Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium — Method by measuring the oxygen demand in a closed respirometer
National foreword
This British Standard is the official English language version of EN ISO 14851:2004. It is identical with ISO 14851:1999.

The UK participation in its preparation was entrusted to Technical Committee PRI/21, Testing of plastics, which has the responsibility to:

— aid enquirers to understand the text;
— present to the responsible international/European committee any enquiries on the interpretation, or proposals for change, and keep the UK interests informed;
— monitor related international and European developments and promulgate them in the UK.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Summary of pages
This document comprises a front cover, an inside front cover, the EN ISO title page, the EN ISO foreword page, the ISO title page, page ii, pages 1 to 19, and a back cover.

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Amendments issued since publication

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Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium — Method by measuring the oxygen demand in a closed respirometer

(ISO 14851:1999)
Foreword

The text of ISO 14851:1999 has been prepared by Technical Committee ISO/TC 61, Plastics, of the International Organization for Standardization (ISO) and has been taken over as EN ISO 14851:2004 by Technical Committee CEN/TC 249, Plastics, the Secretariat of which is held by IBN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by January 2005, and conflicting national standards shall be withdrawn at the latest by January 2005.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: of Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

Endorsement notice

The text of ISO 14851:1999 has been approved by CEN as EN ISO 14851:2004 without any modifications.

NOTE Normative references to International Standards are listed in Annex ZA (normative).
INTERNATIONAL STANDARDB

Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium — Method by measuring the oxygen demand in a closed respirometer

Évaluation de la biodégradabilité aérobie ultime des matériaux plastiques en milieu aqueux — Méthode par détermination de la demande en oxygène dans un respiromètre fermé
Foreword

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

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

International Standard ISO 14851 was prepared by Technical Committee ISO/TC 61, Plastics, Subcommittee SC 5, Physical-chemical properties.

Annex A to Annex G of this International Standard are for information only.

Introduction

With the increasing use of plastics, their recovery and disposal have become a major issue. As a first priority, recovery should be promoted. Complete recovery of plastics, however, is difficult. For example, plastic litter, which comes mainly from consumers, is difficult to recover completely. Additional examples of plastics which are difficult to recover are fishing tackle, agricultural mulches and water-soluble polymers. These plastic materials tend to leak from closed waste-management cycles into the environment. 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 biodegradability in natural environments.
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 International Standard specifies a method, by measuring the oxygen demand in a closed respirometer, for the determination of the degree of aerobic biodegradability of plastic materials, including those containing formulation additives. The test material is exposed in an aqueous medium under laboratory conditions to an inoculum from activated sludge, compost or soil.

If an unadapted activated sludge is used as the inoculum, the test simulates the biodegradation processes which occur in a natural aqueous 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 International Standard do not necessarily correspond to the optimum conditions allowing maximum biodegradation to occur, but the standard is designed to determine the potential biodegradability of plastic materials or give an indication of their biodegradability in natural environments.

The method enables the assessment of the biodegradability to be improved by calculating a carbon balance (optional, see Annex E).

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 e.g. ISO 8192[3]). 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 standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.


ISO 9408:—1), Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in an aqueous medium by determination of oxygen demand in a closed respirometer.


3 Definitions

For the purposes of this International Standard, the following definitions apply:

3.1 ultimate aerobic biodegradation
the 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
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
the 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 biochemical oxygen demand
BOD
the mass concentration of the dissolved oxygen consumed under specified conditions by the aerobic biological oxidation of a chemical compound or organic matter in water, expressed as milligrams of oxygen uptake per milligram or gram of test compound

3.5 theoretical oxygen demand
ThOD
the theoretical maximum amount of oxygen required to oxidize a chemical compound completely, calculated from the molecular formula, expressed as milligrams of oxygen uptake per milligram or gram of test compound

3.6 total organic carbon
TOC
all the carbon present in organic matter which is dissolved or suspended in water

3.7 dissolved organic carbon
DOC
d that part of the organic carbon in water which cannot be removed by specified phase separation, for example by centrifugation at 40 000 m·s⁻² for 15 min or by membrane filtration using membranes with pores of 0,2 µm to 0,45 µm diameter

3.8 lag phase
the time, measured in days, 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

3.9 maximum level of biodegradation
the degree of biodegradation, measured in per cent, of a chemical compound or organic matter in a test, above which no further biodegradation takes place during the test
3.10 biodegradation phase
the time, measured in days, from the end of the lag phase of a test until about 90% of the maximum level of biodegradation has been reached

