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Water quality — Evaluation in an aqueous medium of the "ultimate" aerobic biodegradability of organic compounds — Method by analysis of biochemical oxygen demand (closed bottle test)

Qualité de l'eau — Évaluation en milieu aqueux de la biodégradabilité aérobie «ultime» des composés organiques — Méthode par analyse de la demande biochimique en oxygène (essai en fiole fermée)

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.

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 10707 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Annexes A, B and C 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 — Method by analysis of biochemical oxygen demand (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, by analysis of biochemical oxygen demand, for the evaluation in an aqueous medium of the "ultimate" biodegradability of organic compounds at a given concentration by aerobic microorganisms.

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

The method applies to all organic compounds which are sufficiently water soluble to prepare a stock solution or poorly water soluble when using special dosing techniques.

Due to the low concentration of test compound at the beginning of the test, normally no special precautions for the toxicity of the test compound to the microorganisms of the inoculum is necessary; if required a parallel inhibition test can be performed.

2 Normative references

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

ISO 5813:1983, *Water quality — Determination of dissolved oxygen — Iodometric method.*

ISO 5814:1990, *Water quality — Determination of dissolved oxygen — Electrochemical probe method.*

ISO 6060:1989, *Water quality — Determination of the chemical oxygen demand.*

ISO 9887:1992, *Water quality — Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium — Semi-continuous activated sludge method (SCAS).*

ISO 9888:1991, *Water quality — Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium — Static test (Zahn-Wellens method).*

ISO 10304-2:—[1], *Water quality — Determination of dissolved anions by liquid chromatography of ions — Part 2: Determination of bromide, chloride, nitrate, nitrite, orthophosphate and sulfite in waste water.*

1) To be published.

ISO 10634:—[1], *Water quality — Guidance for the evaluation in an aqueous medium of the "ultimate" biodegradability of poorly soluble organic compounds.*

3 Definitions

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

3.1 ultimate biodegradation: The level of degradation achieved when the test compound is totally utilized by microorganisms resulting in the production of carbon dioxide, water, mineral salts and new microbial cellular constituents (biomass).

3.2 biochemical oxygen demand (BOD): The mass concentration of dissolved oxygen consumed under specified conditions by the biological oxidation of organic and/or inorganic matter in water and is expressed in this case as milligrams of oxygen uptake per milligram or gram of test compound.

3.3 Chemical oxygen demand (COD): The amount of oxygen consumed during oxidation of a test compound with hot, acidic dichromate. It provides a measure of the amount of oxidizable matter present and is expressed in this case as milligrams of oxygen consumed per milligram or gram of test compound.

3.4 theoretical oxygen demand (ThOD): The total amount of oxygen required to oxidize a chemical completely. It is calculated from the molecular formula and is expressed in this case as milligrams of oxygen required per milligram or gram of test compound.

3.5 pre-exposure (or pre-adaptation): The pre-incubation of an inoculum in the presence of the test compound, with the aim of enhancing the ability of the inoculum to degrade the test compound. If the aim is achieved, the inoculum is said to be adapted.

3.6 pre-conditioning (or pre-acclimatization): The pre-incubation of an inoculum under the conditions of the test in the absence of the test compound, to improve the performance of the test.

4 Principle

A solution of the organic test compound in a mineral medium as the sole source of carbon and energy is inoculated with a relatively small number of microorganisms from a mixed population and kept in completely full, closed bottles in the dark at a constant temperature. Biodegradation is followed by analysis of dissolved oxygen over a period of 28 d. The amount of oxygen taken up by the test chemical (BOD), corrected for uptake by the blank inoculum run in parallel, is expressed as percentage of ThOD or COD.

5 Test environment

Incubation shall take place in the dark in an enclosure which is maintained at a constant temperature (within ± 1 °C) between 20 °C and 25 °C.

6 Reagents

Use only reagents of recognized analytical grade.

6.1 Water

Distilled or deionized water free from inhibitory concentrations of toxic substances containing less than 10 % of the initial DOC content introduced by the compounds to be tested. For each series of tests, use only one batch of water.

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 ($Na_2HPO_4.2H_2O$)	33,4 g
Ammonium chloride (NH_4Cl)	0,5 g

Dissolve the ingredients in water (6.1) and make up to 1 000 ml.

NOTE 1 The correct composition of the medium can be checked by the measurement of the pH-value, which should be 7.4.

