

# INTERNATIONAL STANDARD

**ISO  
9408**

First edition  
1991-02-15

---

---

**Water quality — Evaluation in an aqueous  
medium of the “ultimate” aerobic  
biodegradability of organic compounds —  
Method by determining the oxygen demand in a  
closed respirometer**

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



Reference number  
ISO 9408:1991(E)

## 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 9408 was prepared by Technical Committee ISO/TC 147, *Water quality*.

Annexes A, B, C and D of this International Standard are for information only.

STANDARDSISO.COM : Click to view the full PDF of ISO 9408:1991

© ISO 1991

All rights reserved. No part of this publication may be reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying and microfilm, without permission in writing from the publisher.

International Organization for Standardization  
Case Postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

# Water quality — Evaluation in an aqueous medium of the “ultimate” aerobic biodegradability of organic compounds — Method by determining the oxygen demand in a closed respirometer

## 1 Scope

This International Standard specifies a method, by determination of the oxygen demand in a closed respirometer, for the evaluation in an aqueous medium of the “ultimate” biodegradability of organic compounds at a given concentration by aerobic micro-organisms.

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

The method applies to organic compounds which

- a) are soluble in the test conditions;
- b) are insoluble in the test conditions, in which case special measures may be necessary to achieve good dispersion of the compound;
- c) do not reach and react with the CO<sub>2</sub> absorbant;
- d) are volatile, provided that a suitable respirometer is used;
- e) are not inhibitory to the test micro-organisms at the concentration chosen for the test. The presence of inhibitory effects can be determined as specified in 8.3, or by using any other method for determining the inhibitory effect of a compound on bacteria (see, for example, ISO 8192).

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

ISO 6107-2:1989, *Water quality — Vocabulary — Part 2*.

ISO 7827:1984, *Water quality — Evaluation in an aqueous medium of the “ultimate” aerobic biodegradability of organic compounds — Method by analysis of dissolved organic carbon (DOC)*.

ISO 8192:1986, *Water quality — Test for inhibition of oxygen consumption by activated sludge*.

## 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 micro-organisms 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 (see ISO 6107-2).

**3.3 suspended solids (of an activated sludge):** Solids removed by filtration or centrifuging of a known volume of sludge under specified conditions, and,

for the purpose of this International Standard, drying at about 100 °C.

**3.4 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.

**3.5 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

Determination of the biodegradation of organic compounds by aerobic micro-organisms, using a test medium.

The organic compound is the sole source of carbon and energy in the medium. The concentration of the test compound is normally 100 mg/l, but its theoretical oxygen demand (ThOD) shall be at least 100 mg/l.

The inoculated medium is stirred in a closed flask and the consumption of oxygen is determined either by measuring the amount of oxygen required to maintain a constant gas volume in the respirometer flask, or by measuring the change in volume or pressure (or a combination of the two) in the apparatus.

Evolved carbon dioxide is absorbed in a suitable substance in the test vessel.

The degradation is followed over a period of 28 days, 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 the blank test) is expressed as a percentage of the theoretical oxygen demand (ThOD) calculated from the formula of the compound or the chemical oxygen demand (COD). Additionally, the degree of biodegradation may also be calculated from supplemental chemical analyses, for example, dissolved organic carbon (DOC) for sufficiently water-soluble compounds or specific analysis (concerning only primary biodegradation), made at the beginning and the end of incubation. Evaluation of the biodegradability of the test compound is made on the basis of these data.

## 5 Test environment

Incubation shall take place in the dark or in diffused light, in an enclosure which is maintained at a constant temperature (within at least  $\pm 1$  °C) between 20 °C and 25 °C and which is free from toxic vapours.

## 6 Reagents

Use only reagents of recognized analytical grade.

### 6.1 Distilled or de-ionized water.

Containing less than 10 % of the initial DOC content introduced by the organic compound to be tested.

