
**Water quality — Evaluation of ultimate
aerobic biodegradability of organic
compounds in aqueous medium — Method
by analysis of inorganic carbon in sealed
vessels (CO₂ headspace test)**

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*Qualité de l'eau — Évaluation en milieu aqueux de la biodégradabilité
aérobie ultime des composés organiques — Méthode par analyse du
carbone inorganique dans des récipients hermétiquement clos (Essai
au CO₂ dans l'espace de tête)*

ISO 14593:1999

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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 14593 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 of ultimate aerobic biodegradability of organic compounds in aqueous medium — Method by analysis of inorganic carbon in sealed vessels (CO₂ headspace test)

WARNING — Activated sludge and sewage may contain potentially pathogenic organisms and should be handled with appropriate precautions. Hazardous test compounds and those whose properties are unknown should be handled with care.

1 Scope

This International Standard specifies a method, by analysis of inorganic carbon, for the evaluation in an aqueous medium of the ultimate aerobic biodegradability of organic substances at a given concentration of microorganisms.

This International Standard is applicable to organic compounds which are:

- a) water-soluble under the test conditions;
- b) poorly water-soluble under the test conditions, in which case special measures may be necessary to achieve a good dispersion of the compound (see ISO 10634);
- c) volatile;
- d) not inhibitory to the test microorganisms at the concentration chosen for the test.

in situ this test, biogenically produced inorganic carbon is measured in the test vessels in such a manner that the rate measured nearly equals the rate of microbial production.

NOTE 1 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.

NOTE 2 With highly volatile substances, losses to the gaseous phase can be minimized by reducing the volume of the headspace. However, there should be sufficient oxygen in the test system to prevent biodegradation being oxygen-limited.

NOTE 3 The presence of an inhibitory effect can be determined as specified in 8.3, or by using any other method for determining the inhibitory effect of a substance on bacteria (see, for example, ISO 8192).

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the normative document indicated below. For undated reference, the latest edition of the normative document referred to applies. 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 aqueous medium*.

3 Terms and definitions

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

3.1

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

3.2

primary biodegradation

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

3.3

activated sludge

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

3.4

concentration of suspended solids

<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

3.5

total organic carbon

TOC

all that carbon present in organic matter which is dissolved and suspended in the water sample

3.6

dissolved organic carbon

DOC

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

NOTE For example, by centrifugation at 40 000 m · s⁻² for 15 min or by membrane-filtration using membranes with pores of diameter 0,2 µm to 0,45 µm.

3.7

total inorganic carbon

TIC

all that inorganic carbon in the water deriving from carbon dioxide and carbonate

3.8

dissolved inorganic carbon

DIC

that part of the inorganic carbon in water which cannot be removed by specified phase separation

NOTE For example, by centrifugation at 40 000 m · s⁻² for 15 min or by membrane-filtration using membranes with pores of diameter 0,2 µm to 0,45 µm.

3.9

theoretical amount of inorganic carbon

ThIC

theoretical maximum amount of inorganic carbon formed after oxidizing a chemical compound completely

NOTE It is calculated from the molecular formula and expressed in this case as milligrams carbon per milligram (or gram) test compound.

3.10**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 normally recorded in days.

3.11**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 normally recorded in percent.

3.12**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 normally recorded in days.

3.13**plateau phase**

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

NOTE It is normally recorded in days.

3.14**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

3.15**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

4 Principle

The test compound, as the sole source of carbon and energy, is added to a mineral salts medium inoculated with a mixed population of microorganisms and incubated in sealed vessels with a headspace of air. The concentration of the compound used normally yields an initial organic carbon concentration in the medium of 2 mg/l to 40 mg/l, usually 20 mg/l. Biodegradation (mineralization to carbon dioxide) is determined by measuring the net increase in total inorganic carbon (TIC) levels over time compared with unamended blanks. The test generally runs for 28 d. The extent of biodegradation is expressed as a percentage of the theoretical amount of inorganic carbon (ThIC) based on the amount of test compound added initially.

For sufficiently water-soluble substances, dissolved organic carbon (DOC) removal during the test may also be determined (see for example ISO 7827).

If a suitable analytical method is available, the primary biodegradation of the test compound during the test may also be determined.

5 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 ± 2 °C during the test.

6 Reagents

Use only reagents of recognized analytical grade.

6.1 Distilled or deionized water, containing less than 1 mg/l total organic carbon.

6.2 Test medium.

6.2.1 Composition

a) Solution a)

Dissolve:

Anhydrous potassium dihydrogenphosphate (KH_2PO_4) 8,50 g

Anhydrous dipotassium hydrogenphosphate (K_2HPO_4) 21,75 g

Disodium hydrogenphosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) 33,40 g

Ammonium chloride (NH_4Cl) 0,50 g

in water (6.1), quantity necessary to make up to 1 000 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.

b) Solution b)

Dissolve 36,40 g of calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in water (6.1), quantity necessary to make up to 1 000 ml.

c) Solution c)

Dissolve 22,50 g of magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) in water (6.1), quantity necessary to make up to 1 000 ml.

d) Solution d)

Dissolve 0,25 g iron(II) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in water (6.1), quantity necessary to make up to 1 000 ml. To avoid precipitation, prepare this solution freshly before use or add a drop of concentrated hydrochloric acid (HCl).