3.11 plateau phase
the time, measured in days, from the end of the biodegradation phase until the end of a test

3.12 pre-exposure
the 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
the 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

4 Principle
The biodegradability of a plastic material is determined using aerobic 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 2000 mg/l of organic carbon, and activated sludge or a suspension of active soil or compost as the inoculum. The mixture is stirred in closed flasks in a respirometer for a period not exceeding 6 months. The carbon dioxide evolved is absorbed in a suitable absorber in the headspace of the flasks. The consumption of oxygen (BOD) is determined, for example by measuring the amount of oxygen required to maintain a constant volume of gas in the respirometer flasks, or by measuring the change in volume or pressure (or a combination of the two) either automatically or manually. An example of a respirometer is given in Annex C. Alternatively, the two-phase closed-bottle version described in ISO 10708[4] may be used (see Annex D).

The level of biodegradation is determined by comparing the BOD with the theoretical amount (ThOD) and expressed in per cent. The influence of possible nitrification processes on the BOD have to be considered. 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 E).

Unlike ISO 9408, which is used for a variety of organic compounds, this International Standard is specially designed for the determination of the biodegradability 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 ± 1 °C, or at any other appropriate temperature depending on the inoculum used and the environment to be assessed.

NOTE With a compost inoculum, higher temperatures may be appropriate.
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 simulating a natural environment use the standard test medium (6.2.1). 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 in water (6.1) and make up to 1 000 ml.

\[
\begin{align*}
\text{anhydrous potassium dihydrogen phosphate (KH}_2\text{PO}_4) & \quad 8,5 \text{ g} \\
\text{anhydrous dipotassium hydrogen phosphate (K}_2\text{HPO}_4) & \quad 21,75 \text{ g} \\
\text{disodium hydrogen phosphate dihydrate (Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O)} & \quad 33,4 \text{ g} \\
\text{ammonium chloride (NH}_4\text{Cl)} & \quad 0,5 \text{ g}
\end{align*}
\]

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

NOTE 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 (MgSO\(_4\) \cdot 7H\(_2\)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 (CaCl\(_2\) \cdot 2H\(_2\)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 (FeCl\(_3\) \cdot 6H\(_2\)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 litre of test medium, add, to about 500 ml of water (6.1),

- 10 ml of solution A;
- 1 ml of each of solutions B to D.

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

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 test-material concentrations are used, increase the nitrogen content to keep the C:N ratio at about 40:1.
6.2.2.1 Solution A
Dissolve

- anhydrous potassium dihydrogen phosphate (KH₂PO₄) 37,5 g
- disodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O) 87,3 g
- ammonium chloride (NH₄Cl) 2,0 g

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

6.2.2.2 Solution B
Dissolve 22,5 g of magnesium sulfate heptahydrate (MgSO₄·7H₂O) in water (6.1) and make up to 1 000 ml.

6.2.2.3 Solution C
Dissolve 36,4 g of calcium chloride dihydrate (CaCl₂·2H₂O) in water (6.1) and make up to 1 000 ml.

6.2.2.4 Solution D
Dissolve 0,25 g of iron(III) chloride hexahydrate (FeCl₃·6H₂O) in water (6.1) and make up to 1 000 ml (see second paragraph of 6.2.1.4).

6.2.2.5 Solution E (trace-element solution, optional)
Dissolve in 10 ml of aqueous HCl solution (25 %, 7,7 mol/l), in the following sequence:
- 70 mg of ZnCl₂
- 100 mg of MnCl₂·4H₂O
- 6 mg of H₃BO₃
- 190 mg of CoCl₂·6H₂O
- 3 mg of CuCl₂·2H₂O
- 240 mg of NiCl₂·6H₂O
- 36 mg of Na₂MoO₄·2H₂O
- 33 mg of Na₂WO₄ and
- 26 mg of Na₂SeO₃·5H₂O

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

6.2.2.6 Solution F (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
- 3 mg of cyanocobalamine
- 2,0 mg of folic acid
- 5,0 mg of riboflavin
- 5,0 mg of DL-thioctic acid
- 1,0 mg of thiamine dichloride

or use a solution of 15 mg of yeast extract in 100 ml of water (6.1). Filter the solution for sterilization using membrane filters (see 7.4).

