrow-neck flasks with volumes of 200 ml to 300 ml with suitable stoppers (e.g. ground-glass stoppers, screw-caps or butyl rubber stoppers), shielded from light (e.g. made of brown glass).

It is recommended to use stopper clamps. Mark each bottle with waterproof markings. If the oxygen electrode used has no mounted stirrer, provide each bottle with a magnetic stirrer-bar coated with polytetrafluorethylene. Either pre-select bottles of standard volume such that the standard deviation about the mean volume of the batch of bottles is less than 1 ml, or measure and record the volumes of individual, numbered bottles to an accuracy of 1 ml. Carefully grease the stoppers of the bottles with inert silicone grease to assure proper closing and easy removal.

7.2 Oxygen electrode to measure over a range of 0 mg/l to 10 mg/l with a precision of 1 %.

Steady-state shall be reached within about 2 min. Mount the electrode for example in an inert stopper which makes a leak-proof fit in the ground glass neck of the incubation bottle. Preferably use electrodes with mounted stirrers.

7.3 Magnetic stirrer with speed regulation, if required.

Stirrer-rods attached to the oxygen electrode shall be of such a material that no ingredients of any plastic coatings will contaminate the test medium and no adsorption of test compounds will occur. Heating the test vessels by stirring and raising the test temperature shall be avoided.

7.4 Shaking device, if required.

7.5 Water bath, or other device to guarantee an accurate temperature control of ± 0,5 °C in a range of 20 °C to 25 °C.

7.6 pH-meter.

7.7 Dissolved organic carbon (DOC) analyser (only in special cases, see 8.3.4, note 3).

8 Procedure

8.1 Preparation of test and reference compounds

8.1.1 Water-soluble test compounds

Prepare a stock solution of water-soluble test compounds in water (6.1) or test medium (6.2). Dilute a suitable amount of this solution in the test medium (6.2) to obtain a final test concentration which corresponds to 100 mg/l ThOD (see 8.3.3). For calculation of ThOD, see annex A. If ThOD cannot be calculated from the formula, use elemental analyses or determine COD (see annex B). Be aware that COD determination of poorly water-soluble substances may be difficult. If DOC removal is to be determined, measure the equivalent DOC test concentration (in milligrams per litre) or calculate it from the measured stock solution.

8.1.2 Water-insoluble test compounds

Grind dry solids in a mortar, weigh them on a piece of glass and place directly into the test bottles. Smear oily and waxy substances onto a piece of glass and, after reweighing, place directly into the test bottles.

If dispersions or emulsions are used, disperse the test compound in water (6.1) by ultrasonic treatment or emulsify with a non-biodegradable emulsifier, or use both techniques in combination. A suitable emulsifier is nonylphenol ethoxylate (about 10 EO) and propoxylate (about 3 to 7 PO). Ensure that the dispersion or emulsion is homogeneous when an aliquot is used to obtain the desired test concentration.

Ensure that the final amount of test compound in the test vessel is at about 100 mg/l of ThOD (see annex A). It is not important to adjust exactly to 100 mg/l, but record the exact amount for each numbered bottle.

For further information on handling of poorly water-soluble test compounds, see ISO 10634.

NOTE – If the test compound (e.g. an oily or fatty substance) adsorbs on the oxygen electrode, the oxygen measurement may be reduced and the test results could be influenced. Adsorption can also occur on the bottle walls or the stoppers. If this occurs, use another test method [e.g. the respirometer test (ISO 9408) or the CO_2-evolution test (ISO 9439)].

8.1.3 Solution of the reference compound

Prepare a stock solution of the reference compound (an organic compound of known biodegradability such as sodium acetate, sodium benzoate or aniline) in the same way as in 8.1.1 in order to obtain a final test concentration which corresponds to a 100 mg/l ThOD.

8.1.4 Solution to check inhibition

If information on a possible inhibition of the inoculum by the test compound is desired, prepare in the test medium (6.2) a solution containing the test compound and the reference compound in the respective concentrations indicated in 8.1.1, 8.1.2 and 8.1.3.

8.2 Preparation of the inoculum

Prepare the inoculum using the sources as described in 8.2.1 to 8.2.3 or use a mixture of these sources to obtain a microbial population that offers sufficient biodegradative activity. Stabilize the inoculum as described in 8.3.2 before use in the test.

8.2.1 Inoculum from a secondary effluent

Take a sample of a secondary effluent collected from a treatment plant or a laboratory plant dealing with predominantly domestic sewage. If the density of microorganisms in the inoculum is too low so that the requirements for suitable volume [see b)] cannot be fulfilled, concentrate the sample by filtration or centrifugation. Mix well, keep the sample under aerobic conditions and use preferably on the day of collection.

