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**Determination of the ultimate aerobic  
biodegradability of plastic materials  
in an aqueous medium — Method by  
analysis of evolved carbon dioxide**

*Évaluation de la biodégradabilité aérobie ultime des matériaux  
plastiques en milieu aqueux — Méthode par analyse du dioxyde de  
carbone libéré*





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Published in Switzerland

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see [www.iso.org/patents](http://www.iso.org/patents)).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

This document was prepared by Technical Committee ISO/TC 61, *Plastics*, Subcommittee SC 14, *Plastics and environment*.

This second edition cancels and replaces the first edition (ISO 14852:1999), which has been technically revised. It also incorporates the Technical Corrigendum ISO 14852:1999/Cor.1:2005 and contains the following changes:

- the validity criteria has been revised to comply with ISO 14855;
- in the introduction, an obsolete, potentially misleading paragraph has been deleted;
- the normative reference clause has been updated;
- the “Terms and definitions” clause has been revised and updated;
- the test methods have been updated for better comprehension.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

## Introduction

With the increasing use of plastics, their recovery and disposal have become a major issue. As a first priority, recovery should be promoted. Biodegradable plastics are now emerging as one of the options available to solve such environmental problems. Plastic materials, such as products or packaging, which are sent to composting facilities should be potentially biodegradable. Therefore, it is very important to determine the potential biodegradability of such materials and to obtain an indication of their potential biodegradability.



# Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium — Method by analysis of evolved carbon dioxide

**WARNING** — Sewage, activated sludge, soil and compost may contain potentially pathogenic organisms. Therefore, appropriate precautions should be taken when handling them. Toxic test compounds and those whose properties are unknown should be handled with care.

## 1 Scope

This document specifies a method, by measuring the amount of carbon dioxide evolved, for the determination of the degree of aerobic biodegradability of plastic materials, including those containing formulation additives. The test material is exposed in a synthetic medium under standardized laboratory conditions to an inoculum from activated sludge, mature compost or soil under aerobic, mesophilic conditions.

If an unadapted activated sludge is used as the inoculum, the test result can be used to assess the aerobic biodegradation processes which occur in a waste water treatment plant environment. If a mixed or pre-exposed inoculum is used, the method can be used to investigate the potential biodegradability of a test material.

The conditions used in this document do not necessarily correspond to the optimum conditions allowing maximum biodegradation to occur, but this test method is designed to measure the biodegradation of plastic materials and give an indication of their potential biodegradability.

The method enables the assessment of the biodegradation to be improved by calculating a carbon balance (optional, see [Annex C](#)).

The method applies to the following materials:

- natural and/or synthetic polymers, copolymers or mixtures thereof;
- plastic materials which contain additives such as plasticizers, colorants or other compounds;
- water-soluble polymers;
- materials which, under the test conditions, do not inhibit the microorganisms present in the inoculum. Inhibitory effects can be determined using an inhibition control or by another appropriate method (see, for example, ISO 8192[1]). If the test material is inhibitory to the inoculum, a lower test concentration, another inoculum or a pre-exposed inoculum can be used.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8245, *Water quality — Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC)*

## 3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

**3.1**  
**ultimate aerobic biodegradation**  
breakdown of an organic compound by microorganisms in the presence of oxygen into carbon dioxide, water and mineral salts of any other elements present (mineralization) plus new biomass

**3.2**  
**activated sludge**  
mixture of organic materials and biomass produced in the aerobic treatment of waste water by the growth of bacteria and other microorganisms in the presence of dissolved oxygen

**3.3**  
**concentration of suspended solids in 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

**3.4**  
**dissolved inorganic carbon**  
**DIC**  
part of the inorganic carbon in water which cannot be removed by specified phase separation

Note 1 to entry: Phase separation can be achieved for example by centrifugation at 40 000 m·s<sup>-2</sup> for 15 min or by membrane filtration using membranes with pores of 0,2 µm to 0,45 µm diameter.

**3.5**  
**theoretical amount of evolved carbon dioxide**  
**ThCO<sub>2</sub>**  
maximum theoretical amount of carbon dioxide evolved after completely oxidizing a chemical compound, calculated from the molecular formula

Note 1 to entry: It is expressed as milligrams of carbon dioxide evolved per milligram or gram of test compound.

**3.6**  
**total organic carbon**  
**TOC**  
amount of carbon bound in an organic compound

Note 1 to entry: It is expressed as milligrams of carbon per 100 mg of the compound.

[SOURCE: ISO 17556:2012, 3.14]

**3.7**  
**dissolved organic carbon**  
**DOC**  
part of the organic carbon in water which cannot be removed by specified phase separation

Note 1 to entry: Phase separation can be achieved for example by centrifugation at 40 000 m·s<sup>-2</sup> for 15 min or by membrane filtration using membranes with pores of 0,2 µm to 0,45 µm diameter.

**3.8**  
**lag phase**  
time from the start of a test until adaptation and/or selection of the degrading microorganisms is achieved and the degree of biodegradation of a chemical compound or organic matter has increased to about 10 % of the maximum level of biodegradation

Note 1 to entry: It is measured in days.



**3.9****maximum level of biodegradation**

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

Note 1 to entry: It is measured in per cent.

**3.10****biodegradation phase**

time from the end of the lag phase of a test until the plateau phase has been reached

Note 1 to entry: It is measured in days.

**3.11****plateau phase**

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

Note 1 to entry: It is measured in days.

**3.12****pre-exposure**

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

**3.13****pre-conditioning**

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

**3.14****inoculum**

microorganisms or other material used in an inoculation

Note 1 to entry: Also called inoculant.