### 6.2 Test medium

#### 6.2.1 Composition

##### 6.2.1.1 Solution (a).

Anhydrous potassium dihydrogenphosphate ( $\text{KH}_2\text{PO}_4$ )	8,5 g
Anhydrous dipotassium hydrogenphosphate ( $\text{K}_2\text{HPO}_4$ )	21,75 g
Disodium hydrogenphosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ )	33,4 g
Ammonium chloride ( $\text{NH}_4\text{Cl}$ )	0,5 g
Water (6.1) (quantity necessary to make up to)	1000 ml

The pH of this solution should be about 7,4.

##### 6.2.1.2 Solution (b).

Dissolve 22,5 g of magnesium sulphate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) in 1000 ml of the water (6.1).

##### 6.2.1.3 Solution (c).

Dissolve 27,5 g of anhydrous calcium chloride ( $\text{CaCl}_2$ ) or 36,4 g of calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in 1000 ml of the water (6.1).

##### 6.2.1.4 Solution (d).

Dissolve 0,25 g of iron(III) chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) in 1000 ml of the water (6.1). Prepare this solution just before use.

NOTE 1 It is not necessary to prepare this solution just before use if a drop of concentrated hydrochloric acid (HCl) or 0,4 g/l of ethylenediamine-tetraacetic acid (EDTA) is added.

### 6.2.2 Preparation of the test medium.

For 1 litre of test medium, just before use add to 800 ml of the water (6.1)

— 10 ml of solution (a);

— and then 1 ml of each solution (b), (c) and (d), (to avoid the formation of turbidity in the final medium).

Make up to 1000 ml with the water (6.1).

**6.3 Carbon dioxide absorber**, Potassium hydroxide solution (about 10 mol/l), sodium carbonate pellets or another suitable absorbant.

## 7 Apparatus

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

Usual laboratory equipment, and

### 7.1 Closed respirometer.

The principle of a closed respirometer is given in annex D. 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.

**7.2 Water-bath or constant temperature room** (to comply with clause 5).

**7.3 Equipment for measurement of dissolved organic carbon**, instrument of sufficient sensitivity for the measurement of dissolved organic carbon (DOC).

**7.4 Device for determining chemical oxygen demand (COD).**

**7.5 Device for filtration**, with membrane filters of suitable porosity (nominal aperture diameter between 0,2  $\mu\text{m}$  and 0,45  $\mu\text{m}$ ) which adsorb organic compounds or release organic carbon to a minimum degree (see note 3 to 8.3).

**7.6 Centrifuge.**

**7.7 pH-meter.**

## 8 Procedure

### 8.1 Preparation of the test solutions

Prepare the following solutions:

- solution of the test compound in the test medium (6.2) to obtain a concentration of 100 mg/l, but at least 100 mg/l ThOD;
- solution of a known organic compound ("reference" compound), for example, sodium acetate, sodium benzoate, aniline, in the test medium (6.2) to obtain a concentration of 100 mg/l;
- solution containing, in the test medium (6.2), the same concentrations of the test compound and the reference compound as used in a) and b).

NOTE 2 Compounds of low water solubility may be added directly, in solid or liquid form, to the medium in the appropriate flask; an ISO International Standard will be prepared for guidance.

### 8.2 Preparation of the inoculum

Take a sample of activated sludge from the aeration tank of a biological wastewater treatment plant, or a laboratory unit, dealing with predominantly domestic sewage. If the sludge is actively respiring on external substrate, bring it to the "endogenous" phase (i.e. having no external substrate remaining) as follows:

- either aerate for a few hours before use, or
- centrifuge, wash with medium (6.2), recentrifuge and resuspend in the medium (this treatment is recommended if it is suspected that the sludge contains inhibiting matter).

When the sludge is judged to be in the "endogenous" phase, or free from inhibiting matter, mix well, maintain in an aerobic state by stirring or aeration at the required temperature, and use on the day of collection or one day later. Just before use, determine the concentration of suspended solids. If required, concentrate the sludge by settling, so that the volume of sludge added to obtain 30 mg/l of dry matter in the reaction mixture is less than or equal to 1 % of the mixture, that is, the sludge should contain at least 3 g/l of dry solids.