From this sample, prepare an inoculum as follows.

a) Let the sample of effluent settle for 1 h.

b) Take a suitable volume of the supernatant, to be used as inoculum, where suitable volume means

- sufficient to give a population which offers enough biodegradation activity;
- degrades the reference compound by the stipulated percentage;
- gives between 10^4 to 10^8 active cells per millilitre;
- gives not greater than the equivalent of 30 mg/l suspended solids of activated sludge in the final mixture.

8.2.2 Inoculum from an activated sludge plant

Take a sample of activated sludge collected from the aeration tank of a treatment plant or a laboratory plant dealing with predominantly domestic sewage. Mix well, keep the sample under aerobic conditions and use preferably on the day of collection.

Before use, determine the concentration of suspended solids (use e.g. ISO 11923). If the concentration of suspended solids is very low, concentrate the sludge by settling so that the volume of sludge added to the test assay is minimal. If many coarse particles are in the sludge, separation is necessary. Put the sludge through a fine sieve and wash repeatedly with test medium (6.2). Subsequently centrifuge or settle the sludge, discard the liquid phase and resuspended the solids in test medium to obtain a concentration of solids of about 3 g/l. Use a volume which gives no more than 30 mg/l of suspended solids in the final mixture.

8.2.3 Inoculum from a surface water

Take a sample of an appropriate surface water. If the amount of microorganisms in the inoculum is too low and does not fulfil the requirements indicated in 8.2.1, concentrate the sample by filtration or centrifugation. Keep the sample under aerobic conditions and use preferably on the day of collection. Take a suitable volume as inoculum (8.2.1).

NOTE – In certain circumstances, pre-exposed inocula may be used, provided that this is clearly stated in the results (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).

8.3 Test procedure

8.3.1 Preparation of test bottles

Set up a sufficient number of incubation bottles (7.1) in order to have

- at least three bottles containing the test compound (8.1.1 or 8.1.2) and inoculated test medium (6.2 and 8.2) (bottles F_T);
- at least three bottles as blanks containing the inoculated test medium (6.2 and 8.2) (bottles F_B);
- at least three bottles for checking the procedure containing the reference compound (8.1.3) and inoculated test medium (6.2 and 8.2) (bottles F_C);
- if needed, at least one bottle for checking a possible inhibitory effect of the test compound containing solution 8.1.4 and inoculated test medium (6.2 and 8.2) (bottle F_I);
- if needed, at least one bottle for checking a possible abiotic elimination containing the test compound (8.1.1 or 8.1.2) but no inoculum, sterilized by addition of, for example, 1 ml/l of a solution containing 10 g/l of mercury(II)

chloride ($HgCl_2$) or another suitable inorganic toxic compound to prevent microbial activity (bottle F_S). If very easily degradable substances are tested, add the same amount of the toxic substance two weeks after the test was begun.

8.3.2 Stabilization of inoculated medium

Prepare sufficient test medium (6.2) to perform the complete test, inoculate it and distribute it to the test bottles. If for example activated sludge is used as inoculum, take about 800 ml of test medium (6.2), add 10 ml of inoculum (8.2.2) and make up to 1 000 ml with test medium (6.2). The concentration of suspended solids shall be no more than 30 mg/l.

Place a magnetic stirrer-bar in each bottle (if a shaker is not used and/or the oxygen electrode does not contain an integral stirrer) and add a volume of well mixed inoculated medium equal to two-thirds of the free volume of the bottle (e.g. 200 ml liquid in 300-ml bottles). Place the stoppered bottles on the shaking device or stir them and incubate at 20 °C to 25 °C for one week. During this time the bacteria will use their reserve material and the inoculum is stabilized.

8.3.3 Start of the test

Aerate the test bottles containing stabilized inoculated medium (8.3.2) with the help of water-saturated compressed air and an air diffuser for about 15 min. Either measure the initial oxygen concentration, which is recommended, or calculate it (see 8.3.4). Add to the F_T bottles an appropriate amount of the stock solution of the test compound (8.1.1) or add poorly-soluble test substances directly (8.1.2) to get the desired test concentration of normally 100 mg/l ThOD per bottle. Add to the F_C bottles an appropriate amount of the stock solution of the reference chemical (8.1.3) and to the F_I bottle the same amounts of test compound and reference compound (8.1.4). In the case of the F_S bottle, use uninoculated test medium (6.2) and add the same amount of test compound as for the F_T bottles. Ensure that all bottles contain the same volumes of water and air (see 8.3.2); add water (6.1) if required.