**3.15****inoculation**

introduction of microorganisms into a culture medium in order to start a biological process

**4 Principle**

The biodegradability of a plastic material is determined using aerobic, mesophilic microorganisms in an aqueous system. The test mixture contains an inorganic medium, the organic test material (the sole source of carbon and energy) with a concentration between 100 mg/l and 2 000 mg/l of organic carbon, and activated sludge or a suspension of active soil or compost as the inoculum. If higher concentrations of test material are used then an optimised test medium should be applied.

NOTE Lower concentrations such as between 20 mg/l and 40 mg/l of organic carbon have been tested and found suitable.

The mixture is agitated in test flasks and aerated with carbon-dioxide-free air over a period of time depending on the biodegradation kinetics, but not exceeding 6 months. The carbon dioxide evolved during the microbial degradation is determined by a suitable analytical method, examples of which are given in [Annexes A](#) and [B](#).

The level of biodegradation is determined by comparing the amount of carbon dioxide evolved with the theoretical amount ( $\text{ThCO}_2$ ) and expressed in per cent. The test result is the maximum level of biodegradation, determined from the plateau phase of the biodegradation curve. Optionally, a carbon balance may be calculated to give additional information on the biodegradation (see [Annex C](#)).

Unlike ISO 9439, which is used for a variety of organic compounds, this document is specially designed for the determination of the biodegradation of plastic materials. The special requirements necessary affect the choice of the inoculum and the test medium, and there is the possibility of improving the evaluation of the biodegradability by calculating a carbon balance.

## 5 Test environment

Incubation shall take place in the dark or in diffuse light in an enclosure which is free from vapours inhibitory to microorganisms and which is maintained at a constant temperature, preferably between 20 °C and 25 °C, to an accuracy of  $\pm 1$  °C.

## 6 Reagents

Use only reagents of recognized analytical grade.

**6.1 Distilled or deionized water**, free of toxic substances (copper in particular) and containing less than 2 mg/l of DOC.

### 6.2 Test medium.

Depending on the purpose of the test, different test media may be used. For example, if a test material is used at higher concentrations, use the optimized test medium (6.2.2) with higher buffering capacity and nutrient concentrations.

#### 6.2.1 Standard test medium.

##### 6.2.1.1 Solution A.

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

anhydrous potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )	8,5 g
anhydrous dipotassium hydrogen phosphate ( $\text{K}_2\text{HPO}_4$ )	21,75 g
disodium hydrogen phosphate 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

The correct composition of the solution can be checked by measuring the pH, which should be 7,4.

##### 6.2.1.2 Solution B.

Dissolve 22,5 g of magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) in water (6.1) and make up to 1 000 ml.

##### 6.2.1.3 Solution C.

Dissolve 36,4 g of calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in water (6.1) and make up to 1 000 ml.

##### 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 water (6.1) and make up to 1 000 ml.

Prepare this solution freshly before use to avoid precipitation, or add a drop of concentrated hydrochloric acid (HCl) or a drop of 0,4 g/l aqueous solution of ethylenediaminetetraacetic acid (EDTA).

### 6.2.1.5 Preparation.

To prepare 1 l of test medium, add the following, to about 500 ml of water (6.1):

- 10 ml of solution A (6.2.1.1);
- 1 ml of each of solutions B (6.2.1.2), C (6.2.1.3), D (6.2.1.4).

Make up to 1 000 ml with water (6.1).

Prepare the test medium freshly before use. The solutions A up to C may be stored up to 6 months in the dark at room temperature. The same applies for solution D in case HCl or EDTA has been added.

### 6.2.2 Optimized test medium.

This optimized medium is highly buffered and contains more inorganic nutrients. This is necessary to keep the pH constant in the system during the test, even at high concentrations of the test material. The medium contains about 2 400 mg/l of phosphorus and 50 mg/l of nitrogen and is therefore suitable for concentrations in the test material of up to 2 000 mg/l of organic carbon. If higher or lower test-material concentrations are used, increase or decrease respectively the nitrogen content to keep the C:N ratio at about 40:1.

#### 6.2.2.1 Solution E.

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

anhydrous potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )	37,5 g
disodium hydrogen phosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ )	87,3 g
ammonium chloride ( $\text{NH}_4\text{Cl}$ )	2,0 g

#### 6.2.2.2 Solution F (trace-element solution, optional).

Dissolve in 10 ml of aqueous HCl solution (25 %, 7,7 mol/l), in the following sequence:

- a) 70 mg of  $\text{ZnCl}_2$ ;
- b) 100 mg of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ;
- c) 6 mg of  $\text{H}_3\text{BO}_3$ ;
- d) 190 mg of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ;
- e) 3 mg of  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ;
- f) 240 mg of  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ;
- g) 36 mg of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ;
- h) 33 mg of  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ;
- i) 26 mg of  $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ .

Make up to 1 000 ml with water (6.1).

#### 6.2.2.3 Solution G (vitamin solution, optional).

Dissolve in 100 ml of water (6.1) 0,6 mg of biotine, 2,0 mg of niacinamide, 2,0 mg of *p*-aminobenzoate, 1,0 mg of panthotenic acid, 10,0 mg of pyridoxal hydrochloride, 5,0 mg of cyanocobalamine, 2,0 mg of folic acid, 5,0 mg of riboflavin, 5,0 mg of DL-thioctic acid and 1,0 mg of thiamine dichloride or use a

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solution of 15 mg of yeast extract in 100 ml of water (6.1). Filter the solution for sterilization using membrane filters (see 7.6).

Solutions E and F are optional and are not required if a sufficient concentration of the inoculum is used, e.g. activated sludge, soil or compost. It is recommended that 1 ml portions be prepared and kept refrigerated until use.