6.2.2 Preparation of the test medium

For 1 l of test medium, mix 10 ml of solution a) with 800 ml water (6.1) and then add 1 ml each of solutions b), c) and d) and make up to 1 000 ml with water (6.1).

6.3 Concentrated orthophosphoric acid (H_3PO_4) (≥ 85 % mass per volume).

6.4 Sodium hydroxide solution.

Dissolve 280 g of sodium hydroxide (NaOH) in 1 l of water (6.1) to obtain a solution of concentration $c = 7$ mol/l. Use either a freshly prepared solution or determine the concentration of dissolved inorganic carbon (DIC) of this solution and consider this value when calculating the test result (see clause 9).

7 Apparatus

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

7.1 Carbon analyzer, instrument for measuring inorganic and (optionally) organic carbon, such as the appropriate parts of a total organic carbon (TOC) analyser or a gas chromatograph.

7.2 Gas-tight glass vessels of known volume, for example, serum bottles of 160 ml capacity sealed with butyl rubber septa and aluminium crimp seals or any other gas-tight system.

7.3 Orbital shaker.

7.4 Syringes of high precision for aqueous and gaseous samples.

7.5 Glass bottles to take, for example, 5 l medium.

7.6 Centrifuge.

7.7 pH-meter.

7.8 Device for filtration, with membrane filters of suitable porosity (nominal aperture diameter between 0,20 μm and 0,45 μm) which adsorb organic substances or release organic carbon to a minimum degree.

7.9 CO₂-free air production system.

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Prepared by passing air through soda lime granules or a solution of sodium hydroxide (see, for example, ISO 9439). Alternatively, a mixture of 80 % N₂/20 % O₂ may be used. The absence of CO₂ in the air can be confirmed by passage through barium hydroxide solution (a white precipitate indicates the presence of CO₂).

8 Procedure

8.1 Preparation of the test solutions

8.1.1 Test compound

Prepare a stock solution of a sufficiently water-soluble test compound in water (6.1) or the test medium (6.2.2) at a concentration preferably 100-fold greater than the final concentration to be used in the test. Add the stock solution to the test medium (6.2.2) to give a final TOC concentration of between 2 mg/l and 40 mg/l (see 8.3), preferably 20 mg/l TOC.

Add substances of low water-solubility directly, in solid or liquid form, to the inoculated medium in the appropriate test vessels. Liquid test compounds, including those which are volatile, may be injected directly into sealed vessels using a high-precision microsyringe. Determine the added amount exactly.

Poorly soluble substances can often be dispensed as a dispersion produced using a non-biodegradable emulsifying agent and/or ultrasonication. Refer to ISO 10634 for guidance on the preparation and treatment of poorly water-soluble substances.

8.1.2 Reference substance

Prepare a stock solution of the reference substance (an organic substance of known biodegradability such as aniline or sodium benzoate) in water (6.1) in the same way as in 8.1.1, and then dilute in the test medium (6.2.2) to give a final TOC concentration of 20 mg/l.

8.1.3 Solution to check inhibition

If required (when e.g. no information on the toxicity of test compound is available), prepare a solution containing, in the test medium (6.2.2), both the test compound (8.1.1) and the reference compound (8.1.2) preferably at concentrations of organic carbon of 20 mg/l for each.

8.2 Preparation of the inoculum

8.2.1 General

Prepare the inoculum using the sources described in 8.2.2, 8.2.3 and 8.2.4, or using a mixture of these sources, to obtain a microbial population that offers sufficient biodegradative activity. Check this activity by means of the reference substance (8.1.2). Based on experience (clause 12 and annex C), the usual inoculum is activated sludge at a dry solids concentration of 4 mg/l. The carbon dioxide production of the blanks should be as low as possible (see clause 11). To reduce the influence of the blanks, it may be helpful to precondition the inoculum by aeration for up to one week before it is used. Use a suitable volume for inoculation (see note 2 below).

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 results (e.g. *biodegradation = x %, using pre-exposed inocula*) and the method of the pre-exposure is detailed in the test report.

NOTE 1 Pre-exposed inocula can be obtained from laboratory biodegradation tests conducted under a variety of conditions (e.g. ISO 9888 Zahn-Wellens test; ISO 9887 SCAS test) or from samples collected from locations where relevant environmental conditions exist (for example treatment plants dealing with similar substances or contaminated areas).