NOTE 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.

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

- 100 ml of solution A;
- 1 ml of each of solutions B to D and, optionally, E and F.

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

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

6.3 Pyrophosphate solution
Dissolve 2,66 g of anhydrous sodium pyrophosphate (Na₄P₂O₇) in water (6.1) and make up to 1 000 ml.

6.4 Carbon dioxide absorber, preferably soda lime pellets or another suitable absorbant.
7 Apparatus

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

Required is usual laboratory equipment, plus the following:

7.1 Closed respirometer, including test vessels (glass flasks) fitted with stirrers and all other necessary equipment, and located in a constant-temperature room or in a thermostatted apparatus (e.g. water-bath). For an example, see Annex C.

NOTE Any respirometer able to determine with sufficient accuracy the biochemical oxygen demand is suitable, preferably an apparatus which measures and replaces automatically and continuously the oxygen consumed so that no oxygen deficiency and no inhibition of the microbial activity occurs during the degradation process. Instead of an ordinary respirometer, the two-phase closed-bottle version may be used (see Annex D).

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

7.3 Analytical equipment for measuring nitrate and nitrite concentrations

NOTE A qualitative test is recommended first to decide if any nitrification has occurred. If there is evidence of nitrate/nitrite in the medium, a quantitative determination using a suitable method (for example ion chromatography) is required.

7.4 Centrifuge, or filtration device with membrane filters (0,45 μm pore size) which neither adsorb nor release organic carbon significantly.

7.5 Analytical balance (usual laboratory equipment).

7.6 pH meter (usual laboratory equipment).

8 Procedure

8.1 Test material

The test material shall be of known mass and contain sufficient carbon to yield a BOD that can be adequately measured by the respirometer used. Calculate from the chemical formula or determine by elemental analysis the ThOD (see Annex A) and the TOC (using e.g. ISO 8245). Use a test-material concentration of at least 100 mg/l, corresponding to a ThOD of about 170 mg/l or a TOC of about 60 mg/l. Use lower concentrations only if the sensitivity of the respirometer is adequate. The maximum amount of test material is limited by the oxygen supply to the respirometer and the test medium used. When using the optimized test medium (6.2.2), 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 amount of nitrogen in the test medium.

NOTE 1 If biodegradation processes in natural environments are to be simulated, the use of the standard medium and a test-material concentration of 100 mg/l are recommended.

NOTE 2 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 biodegradability. 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.

Optional, 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 e.g. ASTM D 3536-91[1] or any other applicable standard method). Preferably, plastic materials without additives such as plasticizers should be tested. When the material does contain such additives, information on their biodegradability will be needed to assess the biodegradability of the polymeric material itself.

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

8.2 Reference material

Use aniline and/or 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

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 in which a broad range of plastic materials has to be tested. 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 system is to be determined, collect the inoculum from that environment.

The inoculum can be prepared from the sources described in 8.3.1 and 8.3.2, 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 note to Clause 5).

NOTE It may be useful to determine the colony-forming units (cfu) of the inoculum used. The test mixture should preferably contain about $10^6$ cfu/ml.

8.3.1 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[5]). 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.

NOTE 1 When biodegradation processes in a natural environment are to be simulated or when a carbon balance determination (see Annex E) 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.

NOTE 2 An inoculum may be pre-conditioned, but normally no pre-exposed inoculum should be used, especially in the case of standard tests simulating biodegradation behaviour in natural environments. 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 = $\chi \%$, 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).

8.3.2 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).

When a higher inoculum concentration is needed, suspend a higher amount of soil or compost in the test medium and dilute to an appropriate concentration for inoculation.
8.4 Test

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

a) Two test flasks for the test material (symbol FT).

b) Two flasks for the blank (symbol FB).

c) One flask for checking the inoculum activity using a reference material (symbol FC).

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 FS). The test solution in FS 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 (HgCl₂). Add the same amount of the toxic substance during the test if required.

e) One flask as a negative control (symbol FN) 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 FI). 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.

Measure the pH in the flasks and adjust to 7 if necessary. Add carbon dioxide absorber (6.4) to the absorber compartments of the respirometer (see Annex C). 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. If a carbon balance is to be run (see Annex E), 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.