### 6.2.2.4 Preparation.

To prepare 1 litre of test medium, add, to about 800 ml of water (6.1),

- 100 ml of solution E (6.2.2.1), and
- 1 ml of each of solutions B (6.2.1.2), C (6.2.1.3), D (6.2.1.4) and, optionally, F (6.2.2.2) and G (6.2.2.3).

Make up to 1 000 ml with water (6.1) and measure the pH.

The correct composition of the test medium can be checked by measuring the pH, which should be  $7,0 \pm 0,2$ .

### 6.3 Pyrophosphate solution.

Dissolve 2,66 g of sodium pyrophosphate ( $\text{Na}_4\text{P}_2\text{O}_7$ ) in water (6.1) and make up to 1 000 ml.

## 7 Apparatus

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

Required is usual laboratory equipment, plus the following.

**7.2 Test flasks:** glass vessels (e.g. bottles or conical flasks) designed to allow gas purging and shaking or stirring, and fitted with tubing impermeable to  $\text{CO}_2$ . The vessels shall be located in a constant-temperature room or in a thermostatted apparatus (e.g. water-bath).

**7.3  $\text{CO}_2$ -free-air production system,** capable of supplying  $\text{CO}_2$ -free air at a flow rate between 50 ml/min and 100 ml/min to each test flask, held constant to within  $\pm 10\%$  (see example of system, including test vessels, in Annex A).

**7.4 Analytical instrument for determining carbon dioxide,** consisting of any suitable apparatus with sufficient accuracy, e.g. a  $\text{CO}_2$  or DIC analyser or apparatus for titrimetric determination after complete absorption in a basic solution (see examples in Annex B). Note that, if an analyser with an IR detector, for instance, is used,  $\text{CO}_2$ -free air is not necessary.

**7.5 Analytical equipment for measuring total organic carbon (TOC) and dissolved organic carbon (DOC),** see ISO 8245.

**7.6 Analytical balance.**

**7.7 Centrifuge, or filtration device** with membrane filters (0,45  $\mu\text{m}$  pore size) which neither adsorb nor release organic carbon significantly.

**7.8 pH-meter.**

**7.9 Magnetic stirrer or shaking device.**

## 8 Procedure

### 8.1 Test material

The test material shall be of known mass and contain sufficient carbon to yield CO<sub>2</sub> in a quantity that can be adequately measured by the analytical system used. Calculate the TOC from the chemical formula or determine it by a suitable analytical technique (e.g. elemental analysis or measurement in accordance with ISO 8245) and calculate the ThCO<sub>2</sub>. Use a concentration of a test material such that the TOC content is at least 30 mg/l preferably 100 mg/l. The maximum amount of test material is limited by the oxygen supply to the test system and the test medium used. When using higher amount of test material, the optimized test medium (6.2.2) should be used and, in any case the test-material concentration shall be such that the TOC does not exceed about 2 000 mg/l, i.e. a C:N ratio of about 40:1. If higher concentrations are to be tested, increase the nitrogen amount in the test medium.

**NOTE** Lower amount of test materials, corresponding to a TOC content between 20 mg/l and 40 mg/l, have been tested and found suitable.

The test material should preferably be used in powder form, but it may also be introduced as films, pieces, fragments or shaped articles. The form and shape of the test material may influence its biodegradation. Similar shapes should preferably be used if different kinds of plastic material are to be compared. If the test material is used in the form of a powder, particles of known, narrow size distribution should be used. A particle-size distribution with the maximum at 250 µm diameter is recommended. Also, the size of the test equipment used may depend on the form of the test material. It should be ascertained that no substantial mechanical aberrations occur due to the test conditions, for example due to the type of stirring mechanism used. Processing of the test material (e.g. the use of powder in the case of composites) should not influence significantly the degradation behaviour of the material. Optionally, record the hydrogen, oxygen, nitrogen, phosphorus and sulfur contents and the molecular mass of a polymeric test material, using for example liquid exclusion chromatography (see, for example, ASTM D 3536-91<sup>[Z]</sup> or any other applicable standard method).

For details on how to handle poorly water-soluble compounds, see ISO 10634.

### 8.2 Reference material

Use a well-defined biodegradable polymer (for example microcrystalline cellulose powder, ashless cellulose filters or poly-β-hydroxybutyrate) as a reference material. If possible, the TOC, form and size should be comparable to that of the test material.

As a negative control, a non-biodegradable polymer (e.g. polyethylene) in the same form as the test material can optionally be used.

### 8.3 Preparation of the inoculum

#### 8.3.1 General

Activated sludge from a sewage-treatment plant treating predominantly domestic sewage is a suitable source of the inoculum. It is obtained from an active aerobic environment and is available over a wide geographical area. Alternatively, soil and/or compost suspensions can be used for inoculation, as with some plastic materials the activity of fungi is important for biodegradation. When biodegradation in a specific waste-treatment plant is to be determined, collect the inoculum from that environment.

The inoculum can be prepared from the sources described in 8.3.2 and 8.3.3, or from a mixture of these sources in order to obtain a varied and concentrated microbial flora with sufficient biodegradation activity. If the endogenous respiration of the inoculum is too high, stabilize the inoculum by aeration before use. Harmonize the test temperature with the inoculum used (see Clause 5).

### 8.3.2 Inoculum from wastewater-treatment plants

Take a sample of activated sludge collected from a well-operated sewage-treatment plant or a laboratory plant handling predominantly domestic sewage. Mix well, keep the sample under aerobic conditions and use preferably on the day of collection (at least within 72 h).