NOTE 2 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 11);
- gives between 10^2 to 10^5 colony forming units per millilitre in the final mixture;
- gives normally a concentration of 4 mg/l suspended solids of activated sludge in the final mixture (higher concentrations up to 30 mg/l are generally possible but may influence significantly the CO_2 production of the blanks and are therefore not recommended);
- 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 1 000 ml of test solution.

8.2.2 Inoculum from an activated sludge plant

Collect activated sludge from the aeration tank of a full-scale or a laboratory wastewater treatment plant which treats predominantly domestic sewage. If necessary, remove coarse particles by filtration through a sieve (for example, 1 mm² mesh size) and keep the sludge aerobic thereafter. Since it is necessary for the inoculum blank to have as low an evolution of CO_2 as possible, the sludge may need further treatment. For example, settle or centrifuge (e.g. at $10\,800\text{ m}\cdot\text{s}^{-2}$ for 10 min) the sludge, discard the supernatant and resuspend the settled or centrifuged solids in test medium (6.2.2) to give a suspended solids concentration of about 3 g/l (see, for example, ISO 11923). Alternatively or additionally, aerate the sludge overnight before use. To reduce the blank value still further, the sludge can be preconditioned to the test conditions prior to use by diluting in test medium (6.2.2) to give the final concentration and aerating with moist air for up to one week at the test temperature. Use 4 mg/l dry solids as the concentration of the inoculum in a test (see note 2 in 8.2.1).

8.2.3 Inoculum from wastewater

Take a sample of the influent or the effluent from a full-scale or laboratory wastewater treatment plant dealing with predominantly domestic sewage. Keep this sample under aerobic conditions and use on the day of collection (or precondition if necessary). Coarse-filter the effluent to remove gross particulate matter and measure its pH. Before use, sparge the filtrate with CO₂-free air (7.9) for about 1 h while maintaining the pH at 6,5 using orthophosphoric acid (6.3), restore the pH to its original value and finally let the sample settle for 1 h and take a suitable volume of the supernatant for inoculation.

NOTE This sparging procedure reduces the TIC content of the inoculum. For example, when the maximum of 100 ml filtered effluent per litre test volume is used as the inoculum, the amount of TIC produced in blank vessels in 28 d should be in the range 0,4 mg/l to 1,3 mg/l (see annex D in [9]). The TIC values in the blanks may be different for the various inocula used.

8.2.4 Inoculum from a surface water

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

8.3 Test procedure

8.3.1 Provide a number of test vessels (7.2) sufficient for requirements in order to have:

- a) test vessels (denoted F_T) for the test compound;
- b) blank vessels (denoted F_B) containing test medium and inoculum;
- c) vessels for checking the procedure (denoted F_C) containing the reference compound;
- d) if needed, vessels for checking a possible inhibitory effect of the test compound (denoted F_I);
- e) if needed, vessels for checking a possible abiotic elimination (denoted F_S) containing the test compound but no inoculum, sterilized by autoclaving or by the addition of a suitable inorganic toxic compound to prevent microbial activity. Use, for example, 5 ml of a solution containing 10 g/l of mercury(II) chloride (HgCl₂). Add the same amount of the toxic substance two weeks after the test was begun.

To separate large glass bottles (7.5), make the additions as indicated in Table 1.

The number of vessels needed will depend on the frequency of analysis and the confidence limits required for the final extent of biodegradation (see annex B). Usually at least five vessels from sets F_T , F_B and F_C are analysed at the end of the test.

8.3.2 Thoroughly mix the contents of each of the large bottles (7.5) in turn and dispense suitable aliquots (for example, 100 ml) into labelled test vessels (7.2). Add water-soluble test compounds from stock solutions and poorly water-soluble test compounds directly to the test vessels (see 8.1.1) and add water to give the same volume in each vessel. Ensure that the liquid to headspace ratio and the test compound concentration are such that sufficient oxygen is available in the headspace to allow for complete biodegradation (for example, avoid using a high substrate concentration and a small headspace). A headspace to liquid ratio of 1:2 is usual.

Seal the vessels when all additions have been made, for example, with butyl rubber septa and aluminium caps, place on an orbital shaker in the dark or diffuse light at the test temperature (see clause 5) and start the shaker at a speed sufficient to ensure good mixing of the vessel contents (for example, 150 r/min to 200 r/min).

Calibrate the carbon analyser (7.1) as required (8.4) on the days when analysis is to be made. Sacrifice vessels for analysis on the day of sampling but at least weekly or more frequently if a complete degradation curve is required. Remove the requisite number of replicate vessels from the shaker, representing F_T , F_B and F_C , and, if used, F_I and F_S . The test shall normally run for 28 d but can be prolonged if degradation has started. The test may be finished before 28 d have elapsed if biodegradation has reached a plateau.