Place the flasks in a constant-temperature environment (see Clause 5) and allow all vessels to reach the desired temperature. Make any necessary connections, seal the flasks, place them in the respirometer and start the stirrer. Take the necessary readings on the manometers (if manual) and verify that the recorder of oxygen consumption is functioning properly (automatic respirometer). As an alternative, the two-phase closed-bottle version described in Annex D may be used.

When a constant level of BOD 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 must be paid to the technical system (e.g. tightness of the test vessels and connections).

At the end of the test, measure the pH and determine the concentrations of nitrate and nitrite immediately in flasks FT (see note), or take suitably preserved samples. Use the values to correct the calculated degree of biodegradation for nitrification (see Annex E).

NOTE Allylthiourea can only inhibit nitrification during short incubation periods, as it is biodegradable. Therefore addition of allylthiourea to prevent nitrification is not recommended. Experience shows, however, that with low inoculum concentrations [about 1 % (V/V)] nitrification will not occur, even during long incubation periods, when no inhibitor is used.

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<th>Flask</th>
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<th>Reference material</th>
<th>Inoculum</th>
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<td>FT</td>
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<td>-</td>
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9 Calculation and expression of results

9.1 Calculation
Read the oxygen consumption values for each flask, using the method given by the manufacturer for the appropriate type of respirometer. Calculate the specific biochemical oxygen demand \( BOD_s \) of the test material as the difference between oxygen consumption in the test flasks \( F_T \) and the blanks \( F_B \) divided by the concentration of the test material, using equation (1):

\[
BOD_s = \frac{BOD_t - BOD_{Br}}{\rho_{TC}}
\]

where
- \( BOD_s \) is the specific BOD, in milligrams per gram of test material;
- \( BOD_t \) is the BOD of the flasks \( F_T \) containing test material at time \( t \), in milligrams per litre;
- \( BOD_{Br} \) is the BOD of the blank \( F_B \) at time \( t \), in milligrams per litre;
- \( \rho_{TC} \) is the concentration of the test material in the reaction mixture of flask \( F_T \), in milligrams per litre.

Calculate the percentage biodegradation \( D_t \) as the ratio of the specific biochemical oxygen demand to the theoretical oxygen demand (ThOD, in milligrams per gram of test material), using equation (2):

\[
D_t = \frac{BOD_s}{ThOD} \times 100
\]

Calculate in the same way the BOD and percentage biodegradation of the reference material \( F_C \) and, if included, the abiotic degradation check \( F_S \), the inhibition control \( F_I \) and the negative control \( F_N \).

NOTE For calculation of the ThOD, see Annex A. If significant concentrations of nitrite and nitrate are determined, consider the oxygen demand due to nitrification (see Annex B). If a carbon balance is to be calculated, use information given in Annex E.

9.2 Expression and interpretation of results
Compile a table of the BOD values measured and the percentages of biodegradation for each measurement interval and each test flask. For each vessel, plot a BOD curve and a biodegradation curve in per cent as a function of time. If comparable results are obtained for the duplicate 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 biodegradability.

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 BOD of 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 mg/l dry matter, about 60 mg/l as interlaboratory tests have shown).
If in flask F₁ (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 Fₛ (abiotic degradation check, if included) a significant amount (>10 %) of BOD is observed, abiotic degradation processes may have taken place.

If flask Fₕ (negative control) was included, no significant amount of BOD shall be observed.

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 International Standard;

b) all information necessary to identify the test and reference materials, including their TOC, ThOD, 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 the principle of the respirometer and the TOC and nitrate/nitrite determinations;

f) all the test results obtained for the test and reference materials (in tabular and graphical form), including the measured BOD, the percentage biodegradation values, the respective curves of these parameters against time and the nitrate/nitrite concentrations;

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 Fₛ, the inhibition control F₁ and the negative control Fₕ;

i) the results of the carbon balance determination, including for example:

1) the amount of carbon in the test material oxidized to carbon dioxide, estimated from the degree of biodegradation based on the BOD,

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,

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)
Theoretical oxygen demand (ThOD)

A.1 Calculation of the ThOD

The theoretical oxygen demand (ThOD) of a substance $C_cH_hCl_{cl}N_nS_sP_pNa_{na}O_o$ of relative molecular mass $M_r$ can be calculated, if the elemental composition is known or can be determined by elemental analysis, using the equation

$$\text{ThOD} = \frac{16[2c + 0,5(h - cl - 3n) + 3s + 2,5p + 0,5na - o]}{M_r}$$

This calculation assumes that carbon is converted to CO$_2$, hydrogen to H$_2$O, phosphorus to P$_2$O$_5$, sulfur to an oxidation state of +6 and halogens eliminated as hydrogen halides. The oxidation of N, P and S has to be checked by analysis. The calculation also assumes that nitrogen is released as ammonium. For the influence of nitrification, see Annex B.