Before use, determine the concentration of suspended solids (use e.g. ISO 11923[4]). If necessary, concentrate the sludge by settling so that the volume of sludge added to the test assay is minimal. Add a suitable volume to obtain suspended solids in the range 30 mg/l to 1 000 mg/l in the final mixture.

When a carbon balance determination (see [Annex C](#)) is to be carried out, an inoculum concentration of 30 mg/l suspended solids is recommended. As solid matter can interfere with the carbon balance determination, the following procedure for preparing the inoculum is recommended. Take 500 ml of the activated sludge and homogenize for 2 min at medium speed in a blender or in a suitable high-speed mixer. Allow to settle until the supernatant liquid contains no significant amounts of suspended matter, but in any case for at least 30 min. Decant a sufficient volume of the supernatant liquid and add it to the test flasks to obtain a concentration of 1 % (V/V) to 5 % (V/V) in the test medium. Avoid carrying over sludge particles.

An inoculum may be pre-conditioned, but normally no pre-exposed inoculum should be used. Depending on the purpose of the test, a pre-exposed inoculum may also be used, provided this is clearly stated in the test report (e.g. per cent biodegradation =  $x$  %, using pre-exposed inocula) and the method of pre-exposure detailed in the test report. Pre-exposed inocula can be obtained from suitable laboratory biodegradation tests (see ISO/TR 15462) conducted under a variety of conditions or from samples collected from locations where relevant environmental conditions exist (e.g. contaminated areas or industrial treatment plants).

### 8.3.3 Inoculum from soil and/or compost

Suspend 10 g of non-sterile, fertile soil or compost from a composting plant treating predominantly organic waste in 100 ml of the test medium ([6.2.1](#) or [6.2.2](#)) or in a pyrophosphate solution ([6.3](#)) which is commonly used in soil microbiology. Allow to settle for about 30 min. Decant and filter the supernatant liquid through a coarse porous filter and add the inoculum to the test flasks to obtain a concentration of 1 % (V/V) to 5 % (V/V) in the test medium. Higher amounts of inoculum can be used if necessary, but this may cause problems in establishing carbon balances. The use of compost can increase the number of fungi in the test flasks and improve the biodegradation of plastic materials. In this case, indicate the state of the compost used in the test report (e.g. mature compost, compost from the hot phase at about 50 °C).

## 8.4 Test

Provide a number of flasks, so that the test includes at least the following.

- a) Three test flasks for the test material (symbol  $F_T$ ).
- b) Three flasks for the blank (symbol  $F_B$ ).
- c) Three flask for checking the inoculum activity using a reference material (symbol  $F_C$ ).

Two flasks for test material, blank, and reference material may be used instead of three for screening purposes. And, if required:

- d) One flask for checking for possible abiotic degradation or non-biological change in the test material such as by hydrolysis (symbol  $F_S$ ). The test solution in  $F_S$  shall be sterilized, for example by autoclaving or by the addition of a suitable inorganic toxic compound to prevent microbial activity. Use, for example, 5 ml/l of a solution containing 10 g/l of mercury(II)chloride ( $\text{HgCl}_2$ ). Add the same amount of the toxic substance during the test if required.
- e) One flask as a negative control (symbol  $F_N$ ) using a non-biodegradable polymeric substance (e.g. polyethylene) in the same form as the test material.

- f) One flask for checking the possible inhibiting effect of the test material on microbial activity (symbol  $F_I$ ). Take care that the ratio of carbon in the test and reference material to nitrogen in the medium is at least about C:N = 40:1. Add nitrogen if required.

Add appropriate amounts of the test medium (6.2) and the inoculum (see 8.3) to the test flasks as indicated in Table 1.

**Table 1 — Final distribution of test and reference materials**

Flask	Test material	Reference material	Inoculum
$F_T$ Test	+	-	+
$F_B$ Blank	-	-	+
$F_C$ Inoculum check	-	+	+
$F_S$ Abiotic degradation check (optional)	+	-	-
$F_I$ Inhibition control (optional)	+	+	+
$F_N$ Negative control (optional)	-	+	+

Connect the flasks to the CO<sub>2</sub>-free-air production system (see Annex A). Incubate at the desired test temperature (see Clause 5) and aerate the flasks for 24 h to purge carbon dioxide from the system. At higher temperatures, prevent any ingress or loss of liquid by means of suitable equipment. Agitate throughout the test with a magnetic stirrer or shaker. If excessive foaming is observed, replace the air purge by overhead aeration with stirring. After the pre-aeration period, connect the air exit of each flask to the carbon dioxide trapping or measuring system.

If a carbon balance is to be run (see Annex C), remove a known sufficient volume of the inoculated test medium from each flask or from additional separate flasks for DOC and biomass determination at the beginning and the end of the incubation period. Consider the removed volume when adjusting the final volume or when calculating the test results.

Add the test material (see 8.1), the reference material and the material for the negative control (see 8.2) to the respective flasks as indicated in Table 1 and start the test by bubbling CO<sub>2</sub>-free air through the flasks to ensure a sufficient quantity of oxygen throughout the test. A rate of 50 ml/min to 100 ml/min is usually suitable.

Measure at regular intervals, depending on the carbon dioxide evolution rate, the amount of carbon dioxide evolved from each bottle, using a suitable and sufficiently accurate method (see Annex B).

When a constant level of carbon dioxide release is attained (plateau phase reached) and no further biodegradation is expected, the test is considered to be completed. The maximum test period is 6 months. In the case of long test durations, special attention shall be paid to the technical system (e.g. tightness of the test vessels and connections, ensuring no carbon dioxide enters and ensuring there are no leakages).

