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Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Carbon dioxide evolution test

Qualité de l'eau — Évaluation de la biodégradabilité aérobie ultime en milieu aqueux des composés organiques — Essai de dégagement de dioxyde de carbone

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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.

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 9439 has been prepared by Technical Committee ISO/TC 147, *Water quality,* Subcommittee SC 5, *Biological methods.*

This second edition cancels and replaces the first edition (ISO 9439:1990), which has been technically revised.

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

Introduction

The conditions described in this International Standard do not always correspond to the optimal conditions for allowing the maximum degree of biodegradation to occur. With this test system, the microbially derived carbon dioxide (CO_2) is measured in the traps through which gas exhausted from the test vessels is passed. Some of the CO_2 remains in the medium in the vessels as dissolved inorganic carbon (DIC), the concentration of which may increase as biodegradation proceeds. As the organic carbon approaches complete removal, the concentration of DIC gradually falls and tends to reach zero by the end of incubation. It is thus necessary to acidify the medium at the end of the test to measure the biogenically formed CO_2 completely. The measurement of CO_2 in the external traps may differ from the true production of CO_2 and the kinetic rate may also be lower than a rate based on DOC removal measurement. The consequence may be that the biodegradation curves based on the trapped CO_2 may not fully represent the true microbial kinetic rate. For alternative biodegradation methods, see ISO 15462 and in particular ISO 14593, which is based on CO_2 production as well but does not have this defect.

Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Carbon dioxide evolution test

WARNING — Activated sludge and sewage may contain potentially pathogenic organisms. 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 determination of carbon dioxide (CO_2), for the evaluation in an aqueous medium of the ultimate biodegradability of organic compounds at a given concentration by aerobic microorganisms.

The method applies to organic compounds which are:

a) water-soluble under the conditions of the test, in which case removal of DOC may be determined as additional information (see annex D);

b) 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) non-volatile or which have a negligible vapour pressure under the conditions of the test;

NOTE For volatile substances use for example ISO 9408 or ISO 14593.

d) not inhibitory to the test microorganisms at the concentration chosen for the test.

NOTE 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 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
<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
dissolved organic carbon
DOC
that part of the organic carbon in a 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.

2.6
total inorganic carbon
TIC
all that inorganic carbon in the water deriving from carbon dioxide and carbonate

2.7
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.

2.8
theoretical amount of formed carbon dioxide
$ThCO_2$
theoretical maximum amount of carbon dioxide formed after oxidizing a chemical compound completely

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

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 normally recorded 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 normally recorded 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 normally recorded in days.

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

NOTE It is normally recorded 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

The biodegradability of organic compounds by aerobic microorganisms is determined using a static aqueous test system. The test mixture contains an inorganic medium, the organic compound as the nominal sole source of carbon and energy at a concentration of 10 mg/l to 40 mg/l organic carbon and a mixed inoculum obtained from a wastewater treatment plant or from another source in the environment. The mixture is agitated in test vessels and aerated with CO_2-free air normally up to 28 d (for example see annex A). The CO_2 formed during the microbial degradation is trapped in external vessels, determined by an appropriate analytical method (for examples see annex B), compared with the theoretical amount ($ThCO_2$) and expressed as a percentage.

For sufficiently water-soluble compounds, removal of DOC may optionally be measured to obtain additional information on the ultimate biodegradability. This can be done in the method given, but a convenient procedure is described in annex D which allows the use of higher concentrations of the test compound and the inoculum, thus improving the biodegradation potential of the test. If a substance-specific analytical method is available, information on the primary degradability may also 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 ± 2 °C during the test.

5 Reagents

Use only reagents of recognized analytical grade.

5.1 Water, distilled or deionized, containing less than 1 mg/l DOC.

5.2 Test medium.

5.2.1 Composition

a) **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
in water (5.1), 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 about 7,4. If this is not the case, prepare a new solution.

b) **Solution b)**

Dissolve 22,5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in water (5.1), quantity necessary to make up to 1 000 ml.

c) **Solution c)**

Dissolve 36,4 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in water (5.1), quantity necessary to make up to 1 000 ml.

d) **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 1 000 ml. To avoid precipitation, prepare this solution freshly before use or add a drop of concentrated hydrochloric acid (HCl).