6.2.1.2 Solution b)

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

6.2.1.3 Solution c)

Dissolve 27,5 g of anhydrous calcium chloride ($CaCl_2$) or 36,4 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) in water (6.1) and dilute to 1 000 ml.

6.2.1.4 Solution d)

Dissolve 0,25 g of iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) in water (6.1) and dilute to 1 000 ml.

In order to avoid having to prepare this solution immediately before use, add one drop of concentrated hydrochloric acid (HCl) or 0,4 g of ethylenediaminetetraacetic acid (EDTA) (disodium salt) per litre.

6.2.2 Preparation of the test medium

For 1 litre of test medium, add 1 ml of each of the solutions a) to d) (6.2.1) to about 500 ml of water (6.1) and adjust the volume to 1 000 ml.

Strongly aerate the test medium for at least 20 min. Carry out each series of tests with test medium derived from the same batch. Generally, the medium is ready for use after standing for 20 h at the test temperature. Determine the concentration of dissolved oxygen for control purposes; the value should be about 9 mg/l at 20 °C. Conduct all transfer and filling operations of the air-saturated medium bubble-free, for example, by the use of siphons.

7 Apparatus

Usual laboratory equipment, and

7.1 BOD bottles with glass stoppers, of capacity 250 ml to 300 ml. The bottles can be made airtight by greasing. In this case, only grease which is free of organic carbon, for example silicon grease, shall be used.

7.2 Water bath or incubator, for keeping bottles at a constant test temperature with the exclusion of light.

7.3 Large glass bottles, of capacity 2 litres to 5 litres, for the preparation of media and for filling the BOD bottles.

7.4 Oxygen electrode and meter, or equipment for iodometric oxygen determination.

7.5 pH-meter.

8 Procedure

8.1 Preparation of test compound solution

Prepare a stock solution of the test compound in water (6.1) or test medium (6.2.2) (e.g. 1 g/l). Add a sufficient amount of the stock solution to the large bottles (7.3) containing a known volume of test medium (6.2.2) so that the final concentration of the chemical is normally 2 mg/l. This concentration is in general suitable to ensure that the concentration of oxygen does not fall below 0,5 mg/l during the test and the inoculum activity is not limited. For poorly biodegradable compounds and those with a low ThOD, up to 10 mg/l may be used.

In some cases, for example if a poor or partial degradation is expected, it would be advisable to run parallel series of test chemical at two different concentrations, for example 2 mg/l and 5 mg/l. Normally, calculate the ThOD on the basis of formation of ammonium salts but, if nitrification is expected or known to occur, calculate the ThOD on the basis of the formation of nitrate. However, if nitrification is not complete but does occur, correct for the changes in concentration of nitrite and nitrate, determined by analysis (see annex C).

In the case of test compounds that are very poorly soluble in water for which no stock solution can be prepared, add the test compound in the required quantity directly to the BOD bottles (7.1). Use the large bottles (7.3) only to add inoculated test medium (6.2.2). Avoid loss of test medium and test compound when stoppering the bottles. For alternatives or more details see ISO 10634.

8.2 Preparation of reference compound solution

Prepare a stock solution of a known biodegradable organic substance, for example sodium acetate, sodium benzoate or aniline, in the test medium (6.2.2). In the same way as with the test compound, add a sufficient amount of the stock solution to the large bottles (7.3) to give a test concentration of 2 mg/l.

8.3 Preparation of inhibition control

If the toxicity of the test compound is to be investigated (for example in the case of a previous low biodegradability value having been found), another series of bottles is necessary. Prepare another large bottle (7.3) containing aerated mineral medium plus test compound and reference compound at final concentrations that are the same as those in the other large bottles. Add the mixture to the BOD bottles (7.1).

8.4 Preparation of the inoculum

In this test use an inoculum without sludge flocs. It should be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage or from surface water. Mixtures from these different sources may also be used.

Collect a fresh sample and keep it aerobic during transport. If suspended solids are present, allow to settle for 1 h or filter through a coarse filter paper and keep the sample under aerobic conditions until it is required.

Use a suitable volume of these samples, ranging from one drop (about 0,05 ml) up to 5 ml per litre, to inoculate the large bottles. Trials may be needed to discover the optimum volume. If necessary, concentrate the inoculum by filtration or centrifugation.