On the last day of the test, measure the pH, acidify all the bottles with 1 ml of concentrated hydrochloric acid in order to decompose the carbonates and bicarbonates, and purge to remove the carbon dioxide. Continue aeration for 24 h and measure the amount of carbon dioxide evolved in each of the series of flasks ( $F_T$ ,  $F_B$ ,  $F_C$ , ...).

## 9 Calculation and expression of results

### 9.1 Calculation

#### 9.1.1 Theoretical amount of carbon dioxide evolved by the test material

Calculate the theoretical amount of carbon dioxide ( $\text{ThCO}_2$ ) evolved, expressed in milligrams, using [Formula \(1\)](#):

$$\text{ThCO}_2 = m \times X_c \times \frac{\text{MM}_{\text{CO}_2}}{\text{MM}_c} \quad (1)$$

where

- $m$  is the mass of test material introduced into the test system, in milligrams;
- $X_c$  is the carbon content of the test material, determined from the chemical formula or calculated from an elemental analysis and expressed as a mass fraction;
- $\text{MM}_{\text{CO}_2}$  is the molecular mass of carbon dioxide;
- $\text{MM}_c$  is the molecular mass of carbon.

Calculate, in the same way, the theoretical amount of carbon dioxide evolved by the reference material and the mixture of test and reference material in flask  $F_I$ .

#### 9.1.2 Percentage biodegradation from $\text{CO}_2$ evolution

Calculate the percentage biodegradation  $D_t$  for the test flasks  $F_T$  from the amount of carbon dioxide evolved for each measurement interval using [Formula \(2\)](#):

$$D_t = \frac{\sum(\text{CO}_2)_T - \sum(\text{CO}_2)_B}{\text{ThCO}_2} \times 100 \quad (2)$$

where

- $\sum(\text{CO}_2)_T$  is the amount of carbon dioxide evolved in flask  $F_T$  between the start of the test and time  $t$ , expressed in milligrams;
- $\sum(\text{CO}_2)_B$  is the amount of carbon dioxide evolved in the blank flask  $F_B$  between the start of the test and time  $t$ , expressed in milligrams;
- $\text{ThCO}_2$  is the theoretical amount of carbon dioxide evolved by the test material, expressed in milligrams.

If possible, calculate the average for the triplicate flasks. In the same way, calculate the percentage biodegradation of the reference material in the inoculum check flask  $F_C$  and, if included, the percentage biodegradation of the mixture of test and reference material in the inhibition control  $F_I$ , the test material in the abiotic degradation control  $F_S$  and the negative control  $F_N$ .

If a carbon balance is to be run, calculate the degree of biodegradation of the test material from the amount of carbon dioxide evolved and the carbon content of the biomass formed during the test (see [Annex C](#)).

### 9.2 Expression and interpretation of results

Compile a table of carbon dioxide released and the percentage biodegradation for each measurement interval and each test flask. For each vessel, plot a curve of the carbon dioxide evolved and a curve of



the percentage biodegradation as a function of time. If comparable results are obtained for the triplicate flasks, a mean curve may be plotted.

The maximum level of biodegradation determined as the mean value of the plateau phase of the biodegradation curve or the highest value, e.g. when the curve decreases or, further on, slowly increases in the plateau phase, characterizes the degree of biodegradation of the test material. If a carbon balance has been determined, the result of this determination characterizes the total degree of biodegradation.

The wettability and the shape of the test material may influence the result obtained, and hence the test procedure may be limited to comparing plastic materials of similar chemical structure.

Information on the toxicity of the test material may be useful in the interpretation of test results showing a low biodegradation.

## 10 Validity of results

The test is considered valid if

- a) the degree of biodegradation of the reference material (inoculum check  $F_C$ ) is  $> 60\%$  at the end of the test;
- b) the difference between the percentage biodegradation of the reference material in the different vessels is less than  $20\%$  at the end of the test;
- c) the amount of carbon dioxide which has evolved from the blank  $F_B$  at the end of the test does not exceed an upper limiting value obtained by experience (this value depends on the amount of inoculum and is, for example, in the case of  $30\text{ mg/l}$  dry matter, about  $90\text{ mg/l}$  as interlaboratory tests have shown).
- d) the amount of carbon dioxide evolved from the three blanks  $F_B$  are within  $20\%$  of the mean at the plateau phase or at the end of the test.

If in flask  $F_I$  (inhibition check, if included) the percentage biodegradation is  $<25\%$  and no significant degradation of the test material is observed, it can be assumed that the test material is inhibitory.

If in flask  $F_S$  (abiotic degradation check, if included) a significant amount ( $>10\%$ ) of evolved carbon dioxide is observed, abiotic degradation processes may have taken place.

If flask  $F_N$  (negative control) was included, no significant amount of evolved carbon dioxide shall be observed ( $>10\%$ ).

If these criteria are not fulfilled, repeat the test using another pre-conditioned or pre-exposed inoculum.