5.2.2 Preparation of the test medium

For 1 000 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 1 000 ml with water (6.1).

6 Apparatus

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

6.1 Test vessels. Glass vessels (e.g. Erlenmeyer vessels or bottles) allowing gas purging and shaking or stirring, including tubing impermeable to CO_2. Located in a constant-temperature room or in a thermostatically controlled environment (e.g. water bath).

6.2 CO_2-free air production system, capable of supplying each test vessel at a flowrate between about 50 ml/min and 100 ml/min for 3 l of medium, held constant (see example of assembly with the test vessels in annex A).

6.3 Analytical equipment for determining CO_2.

Any suitable apparatus or technique with sufficient accuracy, e.g. CO_2 - or DIC analyzer or device for titrimetric determination after complete absorption in an alkaline solution (see examples in annex B).

6.4 Analytical equipment for measuring dissolved organic carbon (DOC) (optional).

6.5 Centrifuge or device for filtration, with membrane filters (nominal aperture diameter of 0,2 µm to 0,45 µm pore size) which adsorb or release organic carbon to a minimum degree.

6.6 pH meter.

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 water (5.1) or the test medium (5.2) and add a suitable amount of this to obtain an organic carbon concentration in the final test medium of between 10 mg/l and 40 mg/l. 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.

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

7.1.2 Reference compound

Use as reference compound an organic compound of known biodegradability, such as aniline or sodium benzoate. 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 organic carbon concentration of 20 mg/l or a concentration equivalent to that of the test compound.

7.1.3 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 (5.2), both the test compound (7.1.1) and the reference compound (7.1.2) preferably at concentrations of organic carbon of 20 mg/l for each.

7.2 Preparation of the inoculum

7.2.1 General

Prepare the inoculum using activated sludge (7.2.2) or the sources described in 7.2.3 and 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 CO_2 production of the blank should 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 washing with medium (5.2.2) and aerating it, from 1 d to 7 d, before use. Use a suitable volume for inoculation (see note 2 below).

NOTE 1 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).

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 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 1 000 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 wastewater 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 remove coarse particles by filtration through a sieve and concentrate the sludge by settling, so that the volume of sludge added to the test assay is minimal. Keep the sample under aerobic conditions and use preferably on the day of collection. Use a suitable volume to obtain 30 mg/l of suspended solids in the final mixture.

7.2.3 Inoculum from wastewater

Take a sample from the influent or from the effluent of a full-scale or a laboratory wastewater treatment plant dealing with predominantly domestic sewage. If necessary, remove gross particulate matter by coarse filtration and concentrate the sample, e.g. by 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 using a coarse paper filter 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 procedure

Provide a sufficient number of vessels (6.1) in order to have

— at least two test vessels (denoted F_T) for the test compound (7.1.1);

— at least two blank vessels (denoted F_B) containing test medium and inoculum;

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

— if needed, one vessel for checking a possible inhibitory effect of the test compound (denoted F_I) containing solution 7.1.3;

— if needed, one vessel for checking a possible abiotic elimination (denoted F_S) containing the test compound (7.1.1) but no inoculum, sterilized by autoclaving or by addition of a suitable inorganic toxic compound to prevent microbial activity. Use, for example, 1 ml/l of a solution containing 10 g/l of mercury(II) chloride ($HgCl_2$). Add the same amount of the toxic substance two weeks after the test was begun.

Add appropriate amounts of the test medium (5.2), and the inoculum (7.2) to the vessels as indicated in Table 1 to obtain a final test volume of e.g. 3 l. Other final test volumes are possible; adapt in such a case all relevant parameters and the calculation of test results. Connect the vessels to the CO_2-free air production system (see annex A). Incubate at the desired test temperature (see clause 4) and aerate the vessels for 24 h to purge CO_2 from the system. Agitate throughout the test with a magnetic stirrer. If excessive foaming is observed, replace the air sparge by headspace aeration while stirring. After the pre-aeration period, connect the air exit of each vessel to the CO_2 trapping or measuring system.

Add the test sample (7.1.1) and the reference compound (7.1.2) at the desired concentrations to the respective vessels in accordance with Table 1 and start the test by bubbling CO_2-free air through the vessels with 3 l medium at a rate of about 50 ml/min to 100 ml/min.