#### NOTES

3 A concentration of 30 mg/l of suspended solids in the final medium has been found suitable for concentrations of test compound in the range of 50 mg/l to 150 mg/l. The oxygen consumption of the blank solution shall not be greater than 60 mg/l in 28 days and should normally be in the range of 20 mg/l to 30 mg/l. In order to reduce the influence of the blank, the sludge may be pre-conditioned (see 3.5) by aeration for up to one week before it is used.

4 Secondary effluent and surface water may also be used as inoculum, but these inocula may have to be concentrated by filtration or centrifugation to get more biomass.

5 Pre-exposed inocula may be used for certain purposes. When such inocula are used, this should be clearly stated in the test results (e.g. percentage biodegradation = x %, using pre-exposed inocula) 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 (e.g. Zahn-Wellens and SCAS tests) or from samples collected from locations where relevant environmental conditions exist (e.g. treatment plants dealing with identical compounds, contaminated areas, etc.).

### 8.3 Test

Set up the closed respirometer (see 7.1 and the example described in annex D). Assemble a sufficient number of reaction vessels in order to have at least

- a) two test flasks (symbol  $F_T$ ), containing the appropriate volume of test solution [8.1, item a)];
- b) two flasks for the blank test (symbol  $F_B$ ), containing the appropriate volume of the test medium (6.2);
- c) if needed, one flask (symbol  $F_C$ ), for checking the activity of the inoculum, containing the appropriate volume of reference compound solution [8.1, item b)];

and, if necessary,

- d) one flask (symbol  $F_S$ ), for checking a possible abiotic degradation or other non-biological removal of the compound, containing the appropriate volume of test solution [8.1, item a)] 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 toxic compound to prevent microbial activity (see note 6);
- e) one flask (symbol  $F_I$ ), for checking the possible inhibiting effect of the test compound on microbial activity (see note 7).

Measure the pH value and adjust to pH 7.4 if necessary.

Add absorbant (6.3) to the  $CO_2$ -absorber compartments of the respirometer.

Place the respirometer vessels in the water-bath or constant temperature room (7.2) and allow all vessels to reach the desired temperature (see clause 5). Add an appropriate volume of the inoculum (8.2) to flasks  $F_T$ ,  $F_B$ ,  $F_C$  and, if included,  $F_I$ , to give a concentration of suspended solids of 30 mg/l in each flask. Seal the flasks, and in the case of automatic respirometers make any necessary connections and start the stirrer.

Take the necessary readings on the manometers (if manual) and verify that the recorder of oxygen consumption is functioning properly (automatic respirometer).

Stop the test after 28 days, or earlier if a plateau of oxygen consumption has been attained. Extend the test by 1 week to 2 weeks, if degradation has obviously started by the 28th day but has not reached a plateau.

Check the pH value of the final reaction mixtures at the end of the test.

When DOC or the concentration of a specific compound is being monitored, withdraw appropriately sized samples from the respirometric flasks at the beginning and end of the test period (alternatively, the initial value may be calculated). Either filter these samples through a membrane filter (see 7.5) or centrifuge at 4000 g for 15 min (see 7.6). When measurements of organic carbon are not conducted the same day, keep the samples at 4 °C in the dark and in tightly stoppered glass flasks. It should be noted that the maximum allowable storage time is up to 48 h.

If the test compound contains nitrogen, determine the final concentration of nitrate and nitrite immediately at the end of the test, or in suitably preserved samples, so that the calculated degree of biodegradation can be corrected if nitrification has taken place (see annex B). 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.

#### NOTES

6 It can be determined whether or not the test compound is undergoing a degradation process by physico-chemical mechanisms, by comparing the percentage elimination in flasks  $F_T$  and  $F_S$ . Give the result in the test report.

7 In order to check whether or not the test compound has an inhibiting effect on the inoculum, flask  $F_I$  containing the appropriate volume of reference compound solution [8.1, item c)] may be included.

8 The DOC concentration calculated or measured in the tested solution at the beginning of the test (day 0) should be used as the initial concentration and has to be compared to the final concentration to calculate the elimination level. When dealing with mixtures, one should be aware that selective adsorption of different components may occur.