NOTES

2 Suitable volume means:

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

— degrades the reference compound by the stipulated percentage,

— gives between 10^3 to 10^6 active cells/ml.

3 If the oxygen consumption in the blank bottles without test compound is too high (> 1,5 mg/l at the end of the test), a preconditioning by aeration of the inoculum between 1 d and 7 d is recommended. This may help to reduce the oxygen consumption of the microorganisms in the blank.

4 Pre-exposed inocula may be used in certain circumstances. When such inocula are used, this should be clearly stated in the test results (e.g. percentage biodegradation = *x* % using pre-exposed inoculum) and the method of pre-exposure detailed in the test report. Pre-exposed inocula can be obtained from laboratory biodegradation tests run under a variety of conditions as appropriate [e.g. Zahn-Wellens test (ISO 9888) or 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).

8.5 Preparation of test bottles

Prepare parallel groups of BOD bottles (7.1) for the determination involving the test and reference compound, the inhibition control and the blank values in simultaneous experimental series. Assemble a sufficient number of BOD bottles to allow at least duplicate measurements of oxygen consumption to be made at the desired test intervals, generally at least after 0 d, 7 d, 14 d, 21 d and 28 d.

In a typical run the following bottles are used:

— at least 10 bottles containing test compound and inoculum (FT);

— at least 10 bottles containing reference compound and inoculum (FC);

— at least 10 bottles containing only inoculum (blank) (FB);

and if necessary

— at least 6 bottles containing test compound, reference compound and inoculum (inhibition control) (FI).

Add fully aerated mineral medium (6.2.2) to large bottles (7.3) so that they are about one-third full. Then add a sufficient amount of the stock solutions of the test compound and reference compound to separate large bottles to reach the desired final concentration of the chemicals. Do not add any chemicals to the blank control medium contained in another large bottle. Inoculate the large bottles with a suitable volume of inoculum (8.4), dilute the solutions to volume with aerated test medium and mix well.

Dispense each prepared solution immediately into the respective group of BOD bottles from the lower quarter (not the bottom) of the appropriate large bottle, for example by siphoning, so that all the BOD bottles are completely filled. Tap gently to remove any air bubbles.

8.6 Performance of the test

Analyse the zero-time bottles (see 8.5) immediately for dissolved oxygen using an electrode (see ISO 5814).

NOTE 5 Alternatively the Winkler method can be used to measure dissolved oxygen (see ISO 5813). In this case, the contents of the bottles can be preserved for subsequent analysis by adding manganese(II) sulfate and sodium hydroxide. The carefully stoppered bottles should be stored in the dark at 10 °C to 20 °C for no longer than 24 h before proceeding with the remaining steps of the Winkler method.

Stopper the remaining bottles while ensuring that no air bubbles are enclosed, and incubate at the test temperature in the dark. Withdraw at least the duplicate bottles of all series for dissolved oxygen analysis after at least 7 d, 14 d, 21 d and, at the end of the test, after 28 d. Measure the oxygen concentration in the same way as in the zero-time bottles.

For test compounds containing nitrogen, make corrections for uptake of oxygen by any nitrification (see annex C). To do this, withdraw a sample from the large bottle (7.3) at the beginning of the test and use it for analysis of nitrite and nitrate, for example according to ISO 10304-2. Make the same determination with a sample from a BOD bottle at the end of the test. If the Winkler method (see ISO 5813) is used, prepare an additional bottle. From the change in concentration of nitrite and nitrate, calculate the oxygen used for nitrification.

9 Calculation and expression of results

9.1 Calculation

First calculate the oxygen consumption after each time period by subtracting the oxygen concentration of the inoculum blank (mean value of replicates) from that exhibited by the test compound (for each test bottle). Divide this corrected depletion by the concentration of the test compound, to obtain the specific BOD, expressed as milligrams of oxygen per milligram of test compound. Calculate the percentage biodegradability by dividing the specific BOD by the specific ThOD (see annex A). If the ThOD cannot be determined, or as additional information, use the measured COD value (see annex B). These calculation steps are combined in equation (1). It should be noted that these two methods do not necessarily give the same values. COD is often less than ThOD, so that the percentage biodegradation, of the test compound at time t (D_t) using COD can be higher than when ThOD is used. Finally calculate the mean values from the percentages of parallel assays.