Measure the amount of CO_2 released from each vessel at timed intervals, depending on the rate of evolution of CO_2, using an appropriate and sufficiently accurate method (see annex B). If a nearly constant level of CO_2 formation is attained (plateau phase) and no further biodegradation is expected, the test is considered to be completed. Usually the maximum test period should not exceed 28 d. Extend the test by one to two weeks, if degradation has obviously started but has not reached a plateau.

Table 1 — Final distribution of test and reference compounds in the test vessels

Vessel	Test medium (5.2)	Test compound (7.1.1)	Reference compound (7.1.2)	Inoculum (7.2)
F_T Test compound	+	+	–	+
F_T Test compound	+	+	–	+
F_B Blank	+	–	–	+
F_B Blank	+	–	–	+
F_C Inoculum check	+	–	+	+
F_I Inhibition control (optional)	+	+	+	+
F_S Abiotic elimination check (optional)	+	+	–	–

On the last day of the test, measure the pH, acidify all the bottles with 1 ml to 10 ml of concentrated hydrochloric acid in order to decompose the carbonates and bicarbonates and purge the CO_2. Continue aeration for up to 24 h and measure the amount of CO_2 released from each vessel.

NOTE 1 During the handling of samples for the regular measurement of CO_2 in the traps it cannot be excluded that, especially in the case of DIC determinations, small amounts of CO_2 from the air are included and added up during the test. This has normally no effect on the test results as the CO_2 values of the blank vessels, where the same occurs, are subtracted. However, in the case of the abiotic elimination control (vessel F_s) this may lead to an apparent and unjustified impression of degradation. Therefore it is recommended to determine the CO_2 evolution from vessel F_s only at the end of the test.

NOTE 2 If the DOC removal is measured to provide additional information on the biodegradability of a water-soluble test compound, or if a substance-specific analytical method is used to determine the primary biodegradability, use the information given in annex D.

8 Calculation

8.1 Amount of theoretical carbon dioxide from the test compound

The theoretical amount, in milligrams, of released carbon dioxide ($ThCO_2$) in the test vessels is given by equation (1):

$$ThCO_2 = \rho_C \times V_L \times \frac{44}{12} \qquad (1)$$

where

ρ_C is the concentration of organic carbon of the test compound in the test vessel, in milligrams per litre, measured or calculated from the stock solution of the test compound (7.1.1);

V_L is the volume of the test solution in the test vessel, expressed in litres;

44 and 12 are the relative molar and atomic masses of CO_2 and carbon, respectively, to calculate the amount of CO_2 from the measured organic carbon.

Calculate in the same way the $ThCO_2$ of the reference compound and the inhibition solution (7.1.3).

8.2 Percentage biodegradation

Calculate the percentage of biodegradation D_m (%) for each of the test vessels F_T for each measurement interval using equation (2)

$$D_m = \frac{\sum m_{Tt} - \sum m_{Bt}}{ThCO_2} \times 100 \quad (2)$$

where

Σm_{Tt} is the mass, in milligrams, of CO_2 released in vessel F_T between the start of the test and time t;

Σm_{Bt} is the average mass, in milligrams, of CO_2 released in the blank controls F_B between the start of the test and time t.

Calculate in the same way the biodegradation degree of the reference compound in the inoculum check vessel F_C, and, if included, of the mixture of test and reference compound in the inhibition control F_I, and, without subtracting the blanks, of the test compound in the abiotic elimination control F_S.

NOTE If DOC removal and primary biodegradation by substance-specific analyses were measured, it is recommended that results be calculated according to annex D.

8.3 Expression of results

Compile a table of CO_2 released (Σm_{Tt} and Σm_{Bt}) and the percentages of biodegradation (D_m) for each measuring interval and each test vessel. Plot a biodegradation curve in percent as a function of time, and indicate lag phase and degradation phase. Optionally, plot a curve of the net released CO_2 versus time. If comparable results are obtained for the duplicate test vessels F_T (< 20 % difference), plot a mean curve, otherwise plot curves for each vessel (see example in annex C). Plot in the same way a biodegradation curve of the reference compound F_C and, if included of the abiotic elimination check F_S and the inhibition control F_I.