## 9 Calculation and expression of results

### 9.1 Calculation

Calculate the oxygen consumption values for each flask, from the reading obtained, using the method given by the manufacturer for the appropriate type of respirometer. Calculate the biochemical oxygen demand, in milligrams per litre, of the test compound as the difference between oxygen consumption in the test flask  $F_T$  and the blank flask  $F_B$ . Divide this difference by the concentration of the test compound to give the net oxygen consumption expressed as specific BOD, in milligrams of  $O_2$  per milligram of test compound.

$$\text{Specific BOD} = \frac{BOD_t - BOD_{B,t}}{\rho(\text{TC})}$$

where

- $BOD_t$  is the biochemical oxygen demand of the test compound solution at time  $t$ ;
- $BOD_{Bl,t}$  is the biochemical oxygen demand of the blank at time  $t$ ;
- $\rho(TC)$  is the concentration of the test compound.

The degradation is defined as the ratio of the specific biochemical oxygen demand to either the theoretical oxygen demand (ThOD) or the chemical oxygen demand (COD). Determine the percentage degradation ( $D_t$ ) for each test flask, using either of the following equations:

$$D(\text{ThOD})_t = \frac{\text{OD}}{\text{ThOD}} \times 100$$

$$D(\text{COD})_t = \frac{\text{OD}}{\text{COD}} \times 100$$

where

- $D(\text{ThOD})_t$  is the percentage biodegradation of ThOD at time  $t$ ;
- $D(\text{COD})_t$  is the percentage biodegradation of COD at time  $t$ ;
- ThOD is the theoretical oxygen demand, expressed in milligrams per milligram of test compound (for calculation see annex A and annex B);
- COD is the chemical oxygen demand determined experimentally, expressed in milligrams per milligram of test compound;
- OD is the oxygen demand, expressed in milligrams per milligram of test compound.

NOTE 9 Since the COD of a chemical is rarely as much as its ThOD, the percentage degradation of the COD is usually higher than the percentage degradation of the ThOD. The latter value is more accurate.

When determinations of dissolved organic carbon (DOC) are performed (optionally, at the beginning and end of the test), calculate the biodegradation of the test compound as a percentage of DOC removal, using the equation

$$D_t = \left[ 1 - \frac{\rho(\text{DOC})_t - \rho(\text{DOC})_{Bl,t}}{\rho(\text{DOC})_o - \rho(\text{DOC})_{Bl,o}} \right] \times 100$$

where

- $D_t$  is the degradation, expressed as a percentage of DOC removal (at the end of the test);
- $\rho(\text{DOC})_o$  is the measured or calculated initial DOC concentration of the culture medium, in milligrams of DOC per litre;
- $\rho(\text{DOC})_t$  is the DOC concentration of the culture medium at the end of the test, in milligrams of DOC per litre;
- $\rho(\text{DOC})_{Bl,t}$  is the DOC concentration of the blank solution at the end of the test, in milligrams of DOC per litre;
- $\rho(\text{DOC})_{Bl,o}$  is the initial DOC concentration of the blank solution, in milligrams of DOC per litre;

If  $\rho(\text{DOC})_o$  is calculated from the stock solution,  $\rho(\text{DOC})_{Bl,o}$  is neglected.

When specific analyses of the test compound are performed, calculate the percentage primary degradation of the tested compound from the equation

$$D_t = \frac{\rho(\text{TC})_b - \rho(\text{TC})_a}{\rho(\text{TC})_b} \times 100$$

where, at the end of the test,

- $\rho(\text{TC})_a$  is the concentration of the test compound in the test flask,  $F_T$  (experimental result, in milligrams per litre);
- $\rho(\text{TC})_b$  is the concentration of the test compound in the blank flask,  $F_B$  (experimental result, in milligrams per litre).

## 9.2 Expression of results

Plot the percentage degradation,  $D_t$ , for each flask against time to obtain the degradation curve (see example in annex C). Draw an average curve if comparable results in the parallel test flasks are obtained.

If sufficient data are available, indicate clearly on the curve the lag time, the maximum level of degradation and the degradation time.

## 10 Validity of the test

### 10.1 Biodegradation of reference compound

If, in the test with one of the proposed reference compounds, the percentage degradation after 5 days is less than 50 %, the test results are invalid and the series of measurements must be repeated.