## 11 Test report

The test report shall contain at least the following information:

- a) a reference to this document, i.e. ISO 14852:2018;
- b) all information necessary to identify the test and reference materials, including their TOC,  $\text{ThCO}_2$ , chemical composition and formula (if known), shape, form and amount/concentration in the samples tested;
- c) the main test parameters, including test volume, test medium used, incubation temperature and final pH;
- d) the source and amount of the inoculum used, including details of any pre-exposure and the state of the compost used;
- e) the analytical techniques used, including methods of carbon dioxide detection and TOC, DOC and biomass determination;

- f) all the test results obtained for the test and reference materials (in tabular and graphical form), including the measured accumulated carbon dioxide, the percentage biodegradation values and the respective curves of these parameters against time;
- g) the duration of the lag phase, biodegradation phase and maximum level of degradation, as well as the total test duration;

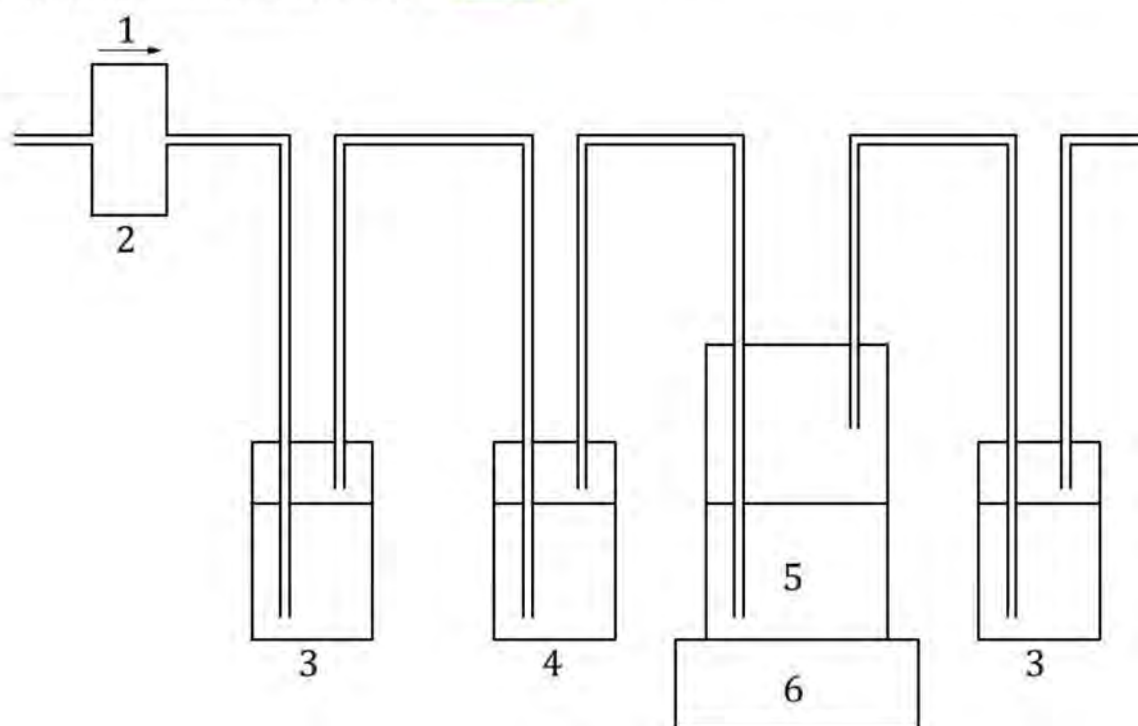
and, optionally, if run or determined:

- h) the results of the abiotic degradation check  $F_S$ , the inhibition control  $F_I$  and the negative control  $F_N$ ;
- i) the results of the carbon balance determination, including, for example,
  - 1) the amount of carbon in the test material oxidized to carbon dioxide,
  - 2) the increase in DOC in the test medium during the incubation period due to water-soluble substances,
  - 3) the increase in organic carbon in the biomass during the test,
  - 4) the carbon content of the residual polymers at the end of the test, and
  - 5) the sum of all the carbon measured, expressed as a percentage of the carbon introduced as the test material;
- j) the colony-forming units (cfu/ml) in the inoculated test mixtures;
- k) any other relevant data (e.g. initial molecular mass of the sample, molecular mass of the residual polymer).

## Annex A (informative)

### Principle of a system for measuring evolved carbon dioxide (example)

The flasks are set up in series as shown in [Figure A.1](#), connected together with gas-impermeable tubing. 50 ml/min to 100 ml/min of CO<sub>2</sub>-free air is passed through the system at a constant low pressure. Count air bubbles or use a suitable flow-rate controller to check the air-flow rate. Use synthetic CO<sub>2</sub>-free air or compressed air. In the latter case, remove the CO<sub>2</sub> by passing the air through a bottle containing dry soda lime or through at least two wash bottles containing e.g. 500 ml of a 10 mol/l aqueous solution of potassium hydroxide (KOH). Use an additional flask containing 100 ml of 0,012 5 mol/l barium hydroxide [Ba(OH)<sub>2</sub>] solution to indicate the presence of any CO<sub>2</sub> in the air by turbidity. An empty flask between the indicator and the following test flask can be used to prevent liquid carryover. CO<sub>2</sub> is produced in the test flask if biodegradation takes place and absorbed in the subsequent absorber bottles for determination as described in [Annex B](#).



#### Key

- 1 compressed air
- 2 flow-rate controller
- 3 carbon dioxide trap (e.g. two wash bottles containing alkali)
- 4 carbon dioxide indicator [Ba(OH)<sub>2</sub>]
- 5 test vessel
- 6 stirrer

**Figure A.1 — Example of system for measuring evolved carbon dioxide**

## Annex B (informative)

### Examples of methods for the determination of evolved carbon dioxide

#### B.1 CO<sub>2</sub> determination by DIC measurement

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

Prepare a solution of 0,05 mol/l NaOH in deionized water. Measure the DIC of this solution and use this blank value when calculating the CO<sub>2</sub> production. Connect in series with the test flask two absorber bottles each containing 100 ml of the NaOH solution. Close the outlet of the last bottle with a small syphon to prevent CO<sub>2</sub> from the air from entering the NaOH solution. On the days of when the CO<sub>2</sub> is determined, remove the absorber bottle next to the test flask and take a sample large enough for DIC measurement (e.g. 10 ml). Replace the bottle by the second and add a new one with freshly prepared NaOH solution. On the last day, after acidification of the test solution, measure the DIC in both bottles.