Determine the mean value of percent biodegradation in the plateau phase or use the highest value, e.g. when the curve decreases in the plateau phase and indicate this maximum level of biodegradation as "degree of biodegradation of the test compound" in the test report.

Information on the toxicity of the test compound may be useful in the interpretation of test results showing a low biodegradation. If in vessel F_I the degradation percentage is < 25 % and insufficient degradation of the test compound is observed, it can be assumed that the test compound is inhibitory. In this case, the test should be repeated using a lower test concentration or another inoculum. If in vessel F_S (abiotic elimination check, if included) a significant amount (> 10 %) of released CO_2 is observed, abiotic degradation processes may have taken place.

9 Validity of results

9.1 Validity criteria

The test is considered as valid if

a) the percentage degradation in vessel F_C (inoculum check) is greater than 60 % on the 14th day;

b) the concentration of CO_2 which has evolved from the blank F_B at the end of the test at a test volume of 3 l is about 40 mg/l and does not exceed 70 mg/l;

c) the amount of DIC at the beginning of the test is < 5 % of the organic carbon of the test compound.

If a) and b) are not fulfilled, the test should be repeated using another or a better preconditioned inoculum. If c) is not fulfilled, verify that the air for aerating the vessels is really free from CO_2.

9.2 Inhibition

If the vessel F_I (inhibition control) was included, the test compound is assumed to be inhibiting if the degradation percentage of the reference compound in vessel F_I is lower than 40 % at the end of the test. In this case, it is advisable to repeat the test with a lower concentration of the test compound.

9.3 pH value

If the pH value at the end of the test is outside the range 6 to 8,5 and if the percentage degradation of the test compound is less than 60 %, it is advisable to repeat the test with a lower concentration of the test compound or to use the test modification described in annex D of this method.

10 Test report

The test report shall contain at least the following information:

a) a reference to this International Standard and the annex if a variation was used;

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

c) all the data (for example in tabular form) obtained and the degradation curve;

d) the concentration of the test compound used and the $ThCO_2$ and, in the case of water-soluble test compounds, the DOC of this concentration;

e) the name of the reference compound used and the degradation 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 main characteristics of the CO_2 analysing system used;

h) the incubation temperature of the test;

i) if included the percentage of DOC removal or primary biodegradation;

j) if included, the percentage of degradation obtained in vessel F_S (abiotic elimination);

k) if included, the percentage of degradation in vessel F_I (inhibition check) and a statement on the toxicity of the test compound;