$$D_t = \frac{(\rho_{\mathrm{O}} - \rho_{\mathrm{O},t}) - (\rho_{\mathrm{O,b}} - \rho_{\mathrm{O},t,\mathrm{b}})}{\mathrm{ThOD} \times \rho_{\mathrm{c}}} \qquad \ldots (1)$$

where

D_t is the percentage biodegradation of the test compound at time t;

ρ_{O} is the oxygen concentration, in milligrams per litre, at time zero in the test bottles;

$\rho_{\mathrm{O},t}$ is the oxygen concentration, in milligrams per litre, at time t in the test bottles;

$\rho_{\mathrm{O,b}}$ is the mean oxygen concentration, in milligrams per litre, at time zero in the blank bottles;

$\rho_{\mathrm{O},t,\mathrm{b}}$ is the mean oxygen concentration, in milligrams per litre, at time t in the blank bottles;

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

ρ_{c} is the concentration, in milligrams per litre, of test compound in the test bottles.

Round the percentage results to the nearest whole number.

Perform the same calculation for the reference compound and, if used, the inhibition control.

For test compounds containing nitrogen, use the appropriate ThOD according to what is known or expected about the occurrence of nitrification. If nitrification occurs but is not complete, calculate a correction for the oxygen consumed by nitrification from the changes in concentration of nitrite and nitrate during the test (see annex C).

9.2 Expression of results

Plot the percentage average biodegradation (D_t) versus time (biodegradation curve). From this curve, determine parameters to describe the biodegradation; in particular the lag time, the degradation time and the maximum level of degradation.

NOTES

6 In most of the degradation curves a so-called lag time can be observed. This is defined as the time from the beginning of inoculation until the degradation percentage has increased to about 10 % of the theoretical maximum degradation (ThOD or COD). The lag time is often highly variable and poorly reproducible. It should be noted in days.

The maximum level of degradation should be defined as the approximate level above which no further degradation takes place during the test.

The degradation time should be defined as the time from the end of the lag time till the time that about 90 % of the maximum level of degradation has been reached. Note the degradation time in days.

7 Due to the restricted amount of measured values in the closed bottle test, the lag and the degradation time may often be only estimated.

10 Validity of the test

Oxygen depletion in the inoculum blank control shall not exceed 1,5 mg/l after 28 d. Values higher than this require investigation of the experimental techniques and the used inoculum. Preconditioning by aeration of the inoculum during 1 d to 7 d can be helpful to reduce the blank value.

The residual concentration of oxygen in the test bottles shall not fall below 0,5 mg/l at any time.

Consider a test valid if the difference of extremes of replicate values at the end of the test is less than 20 %. If 1 of 3 replicates is outside this range, consider it as an outlier and use the remaining values. The percentage degradation of the reference compound shall have reached 60 % after 14 d. If either of these conditions is not met, repeat the test. Because of the extreme stringency of this method, low biodegradation values do not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

In an inhibition test containing both the test and the reference compound, if less than 25 % based on total ThOD or COD occurred after 14 d, assume that the test compound is inhibitory. Repeat the test series, if possible using a lower concentration of the test compound.

11 Test report

The test report shall contain at least the following information:

a) a reference to this International Standard;

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

c) all the data obtained (for example in tabular form), the degradation curve and the percentage of degradation of the test compound;

d) the name of the reference compound used, the data obtained, the degradation curve and the degradation percentage;

e) the source, the characteristics, the volume and any pretreatment of the inoculum used, for example pre-exposure or pre-conditioning;

f) the method of oxygen measurement used;

g) the incubation temperature of the test;

h) the percentage of degradation in the inhibition control (if used);

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

j) any alteration of the standard procedure or any other circumstances that may have affected the results.

Annex A
(informative)

Determination of the theoretical oxygen demand (ThOD)

A.1 Calculation

The ThOD may be calculated if the elemental composition is known or determined.

EXAMPLE

$C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ of relative molecular mass M_r

A.1.1 Without nitrification

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

The following assumptions are made:

H to H_2O, C to CO_2, P to P_2O_5, Na to Na_2O, Cl to HCl and N to NH_3

A.1.2 With nitrification

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

It is assumed in this case that N is finally transformed to NO_3.

A.2 Example: glucose ($C_6H_{12}O_6$) M_r = 180 g

$$\text{ThOD} = \frac{16\left(2 \times 6 + \frac{1}{2} \times 12 - 6\right)}{180} = 1{,}07 \text{ mg } O_2/\text{mg glucose}$$