Calculate the CO<sub>2</sub> produced using [Formula \(B.1\)](#):

$$(\text{CO}_2)_T = \frac{(\text{DIC}_T - \text{DIC}_B) \times 3,67}{10} \quad (\text{B.1})$$

where

- (CO<sub>2</sub>)<sub>T</sub> is the mass of CO<sub>2</sub> evolved, in milligrams;
- DIC<sub>T</sub> is the measured DIC, in milligrams;
- DIC<sub>B</sub> is the blank DIC measured for the NaOH solution, in milligrams;
- 3,67 is the ratio of the molecular mass of CO<sub>2</sub> (44) to the atomic mass of carbon (12);
- 10 is a correction factor to allow for the fact that 100 ml of NaOH solution was used.

#### B.2 Titrimetric method using a barium hydroxide solution

The CO<sub>2</sub> produced reacts with the barium hydroxide [Ba(OH)<sub>2</sub>] and is precipitated as barium carbonate (BaCO<sub>3</sub>) [see reaction (B.2)]. The amount of CO<sub>2</sub> evolved is determined by titrating the remaining Ba(OH)<sub>2</sub> with hydrochloric acid (HCl) [see [Formula \(B.3\)](#)].



Dissolve 4,0 g of Ba(OH)<sub>2</sub> · 8H<sub>2</sub>O in deionized or distilled water and make up to 1 000 ml to obtain a 0,012 5 mol/l solution. It is recommended that a sufficient amount, e.g. 5 l, be prepared at a time when running a series of tests. Filter free of solid material and determine the exact concentration by titration with a standard HCl solution. Use phenolphthalein as indicator or an automatic titrator to determine the end-point. Store as a clear solution in a sealed flask to prevent absorption of CO<sub>2</sub> from the air.

Dilute 50 ml of a 1 mol/l HCl solution (36,5 g/l) to 1 000 ml with deionized or distilled water to obtain a 0,05 mol/l solution.

At the start of the test, dispense exactly 100 ml of Ba(OH)<sub>2</sub> solution into each of three absorber bottles. Depending on the character and amount of the test material, use modifications of the trapping volumes. Periodically remove the bottle nearest the test vessel for titration. This should take place as needed, e.g. when the first bottle is turbid and before any precipitation of BaCO<sub>3</sub> can be observed in the second bottle. 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 bottle, immediately seal it with a plug to avoid CO<sub>2</sub> entering from the air. Move the remaining two bottles one position closer to the test bottle and place at the end of the series a new bottle filled with fresh Ba(OH)<sub>2</sub> solution. Especially if longer test periods are used, determine the exact concentration of the solution. Handle all flasks containing test material, reference material, blank, inhibition control and inoculum control in exactly the same way.

Immediately after removing the bottle, titrate two or three aliquot portions of the Ba(OH)<sub>2</sub> solution with the HCl solution. Note the volumes of the HCl solution needed for neutralization.

Calculate the mass of CO<sub>2</sub> trapped in the absorber bottle using [Formula \(B.4\)](#):

$$m = \left( \frac{2c_B \times V_{B0}}{c_A} - V_A \times \frac{V_{Bt}}{V_{BZ}} \right) \times c_A \times 22 \quad (\text{B.4})$$

where

- $m$  is the mass of CO<sub>2</sub> trapped in the absorber bottle, in milligrams;
- $c_A$  is the exact concentration of the HCl solution, in moles per litre;
- $c_B$  is the exact concentration of the Ba(OH)<sub>2</sub> solution, in moles per litre;
- $V_{B0}$  is the volume of the Ba(OH)<sub>2</sub> solution at the beginning of the test, in millilitres;
- $V_{Bt}$  is the volume of the Ba(OH)<sub>2</sub> solution at time  $t$ , before titration, in millilitres;
- $V_{BZ}$  is the volume of the aliquots of Ba(OH)<sub>2</sub> solution used for titration, in millilitres;
- $V_A$  is the volume of the HCl solution used for titration, in millilitres;
- 22 is half the molecular mass of CO<sub>2</sub>.

When the following conditions apply:

- the volume of the Ba(OH)<sub>2</sub> solution before and after absorption is exactly 100 ml;
- the complete solution is used for the titration ( $V_{B0} = V_{Bt} = V_{BZ}$ );
- the concentration  $c_B$  of the Ba(OH)<sub>2</sub> solution is exactly 0,012 5 mol/l;
- the concentration  $c_A$  of the HCl solution is exactly 0,05 mol/l;

use [Formula \(B.5\)](#)

$$m = 1,1 \times (50 - V_A) \quad (\text{B.5})$$

## Annex C (informative)

### Example of the determination of a carbon balance

#### C.1 Principle

Plastic materials are normally of more complex composition than substances with low molecular masses. The determination of CO<sub>2</sub> evolution or of biochemical oxygen demand (BOD) alone is often not sufficient to characterize and quantify their biodegradation. During biodegradation, new biomass is built up by the microorganisms and a part of the carbon in the test material is transformed to biomass but not biochemically oxidized. Therefore, analytical parameters such as CO<sub>2</sub> evolution and BOD will often not reach 100 % of the respective theoretical values even in the case of complete biodegradation of a test material, and insufficient degradation could falsely be deduced from the test results. The determination of a total carbon balance, as described in this annex, may be helpful in such cases to confirm complete biodegradability. Such a balance is based on the summation of the amounts of carbon deriving from the following measurements: the carbon evolved as carbon dioxide, the carbon produced as new biomass, the carbon transformed into water-soluble organic metabolites, the carbon determined as DOC and the carbon remaining in the undegraded polymer material. The carbon sum is compared with the amount of organic carbon in the test material introduced into the test system.