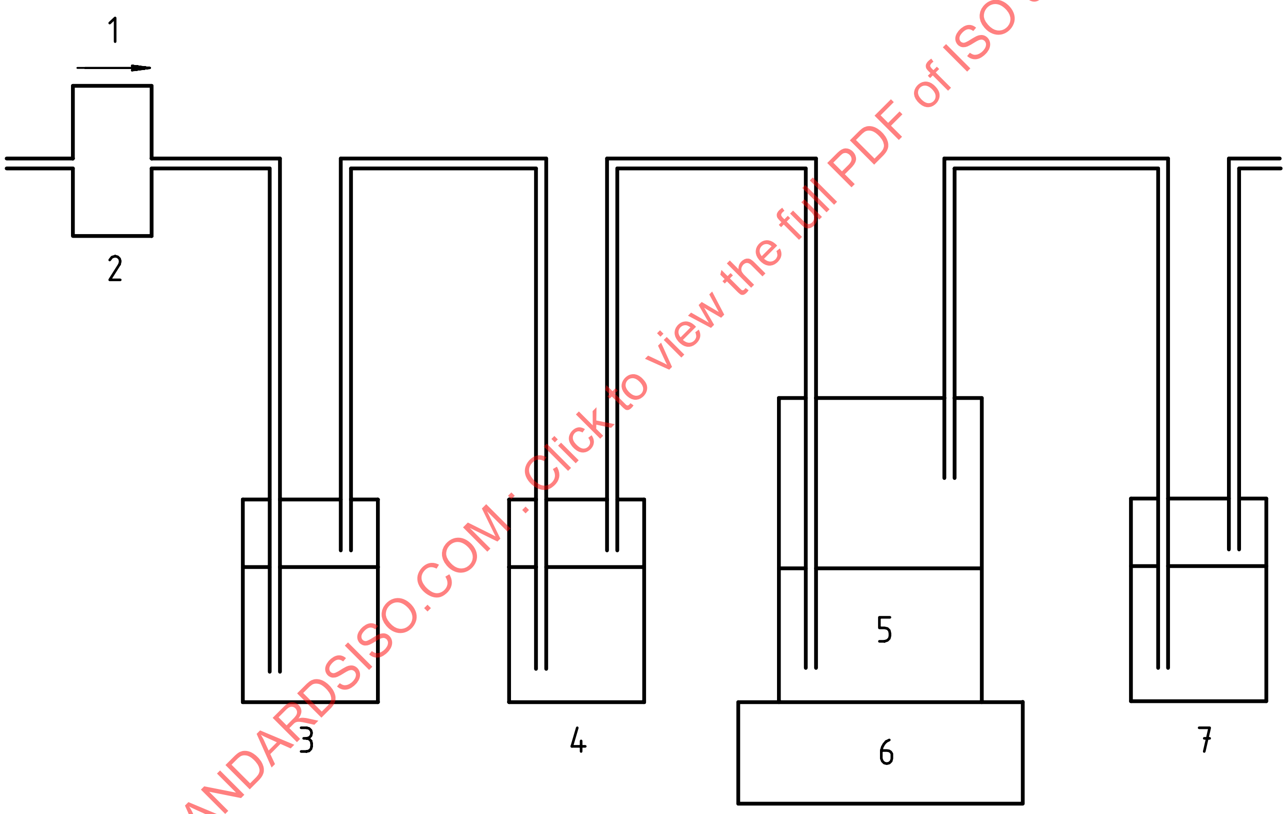
l) the reasons, in the event of rejection of the test;

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

Annex A
(informative)

Principle of a test system for measuring carbon dioxide (example)

Set up vessels in series as shown in Figure A.1 and connect them with gas-impermeable tubing. Aerate the test system with CO_2-free air at 50 ml/min to 100 ml/min and a constant low pressure. Count air bubbles or use a suitable gas-flow controller to check the flowrate. Use synthetic CO_2-free air or compressed air. In the latter case, remove CO_2 by passing the air through a vessel with dry soda lime or through at least two gas-washing vessels containing e.g. 500 ml of an aqueous NaOH solution (c = 10 mol/l). A second vessel containing 100 ml $Ba(OH)_2$ solution (c = 0,0125 mol/l) is used to indicate any CO_2 in the air by turbidity. An empty vessel between the indicator and the following test vessel prevents liquid carry-over. In the test vessel, CO_2 is produced if biodegradation takes place and absorbed in the subsequent absorber vessels as described in annex B.



Key

1 Compressed air
2 Flow controller
3 Carbon dioxide trap (NaOH)
4 Carbon dioxide indicator [$Ba(OH)_2$]
5 Test vessels
6 Stirrer
7 Carbon dioxide trap [$Ba(OH)_2$ or (NaOH)]

Figure A.1

Annex B
(informative)

Examples of the determination of released carbon dioxide

B.1 CO_2 determination by DIC measurement

The CO_2 released is absorbed in sodium hydroxide (NaOH) solution and determined as dissolved inorganic carbon (DIC) using e.g. a DOC analyser without incineration or oxidation.

Prepare a solution of NaOH (c = 0,05 mol/l) in deionized water. Measure the DIC of this solution and consider this blank value (ρ_B) when calculating the CO_2 production. Connect two absorber vessels in series with the test vessel, each containing at least 100 ml of the NaOH solution. Close the outlet of the last vessel with a small syphon to prevent introduction of CO_2 from the air to the NaOH solution. On the days of CO_2 determination, remove the vessel closest to the test vessel and take a sufficient sample for DIC measurement (e.g. 10 ml). Replace the vessel by the second and add a new one with freshly prepared NaOH solution. On the last day measure, after acidification of the test solution, DIC in both vessels.