Express the ThOD in milligrams per gram of substance or in milligrams per milligram of substance.

A.2 Example: Poly(β-hydroxybutyric acid) (PHB)

Summary formula$^2$: $C_4H_6O_2$, $c = 4$, $h = 6$, $o = 2$; relative molecular mass $M_r = 86$.

$$\text{ThOD} = \frac{16[2 \times 4 + 0,5 \times 6 - 2]}{86}$$

$$\text{ThOD} = 1,674,4 \text{mg/mg PHB} = 1 \, 674,4 \text{mg/g PHB}$$

A.3 Example: Blend of polyethylene/starch/glycerol

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>ThOD mg/g</th>
<th>Amount of component</th>
<th>ThOD mg/flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethylene</td>
<td>$C_2H_4n$</td>
<td>3 400</td>
<td>50</td>
<td>1 700</td>
</tr>
<tr>
<td>Starch</td>
<td>$C_6H_{10}O_5n$</td>
<td>1 190</td>
<td>40</td>
<td>476</td>
</tr>
<tr>
<td>Glycerol</td>
<td>$C_3H_8O_3$</td>
<td>1 200</td>
<td>10</td>
<td>120</td>
</tr>
<tr>
<td>Total blend</td>
<td></td>
<td>1 000</td>
<td>100</td>
<td>2 296</td>
</tr>
</tbody>
</table>

Annex B (informative)
Correction of BOD values for interference by nitrification

B.1 Influence of nitrification

BOD values can be influenced by nitrification. They have to be corrected if serious errors are to be avoided in the calculation of degrees of biodegradation based on the oxidation of the carbon in a nitrogen-containing test material. Errors in the case of nitrogen-free substances are normally negligible, because the oxidation of the ammonium in the medium is taken into account by the subtraction of the blank.

Ammonium salts and nitrogen-containing test compounds can be oxidized to nitrite or nitrate during the incubation period of a biodegradation test. Since the reactions are sequential (carried out by different bacterial species), it is possible for the nitrite concentration to increase or decrease. In the latter case, an equivalent concentration of nitrate is formed. The chemical reactions follow equations (B.1) to (B.3):

$$2\text{NH}_4\text{Cl} + 3\text{O}_2 = 2\text{HNO}_2 + 2\text{HCl} + 2\text{H}_2\text{O}$$  \hspace{1cm} (B.1)

$$2\text{HNO}_2 + \text{O}_2 = 2\text{HNO}_3$$  \hspace{1cm} (B.2)

Overall:

$$2\text{NH}_4\text{Cl} + 4\text{O}_2 = 2\text{HNO}_3 + 2\text{HCl} + 2\text{H}_2\text{O}$$  \hspace{1cm} (B.3)

$^2$ PHB is a polymer consisting of the β-hydroxybutyrate monomer. For polymerization (ester formation), water is removed, so that the summary formula for PHB is equivalent to that of the monomer minus one H$_2$O, which is eliminated in the chemical reaction.
From these equations, it can be concluded that
— for the oxidation of 2 moles (28 g) of ammonia nitrogen (added as NH₄Cl with the inorganic medium) to nitrite, 3 moles (96 g) of oxygen (BOD₉₂) are needed, resulting in a factor of 3,43 (96/28) mg of oxygen demand per mg of nitrogen;
— for the oxidation of 2 moles (28 g) of ammonia nitrogen to nitrate, 4 moles (128 g) of oxygen (BOD₉₃) are needed, resulting in a factor of 4,57 (128/28) mg of oxygen demand per mg of nitrogen.

The amount of nitrification can be determined by measuring the nitrate and nitrite concentrations at the end of the test in the medium in flasks Fₚ. A qualitative test is recommended first to decide if any nitrification has occurred. If there is evidence of nitrate or nitrite, a quantitative determination is required.