#### C.2 Test procedure

Determine the amount of carbon dioxide evolved as described in [8.4](#).

Take samples of the inoculated medium at the beginning, before adding the test material, and at the end of the incubation period. Sampling shall be done carefully to obtain representative samples. Pass the samples through a membrane filter or centrifuge them at about 40 000 m·s<sup>-2</sup>.

Determine, for each sample, the amount of biomass on the filter or in the residue using suitable methods, e.g. by protein measurement. Determine or assume the amount of carbon in the biomass and calculate from the difference the increase in organic carbon in the biomass.

Determine, in accordance with ISO 8245, the DOC in the filtrate from each sample, and calculate the increase in organic carbon. If possible, identify the substances forming the DOC in order to confirm the production of water-soluble metabolites.

Determine the amount of carbon in the residual polymers at the end of the test using the whole of each remaining sample. This is normally a difficult procedure and can be done either directly if a polymer-specific analysis is available (see [Annex D](#)) or indirectly. In the first case, extract and weigh the residual polymers and calculate from their known composition the amount of carbon. One possible method for indirect determination is to wash, dry and weigh the residue and determine the total organic carbon (TOC). Then subtract the biomass carbon (see above) from the TOC to obtain the amount of carbon in the residual polymers. Another possibility is to weigh the residue exactly and then treat it, using a suitable method, to destroy the biomass but not the polymers (this needs to be checked in advance). Use, for example, sodium hypochlorite, remove the soluble part and weigh the sample again. Assume that all biomass has been removed and calculate from the mass obtained the polymer content of the residue.

### C.3 Calculation of the carbon balance

Calculate the biochemically oxidized amount of carbon  $C_{CO_2}$  (mg/l) in the test material introduced into the test system (carbon content  $C_{MAT}$ ) from the percentage biodegradation  $D_t$  obtained in the  $CO_2$ -evolution test (see 9.1.2), using [Formula \(C.1\)](#):

$$C_{CO_2} = \frac{C_{MAT} \times D_t}{100} \quad (C.1)$$

Calculate the increase in biomass carbon in the test flasks containing test material  $C_{BIO}$  (mg/l) by comparing the biomass at the beginning and the end of the incubation test period, considering the measured or estimated amount of carbon in the biomass  $C_{B(start)}$  and  $C_{B(end)}$ , as shown in [Formula \(C.2\)](#):

$$C_{BIO} = C_{B(end)} - C_{B(start)} \quad (C.2)$$

Determine the increase in DOC during the incubation period  $C_{DOC}$  (mg/l) by comparing the DOC concentrations at the beginning  $DOC_{(start)}$  and the end  $DOC_{(end)}$  as shown in [Formula \(C.3\)](#):

$$C_{DOC} = DOC_{end} - DOC_{start} \quad (C.3)$$

Determine the amount of organic carbon in the residual polymers at the end of the test  $C_{POL}$ .

Calculate the different amounts of transformed carbon as percentages of the introduced carbon  $C_{MAT}$  and sum to obtain the calculated carbon  $C_{CALC}$  (%) as shown in [Formula \(C.4\)](#):

$$C_{CALC} = C_{CO_2} + C_{BIO} + C_{DOC} + C_{POL} \quad (C.4)$$

### C.4 Example: Carbon balance of poly(*b*-hydroxybutyrate)<sup>1)</sup>

Input of test material:  $C_{MAT} = 600 \text{ mg/l} = 334,8 \text{ mg/l of carbon}$

Degree of biodegradation:  $D_t = 78 \%$

	$C_{B(start)}$	$C_{B(end)}$	$C_{BIO}$	$DOC_{(start)}$	$DOC_{(end)}$	DOC	$C_{CO_2}$
mg/l	3,2	61,0	57,8	2,0	22,0	20,0	261
% of $C_{MAT}$			17,2			6,0	78

Calculated carbon balance:  $C_{CALC} = 78 \% + 17 \% + 6 \% = 101 \% \text{ of } C_{MAT}$

1) Taken from Reference [9].

## Annex D (informative)

### Example of a determination of the amount of water insoluble polymer remaining at the end of a biodegradation test and the molecular mass of the polymer

It may be helpful to use a procedure for measuring the amount and the molecular mass of polymers remaining at the end of a biodegradation study. The following method or another appropriate one can be used to analyse water-insoluble polymers that dissolve in organic solvents which are not miscible with water.

- a) Transfer the test mixture to a funnel, add a suitable organic solvent and shake for 10 min to 20 min to extract the remaining polymers. Separate the organic solvent layer from the aqueous layer. Add fresh solvent and repeat the procedure.
- b) Combine the organic extracts and evaporate the solvent until dry. Dissolve the solid sample in an appropriate volume of a suitable eluate.
- c) Using a microsyringe, inject a suitable amount into a high-performance liquid chromatography (HPLC) apparatus having a column packed with a size-exclusion chromatographic gel. Start the analysis and record the chromatogram.
- d) Determine the amount of polymer present using a calibration curve.
- e) Determine the molecular mass of the polymer by injecting into the chromatograph the same polymer, or polymers of structure similar to that of the test polymers whose molecular masses are known. The relationship between the retention time and the molecular mass is obtained from the resulting chromatogram. Calculate the molecular mass using this relationship.

The absolute molecular mass of the test polymer can also be determined by HPLC with a combined low-angle laser-light scattering (LALLS) and differential refractive index (RI) detector.



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