Calculate the CO_2 produced using equation (B.1):

$$m_{Tt} = \frac{(\rho_T - \rho_B) \times 3,67}{10} \tag{B.1}$$

where

m_{Tt} is the mass, in milligrams, of CO_2 released in vessel F_T between the start of the test and time t;

ρ_T is the measured DIC concentration, in milligrams per litre, of the NaOH in vessel F_T solution at time t;

ρ_B is the measured DIC concentration, in milligrams per litre, of the NaOH in the blank F_B solution at time t;

3,67 is the ratio of the relative molecular/atomic masses CO_2/C (44/12);

10 is a correction factor, expressed in reciprocal litres, for 100 ml volume of the NaOH solution. If other volumes are used adapt this factor.

B.2 Titrimetric method using barium hydroxide

The CO_2 produced reacts with the barium hydroxide [$Ba(OH)_2 \cdot 8H_2O$] and is precipitated as barium carbonate ($BaCO_3$) [equation (B.2)]. The amount of released CO_2 is determined by titrating the remaining $Ba(OH)_2$ with hydrochloric acid (HCl) [equation (B.3)].

$$CO_2 + Ba(OH)_2 \rightarrow BaCO_3 + H_2O \tag{B.2}$$

$$Ba(OH)_2 + 2HCl \rightarrow BaCl_2 + 2H_2O \tag{B.3}$$

Dissolve 4,0 g $Ba(OH)_2 \cdot 8H_2O$ in 1 000 ml deionized or distilled water to obtain a 0,0125 mol/l solution. It is recommended that a sufficient amount, e.g. 5 l, be prepared at one time when running a series of tests. Filter free of solid material and determine the exact normality by titration with a standard HCl solution for calculation of the test results. Store sealed as a clear solution to prevent absorption of CO_2 from the air.

Dissolve 50 ml of HCl solution (c = 1 mol/l) (36,5 g/l) in 1 000 ml deionized or distilled water to obtain a 0,05 mol/l solution. Use phenolphthalein as an indicator or an automatic titrator to determine the endpoint.

At the start of the test, dispense exactly 100 ml of the $Ba(OH)_2$ solution into each of the three absorber vessels. Depending on the character and amount of the test compound, use modifications of the trapping volumes. Periodically, on each day of measurement, remove the vessel nearest the test vessel for titration. This should take place as needed, e.g. when the first absorber vessel is turbid due to precipitation of $BaCO_3$ and before any turbidity can be observed in the second vessel. Usually, at the beginning of the test, titration may be required every other day and then every fifth day when the plateau phase is reached. After removing the absorber vessel, immediately seal it with a plug to avoid CO_2 input from air. Move the remaining two vessels one position closer to the test vessel and place at the end of the series a new vessel filled with fresh $Ba(OH)_2$ solution. Handle all vessels containing test compound, reference compound, blank, inhibition and inoculum control in exactly the same way.

Immediately after removing the vessel, titrate either the whole amount (100 ml) or two or three portions of the $Ba(OH)_2$ solution with the HCl solution. Note the volumes of the HCl solution needed for neutralization.

The mass of CO_2 trapped in the absorber vessel is given by equation (B.4):

$$m_T = \left\{ \frac{2 c_{Ba} \cdot V_{B0}}{c_{HCl}} - V_A \cdot \frac{V_{BT}}{V_{BZ}} \right\} \cdot c_{HCl} \times 22 \qquad (B.4)$$

where

m_T is the mass of CO_2, in milligrams, trapped in the absorber of vessel F_T;

c_{HCl} is the exact concentration, in moles per litre, of the HCl solution;

c_{Ba} is the exact concentration, in moles per litre, of the $Ba(OH)_2$ solution;

V_{B0} is the volume, in millilitres, of the $Ba(OH)_2$ solution at the beginning of the test;

V_{BT} is the volume, in millilitres, of the $Ba(OH)_2$ solution at time t before filtration;

V_{BZ} is the volume, in millilitres, of the aliquots of the $Ba(OH)_2$ solution used for titration;

V_A is the volume, in millilitres, of the HCl solution used for titration of the $Ba(OH)_2$ solution;

22 is the half-molarity of CO_2.