### 10.2 Inhibition

If the flask  $F_1$  (inhibition control) was included, the test compound is assumed to be inhibiting if the degradation percentage of the reference compound in flask  $F_1$  is lower than 40 %. In this case, it is advisable to repeat the test with lower concentrations of the test compound.

### 10.3 pH value

If the pH value at the end of the test is outside the range 6 to 8 and if the percentage degradation is less than 50 %, it is advisable to repeat the test with a lower concentration of the test compound and/or a higher concentration of the buffer solution.

## 11 Test report

The test report shall contain at least the following information:

- a) reference to this International Standard;
- b) all information necessary to identify the test compound and the reference compound;
- c) main characteristics of the respirometer and the DOC analyser;
- d) all the data obtained (for example, in tabular form) and the degradation curve (see 9.1 and 9.2);
- e) the concentration of the test compound and the reference compound used;
- f) the source, characteristics and volume of the inoculum used, including details of any pre-exposure treatment;
- g) the incubation temperature of the test;
- h) the ThOD (or COD) and the theoretical or determined DOC of the test compound and reference compound;
- i) the BOD of the test compound and the percentage of the ThOD;
- j) the BOD of the reference compound and the percentage of the ThOD;
- k) the percentage degradation by DOC or chemical analysis of the test compound and reference compound (if carried out);
- l) results of physical-chemical degradation tests and toxicity control by oxygen consumption and/or DOC (if included);
- m) the reasons in the event of rejection of the test (clause 10);
- n) any other facts that are relevant to the procedure followed.

## Annex A (informative)

### Example of the calculation of the theoretical oxygen demand

The ThOD of the 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

$$\text{ThOD}_{\text{NH}_3} = \frac{16 \left[ 2c + \frac{1}{2} (h - cl - 3n) + 3s + \frac{5}{2} p + \frac{1}{2} na - o \right]}{M_r}$$

This calculation implies that C is mineralized to  $\text{CO}_2$ , H to  $\text{H}_2\text{O}$ , P to  $\text{P}_2\text{O}_5$  and Na to  $\text{Na}_2\text{O}$ . The halogen is eliminated as hydrogen halide and nitrogen as ammonia.

#### A.1 Example: glucose ( $\text{C}_6\text{H}_{12}\text{O}_6$ ) $M_r = 180 \text{ g}$

$$\text{ThOD} = \frac{16 \left( 2.6 + \frac{1}{2} \cdot 12 - 6 \right)}{180} = 1,07 \text{ mg O}_2/\text{mg glucose}$$

Relative molecular masses of salts other than those of the alkali metals are calculated on the assumption that these salts have been hydrolyzed.

Sulfur is assumed to be oxidized to the state of + VI.

#### A.2 Example: sodium *n*-dodecylbenzenesulfonate ( $\text{C}_{18}\text{H}_{29}\text{SO}_3\text{Na}$ ) $M_r = 348 \text{ g}$

$$\text{ThOD} = \frac{16 \left( 36 + \frac{29}{2} + 3 + \frac{1}{2} - 3 \right)}{348} = 2,34 \text{ mg O}_2/\text{mg compound}$$

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

$$\text{ThOD}_{\text{NO}_2} = \frac{16 \left[ 2c + \frac{1}{2} (h - cl) + 3s + \frac{3}{2} n + \frac{5}{2} p + \frac{1}{2} na - o \right]}{M_r}$$

$$\text{ThOD}_{\text{NO}_3} = \frac{16 \left[ 2c + \frac{1}{2} (h - cl) + 3s + \frac{5}{2} n + \frac{5}{2} p + \frac{1}{2} na - o \right]}{M_r}$$

Suppose full nitrate formation was observed by analysis in the case of a secondary amine, then

$(\text{C}_{12}\text{H}_{25})_2 \text{NH}$ ,  $M_r = 353 \text{ g}$

$$\text{ThOD}_{\text{NO}_3} = \frac{16 \left( 48 + \frac{50}{2} + \frac{5}{2} \right)}{353} = 3,42 \text{ mg O}_2/\text{mg compound}$$