The part of the BOD deriving from nitrogen oxidation at the end of the test, BODₙ, is calculated, in milligrams per litre, using equation (B.4):

\[ \text{BOD}_n = (\rho_{NO_3} \times 4.57) + (\rho_{NO_2} \times 3.43) \]  
(B.4)

where
- \( \rho_{NO_3} \) is the measured concentration of nitrate nitrogen in flasks Fₚ at the end of the test, in milligrams per litre;
- \( \rho_{NO_2} \) is the measured concentration of nitrite nitrogen in flasks Fₚ at the end of the test, in milligrams per litre;
- 4.57 is the factor for the oxygen demand for the formation of nitrate;
- 3.43 is the factor for the oxygen demand for the formation of nitrite.

The part of the BOD deriving from carbon oxidation at the end of the test, BODₙ, is calculated, in milligrams per litre, using equation (B.5):

\[ \text{BOD}_c = \text{BOD}_G - \text{BOD}_n - \text{BOD}_B \]  
(B.5)

where
- \( \text{BOD}_G \) is the measured BOD of flasks Fₚ at the end of the test, in milligrams per litre;
- \( \text{BOD}_B \) is the BOD of the blank Fₚ at the end of the test, in milligrams per litre.

BODₙ corresponds to BODₚ and is used for calculating BODₛ and \( D_t \) [see equations (1) and (2) in 9.1].

### B.2 Example

Test substance \( p \)-aminobenzoic acid 2-ethylhexyl ester at a concentration of 100 mg/l in Fₚ.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ThOD</td>
<td>239 mg/l</td>
</tr>
<tr>
<td>Measured BODₚ at the end of the test</td>
<td>199 mg/l</td>
</tr>
<tr>
<td>Measured blank BODₚ</td>
<td>8 mg/l</td>
</tr>
<tr>
<td>( D_t ) without correction for nitrification</td>
<td>80 %</td>
</tr>
<tr>
<td>Nitrate at the end of the test 15 mg/l</td>
<td>( \rho_{NO_3} = 3.5 \text{ mg/l} )</td>
</tr>
<tr>
<td>Nitrite at the end of the test 1 mg/l</td>
<td>( \rho_{NO_2} = 0.3 \text{ mg/l} )</td>
</tr>
<tr>
<td>BODₙ at the end of the test</td>
<td>17 mg/l</td>
</tr>
<tr>
<td>BODₙ</td>
<td>174 mg/l</td>
</tr>
<tr>
<td>( D_t ) corrected for nitrification</td>
<td>73 %</td>
</tr>
</tbody>
</table>
Annex C (informative)
Principle of a closed manometric respirometer

The respirometer is set up in a temperature-controlled environment (e.g. a water bath) and contains test vessels each fitted with a magnetic stirrer rod and a container in the headspace for a CO₂ absorber, a coulometric oxygen production unit, a manometer, magnetic stirrers for each vessel, and an external monitoring device and recorder (printer, plotter or computer). The test vessels are filled to about one-third of their volume with the test mixture. Continuous stirring guarantees an equilibrium of oxygen between the aqueous and the gaseous phase. If biodegradation takes place, the microorganisms consume oxygen and produce carbon dioxide which is totally absorbed. The total pressure in the vessels decreases. The pressure drop is detected by the manometer and used to initiate the electrolytic generation of oxygen. When the original pressure is re-established, electrolysis is stopped and the quantity of electricity used, which is proportional to the oxygen consumption, is continuously measured and utilized to indicate the oxygen consumption, in mg/l BOD, on the recorder.

Figure C.1 — Schematic drawing of a manometric respirometer
Annex D (informative)
Two-phase closed-bottle version of the respirometric test

D.1 Principle
This version can be used as an alternative, e.g. if no respirometer is available. The inoculated medium and
the test and reference materials are shaken or stirred at 20 °C to 25 °C in closed bottles containing known
volumes of aqueous medium and air to assure steady-state oxygen partitioning between the aqueous and
the gas phase. The biodegradation is followed by means of regular measurements of the dissolved-oxygen
concentration in the aqueous phase. The total oxygen uptake in the test flasks is calculated from the
difference in the measured dissolved-oxygen concentrations in the blank and test flasks divided by the
oxygen saturation value under normal conditions and multiplied by the total oxygen content originally
present in the aqueous and gas phases. Biodegradability is calculated as the total oxygen uptake divided
by the theoretical oxygen demand (ThOD) and expressed as a percentage.