When the following conditions apply:

— volume of the $Ba(OH)_2$ solution before and after absorption is exactly 100 ml and the complete solution is used for titration ($V_{B0} = V_{BT} = V_{BZ}$);

— concentration of the $Ba(OH)_2$ solution is exactly c_{Ba} = 0,0125 mol/l;

— concentration of the HCl solution is exactly c_{HCl} = 0,05 mol/l;

use equation (B.5):

$$m_T = 1{,}1\,(50 - V_A) \qquad (B.5)$$

Annex C
(informative)

Example of a biodegradation curve

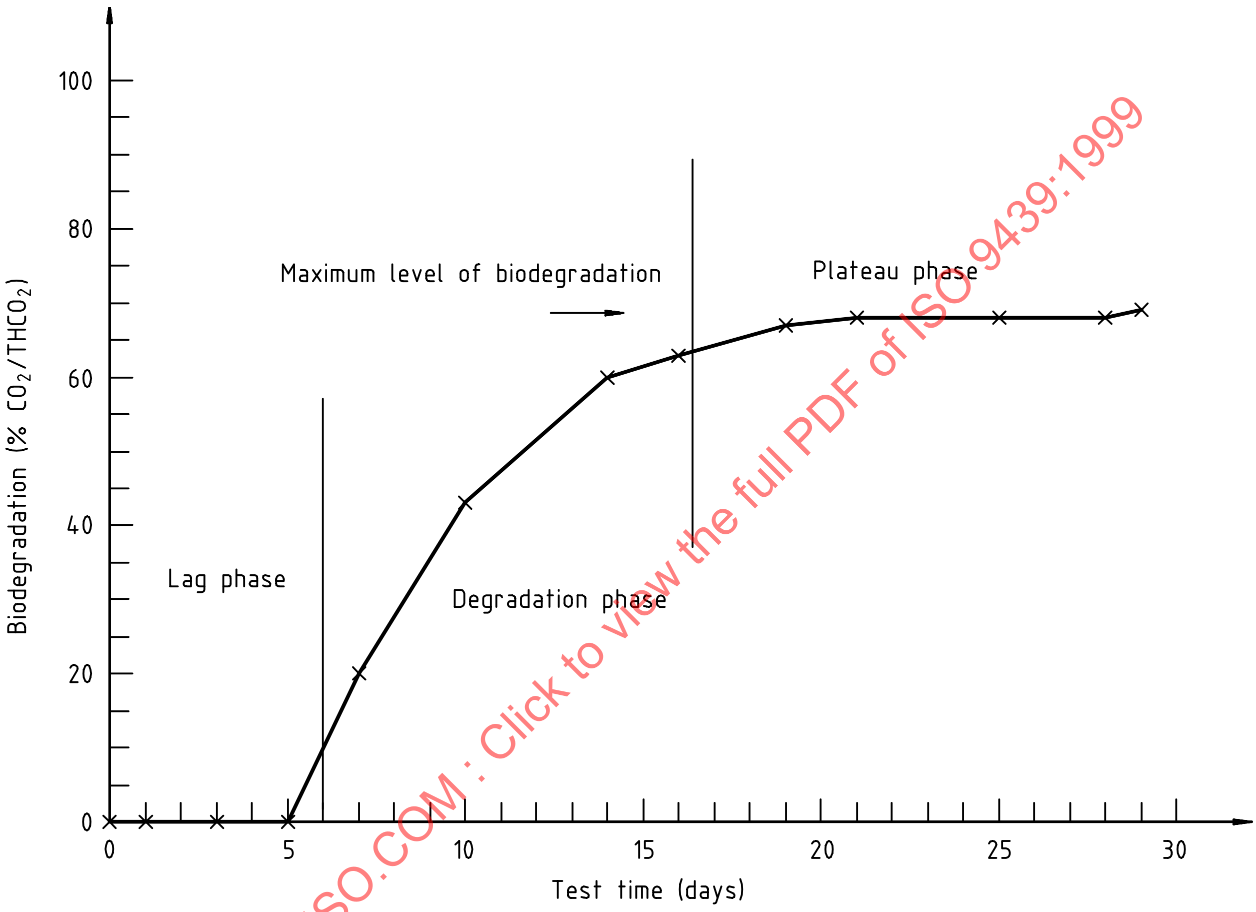


Figure C.1 — Biodegradation of aniline in the CO_2 evolution test