Stopper all bottles tightly (e.g. using stopper clamps), put them on a shaker and incubate at a constant temperature ($\pm$ 0,5 °C) in a range between 20 °C and 25 °C. Instead of using a shaker, the bottles may be stirred with a magnetic stirrer.

8.3.4 Analysis

Calibrate accurately the oxygen electrode according to the manufacturer's instructions.

The zero point, saturation value and drift should be calibrated, for example by placing the electrode in air-saturated water at 20 °C $\pm$ 0,5 °C. The reading of the saturation value for dissolved oxygen in water should be adjusted to 9,08 mg/l, the theoretical value at normal atmospheric pressure (1 013 hPa) and 20 °C. The oxygen values are normally read from a scale of 0 mg/l to 10 mg/l with an accuracy of 0,1 mg/l. Correction for changes in atmospheric pressure is unnecessary.

NOTE 1 If the test compound is suspected to be affecting the oxygen electrode (e.g. in the case of oily or fatty substances or as a result of a visual check), it is recommended to establish in separate experiments whether the test compound adheres to the electrode or whether or not the functioning and calibration of the oxygen electrode may be affected.

NOTE 2 In this International Standard, determination of the oxygen concentration in the test bottles is described using an oxygen electrode. However other appropriate techniques may also be used, which require other apparatus and test procedures.

After incubation periods of one week maximum, or more frequently if more exact degradation curves are desired, determine the concentration of dissolved oxygen in each bottle. Maintain the bottles at the incubation temperature kept at a constant value ($\pm$ 0,5 °C) during the measurements.

Take each bottle successively and shake vigorously by hand for about 30 s. Place the bottle on a stirrer, without stirring. Remove the stopper and immediately put the oxygen electrode through the neck of the bottle so that the electrode stopper effectively closes the bottle and the electrode tip is well below the liquid surface. Start the stirrer at such a speed that oxygen measurements are possible but no vortex is formed. Use the same stirrer speed throughout a set of measurements and electrode calibrations. Record the oxygen value when it is stable, which should be reached in about 2 min.

Then measure the pH of the solution in each bottle and record it. If the pH is below 6,0, adjust to about 7,5 with sodium hydroxide solution (6.3). If the pH is above 8,0 adjust to about 7,5 with hydrochloric acid solution (6.4).

Finally, aerate the medium in each bottle with an air-diffuser for 15 min and again measure the oxygen concentration as described previously. Restopper the bottles, put them on the shaker and continue the incubation.

Normally the test is finished after 28 days. If a sufficient degree (> 60 %) and a constant level of biodegradation is attained before the end of the 28-day test period, consider that the test is finished. Extend the test by 1 to 2 weeks if biodegradation has obviously started but has yet reached a plateau.

If the test compound contains nitrogen, determine the final concentration of nitrate and nitrite immediately at the end of the test, or in preserved samples, so that the calculated degree of biodegradation can be corrected if nitrification has taken place (see annex C). Alternatively, use a qualitative spot test procedure for nitrite and nitrate on a small volume of reaction mixture taken from each flask; apply a quantitative method only if positive results are obtained.

NOTE 3 When a water-soluble compound is tested, the removal of dissolved organic carbon (DOC) can be determined to obtain additional information on ultimate biodegradation. In this case, appropriate test vessels should be used containing a sufficient test volume to enable DOC analyses. Samples can be taken either after each oxygen measurement or at the end of the test. The change in volume should be taken into account in the calculation of the biodegradation degree [see equation (2) in 9.1].

NOTE 4 When a substance-specific analytical method is available for a test compound, additional information on primary degradation can be obtained even for water-insoluble substances (e.g. after extraction with a suitable solvent). In this case samples should be taken at the end of the test from bottles F_T and F_S.

9 Calculation and expression of results

9.1 Calculation

Determine the relative oxygen uptake OR measured in the water phase in each bottle according to equation (1):

$$OR = \frac{\rho_{oBt} - \rho_{ot}}{\rho_{os}} \quad \ldots (1)$$

where

ρ_{oBt} is the mean value of the dissolved oxygen concentration in blank bottles after incubation at time t (mg/l);

ρ_{ot} is the dissolved oxygen concentration in each test bottle after incubation at time t (mg/l);

ρ_{os} is the saturation value for dissolved oxygen (mg/l).

A mean ρ_{os} value after each aeration or re-aeration measurement series of the blank and test bottles should be calculated.

NOTE 1 The theoretical saturation value for dissolved oxygen at normal atmospheric pressure (1 013 hPa) and 20 °C is 9,08 mg/l.

