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

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

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 9408 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods.*

This second edition cancels and replaces the first edition (ISO 9408:1991), which has been technically revised.

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

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

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

1 Scope

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

The method applies to organic compounds which

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

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

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

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

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

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

2 Terms and definitions

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

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

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

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

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

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

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

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

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

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

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

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

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

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

NOTE It is expressed in days.

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

NOTE It is expressed in percent.

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

NOTE It is expressed in days.

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

NOTE It is expressed in days.

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

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

3 Principle

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

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

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

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

4 Test environment

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

5 Reagents

Use only reagents of recognized analytical grade.

5.1 Water

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

5.2 Test medium

5.2.1 Composition

5.2.1.1 Solution a)

Dissolve

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

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

5.2.1.2 Solution b)

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

5.2.1.3 Solution c)

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

5.2.1.4 Solution d)

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

5.2.2 Preparation of the test medium

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

— 10 ml of solution a);

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

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

5.3 Carbon dioxide absorber

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

5.4 Mercury chloride solution

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

5.5 Sodium hydroxide solution

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

5.6 Hydrochloric acid solution

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

6 Apparatus

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

6.1 Closed respirometer

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

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

6.3 Equipment for measurement of dissolved organic carbon

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

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

6.5 Centrifuge or device for filtration

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

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

6.6 pH meter (usual laboratory equipment)

7 Procedure

7.1 Preparation of the test solutions

7.1.1 Test compound

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

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

7.1.2 Solution of the reference compound

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

7.1.3 Solution to check inhibition

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

7.2 Preparation of the inoculum

7.2.1 General

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

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

Based on experience, suitable volume means:

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

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

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

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

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

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

7.2.2 Inoculum from an activated sludge plant

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

7.2.3 Inoculum from waste water

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

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

7.2.4 Inoculum from a surface water

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

7.3 Test

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

— at least 2 test vessels (symbol F_T) for the test compound (7.1.1);

— at least 2 blank vessels (symbol F_B) containing test medium and inoculum;

— at least 1 vessel, for checking the procedure (symbol F_C) containing the reference compound (7.1.2);

— if needed, 1 vessel for checking a possible inhibitory effect of the test compound (symbol F_I) containing solution (7.1.3).

— if needed 1 vessel for checking a possible abiotic elimination (symbol F_S) containing the test compound (7.1.1) but no inoculum, sterilized by addition of a suitable inorganic toxic compound to prevent microbial activity. Use, for example, 1 ml/l of the mercury(II) chloride solution (5.4). Add the same amount of the toxic substance two weeks after the test is begun, if required.

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

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

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

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

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

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

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