Determine from equation (2) the total oxygen capacity OC (mg/bottle) of a bottle from the maximum oxygen content in the gas phase and the oxygen content in the liquid phase at normal pressure and at 20 °C.

$$OC = (0{,}28 \times V_g) + (0{,}009 \times V_l) \quad \ldots (2)$$

where

0,28 is the oxygen content of normal air (mg/ml);

V_g is the volume of gas in an incubation bottle (ml);

0,009 is the oxygen content of saturated water (mg/ml);

V_l is the volume of liquid in an incubation bottle (ml).

NOTE 2 Normally V_l will be constant in a test series, except if samples for analysis are taken, but V_g may differ depending on the test bottles used. If the differences among the individual bottles are small, a constant OC may be used. If the differences are significant (e.g. > 2 ml for bottles with 200 ml volume), OC for each bottle should be calculated. If V_l decreases according to the volume of the sample taken, V_g increases correspondingly.

Then calculate the oxygen uptake B (mg/bottle) from equation (3):

$$B = \mathrm{OR} \times \mathrm{OC} \quad \ldots (3)$$

Sum the oxygen uptakes $\sum B$ (mg/bottle) for all (n) incubation periods using equation (4) to obtain the value of at the end of the test:

$$\sum B = B_1 + B_2 + \cdots + B_n \quad \ldots (4)$$

Finally, calculate the percentage of biodegradation D_{ThOD} (%) from equation (5):

$$D_{\mathrm{ThOD}} = \frac{\sum B}{B_{\mathrm{ThOD}}} \times 100 \quad \ldots (5)$$

where B_{ThOD} is the theoretical oxygen demand, in milligrams per bottle. For calculation, see annex A.

NOTE 3 If, instead of ThOD, the COD is used (see 8.1.1), D_{COD} can be calculated in the same way using equation (5). Be aware that different test results may be observed if these values are compared. COD will normally be lower than the ThOD if the test compound is not fully oxidized.

NOTE 4 The abiotic elimination (bottle F_S) can be calculated according to equation (5) but without considering the blank values in equation (1).

NOTE 5 The degree of elimination of the mixture of test compound and reference substance in the inhibition control (bottle F_I) can be calculated using equation (5).

When, as additional information for water-soluble compounds, DOC is determined, calculate the elimination D_c (%) using equation (6):

$$D_c = \left[1 - \frac{\rho_{ct} - \rho_{cBt}}{\rho_{co}}\right] \times 100 \quad \ldots (6)$$

where

ρ_{co} is the calculated DOC test concentration of the test compound (mg/l);

ρ_{ct} is the measured mean DOC concentration at the end of the test in bottles F_T (mg/l);

ρ_{cBt} is the measured mean DOC concentration at the end of the test in bottles F_B (mg/l).

When specific analyses of the test compound are performed, calculate the primary degradation degree D_S (%) using equation (7):

$$D_S = \frac{\rho_S - \rho_T}{\rho_S} \times 100 \quad \ldots (7)$$

where

ρ_T is the concentration of the test compound in bottle F_T at the end of the test (mg/l);

ρ_S is the concentration of the test compound in bottle F_S at the end of the test (mg/l).

The results D_c and D_S are based on different test principles and may therefore not be the same as D_{ThOD}.

9.2 Expression of results

Compile a table of all measured values and biodegradation percentages for each determination interval and each incubation bottle. If results obtained for the triplicates differ more than 20 %, plot a mean elimination curve as a function of time (see an example in annex D). If the differences are less than 20 %, use the curve obtained from a representative bottle.

If possible, indicate clearly on the curve the lag time, the degradation time and the maximum level of degradation.

10 Validity of the test

10.1 Consider the test to be valid if, in the test flasks with the same test concentration and inoculum, the difference between the extreme degradation values found is less than 20 % at the end of the test.

10.2 Consider the test to be valid if, in the test with one of the proposed reference compounds, the percentage of degradation after 14 days is more than 60 %.

10.3 The total oxygen uptake in the blank bottles after the first week of the test shall be lower than 3 mg of oxygen per litre and in the following weeks it shall be lower than 1 mg/l per week. If the oxygen uptake is higher, the test is not valid. If the test is repeated, use a longer period for stabilizing the inoculum (8.3.2) or use another inoculum.

10.4 Assume the test compound to be toxic if the biodegradation degree of the mixture in the bottles of the inhibition control is less than 25 % in 14 days, which indicates that the biodegradation of the reference substance is inhibited by the test compound. If in such a case the test compound has not been biodegraded and the test is repeated, use a lower concentration of the test compound. If in such a case the test compound has not been biodegraded and the test is repeated, use a lower concentration of the test compound or a pre-exposed inoculum.

If these criteria are not fulfilled, repeat the test, for example with a more active or better pre-aerated inoculum.

11 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 data (e.g. in tabular form) obtained and the degradation curve;

d) the concentration of the test compound used, the ThOD content of this concentration and the treatment given to poorly water-soluble compounds;

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

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

g) the incubation temperature of the test;

h) if included the percentage of degradation obtained in bottle F_S (monitoring the abiotic elimination);

i) if included, the percentage of degradation in bottle F_I (toxicity test) and a statement on the toxicity of the test compound;

j) the reasons in the event of rejection of the test;

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

Annex A
(informative)

Calculation of the theoretical oxygen demand (ThOD)

The theoretical oxygen demand (ThOD) may be calculated if the elemental composition and molecular mass are known or determined.

EXAMPLE

$C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$

Without nitrification

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

where M is the molecular mass.

Or with nitrification

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

Indicate the ThOD in milligrams per milligram of substance. To calculate the biodegradation degree according to equation (5) in 9.1, the ThOD is indicated in milligrams per bottle.

For a water-soluble substance, calculate the amount to prepare a stock solution from the amount of substance multiplied by the ThOD (mg/mg). If as an example, the volume of the liquid in the bottles is 200 ml and the concentration of the stock solution is 40 g ThOD per litre, use 0,5 ml per bottle to obtain a ThOD of 100 mg/l or 20 mg per bottle.

The amount of ThOD of a poorly water-soluble substance in the test bottles (mg/bottle) is calculated from the amount of substance multiplied by the ThOD (mg/mg).

Annex B
(informative)

Determination of the chemical oxygen demand (COD)

The chemical oxygen demand of water-soluble organic substances is determined by established procedures (see for example, ISO 6060).

The COD is often, and especially in the case of poorly soluble substances, determined advantageously using a variant of the above analyses, i.e. in a closed system with a pressure equalizer (see reference [8]). In this variant, compounds which are only with difficulty determined by the conventional method, for example acetic acid, may often be successfully quantified. The method may also fail, for example in the case of pyridine. If the potassium dichromate concentration is raised from 0,016 N (0,002 6 mol/l) as prescribed by Kelkenberg to 0,25 N (0,041 6 mol/l), the direct weighing-out of 5 mg to 10 mg of substance is facilitated, which is essential for the COD determination of substances that are poorly soluble in water.

Annex C
(informative)

Correction for oxygen uptake for interference by nitrification

Nitrification errors in the case of nitrogen-free substances are negligible (< 5 %), even if oxidation of the ammonium nitrogen in the medium occurs erratically between the test and blank vessels, provided that the concentration of ammonium in the medium is not significantly increased. However, for test substances containing large fractions of nitrogen, serious errors can arise.

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 nitrate, if the changes in concentration of nitrite and nitrate during incubation are determined and taken into account under consideration of the following equations:

$$2NH_4Cl + 3O_2 \longrightarrow 2HNO_2 + 2HCl + 2H_2O \quad \text{... (C.1)}$$

$$2HNO_2 + O_2 \longrightarrow 2HNO_3 \quad \text{... (C.2)}$$

Overall:

$$2NH_4Cl + 4O_2 \longrightarrow 2HNO_3 + 2HCl + 2H_2O \quad \text{... (C.3)}$$

From equation (C.1), the oxygen uptake for the oxidation of 28 g of nitrogen (added as NH_4Cl) to nitrite is 96 g; i.e. a factor of 3,43 (96/28). In the same way, from equation (C.3) the oxygen uptake for the oxidation of 28 g of nitrogen (added as NH_4Cl) 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 multiplied by the increase in concentration of nitrate nitrogen, whereas the oxygen associated with the formation of nitrite is 3,43 multiplied by the increase in the concentration of nitrite nitrogen or, with the decrease in its concentration the oxygen "loss" is 3,43 multiplied by the decrease in concentration:

O_2 consumed in nitrate formation equals 4,57 times increase in nitrate-N conc.

and

O_2 consumed in nitrite formation equals 3,43 times increase in nitrite-N conc.

and

O_2 "lost" in nitrite disappearance equals – 3,43 times decrease in nitrite-N conc.

so that

O_2 uptake due to nitrification equals ± 3,43 times change in NO_2-N-conc. + 4,57 times increase in NO_3-N-conc.

and thus

O_2 uptake due to carbon oxidation equals BOD minus uptake due to nitrification

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

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