D.2 Special apparatus
D.2.1 Incubation bottles: gas-tight bottles, e.g. narrow-necked flasks with volumes of 200 ml to 300 ml
and with suitable stoppers (e.g. ground-glass stoppers, butyl-rubber stoppers or screw caps), providing
shielding from the light (e.g. made of brown glass). Stopper clamps are recommended. Mark each bottle
with waterproof markings. If oxygen electrodes with mounted stirrers are not used, provide the bottles with
a magnetic stirrer with a PTFE-coated stirrer bar. Either use bottles of standard volume such that the
standard deviation from the mean volume for the batch of bottles is less than 1 ml or measure and record
the volumes of individual, numbered bottles with an accuracy of 1 ml. Carefully grease the stoppers of the
bottles with inert silicone grease to assure proper closing and easy removal.

D.2.2 Oxygen electrode, preferably with a mounted stirrer, capable of measuring in the range 0
to 10 mg/l to an accuracy of 1 %. The steady state should be reached within about 1.5 min. Mount the
electrode e.g. in an inert stopper which makes a leakproof fit in the ground-glass neck of the incubation
bottle or use a technique to measure the oxygen concentration in a circular bypass.

D.2.3 Magnetic stirrer or shaking device

D.3 Procedure
Set up the incubation bottles as described in 8.4, but with three of each of bottles F_T, F_B and F_C. Place a
stirrer bar in each bottle if they are to be stirred rather than shaken. Prepare sufficient test medium,
preferably the standard test medium (6.2.1), to perform the complete test. To guarantee a sufficient
supply of nutrients, increase the amount of ammonium chloride in solution A (6.2.1.1) by a factor of three
to 1.5 g/l. Inoculate the medium in accordance with 8.3, preferably using activated sludge at a
concentration of 30 mg/l of suspended solids, mix well and add the mixture to the bottles. Add a volume
equal to two-thirds of the volume of the bottle (e.g. 200 ml of liquid to 300 ml bottles). Place the bottles on
the shaking device or stir them, and incubate at 20 °C to 25 °C for one week. During this time, the bacteria
will use their reserve material and the inoculum will be stabilized. Then aerate the bottles with the help
of water-saturated compressed air and an air diffuser for about 15 min. Measure the initial oxygen
concentration. Add to the relevant bottles the test or reference material as specified in 8.1 and 8.2. The
maximum test material concentration in this test should correspond to 150 mg/l ThOD which corresponds
to about 90 mg/l TOC. Stopper all bottles tightly and continue the incubation.

After incubation periods of one week, or more frequently, determine the concentration of dissolved oxygen
in each bottle. Maintain the bottles at the incubation temperature kept at a constant value (± 0.5 °C)
during the measurements. Calibrate accurately the oxygen electrode in accordance with the
manufacturer’s instructions. For oxygen measurements, take each bottle successively and shake vigorously
by hand for about 30 s. Place the bottle on a stirrer, without stirring. Remove the stopper and immediately
put the oxygen electrode through the neck of the bottle so that the electrode stopper effectively closes the
bottle and the electrode tip is well below the liquid surface. Start the stirrer at such a speed that oxygen
measurements are possible but no vortex is formed. Use the same stirrer speed throughout a set of
measurements and electrode calibrations. Record the oxygen value when it is stable, which should be
reached in about 2 min. Use for the calculation of the test result only those bottles in which the
oxygen concentration was > 1.5 mg/l. Alternatively, other appropriate techniques for measuring the
dissolved-oxygen concentration in the aqueous phase can be used, such as an oxygen electrode positioned
in a closed circular bypass.
Then measure the pH in each bottle and record it. If the pH is below 6.0, adjust to about 7.5 with 0.1 mol/l to 0.5 mol/l sodium hydroxide solution. If the pH is above 8.0, adjust to about 7.5 with 0.1 mol/l to 0.5 mol/l hydrochloric acid solution. Finally, aerate the medium in each bottle with an air-diffuser for 15 min and measure the oxygen concentration again as described previously. Re-stopper the bottles, put them on the shaker and continue the incubation. Correct the measured BOD values at the end of the test for nitrification (see 8.4 and Annex B).

D.4 Calculation of results

Determine the relative oxygen uptake $U_{r}$ measured in the aqueous phase in each bottle, using equation (D.1)

$$U_{r} = \frac{C_{Bt} - C_{t}}{C_{s}}$$

where

- $C_{Bt}$ is the mean value, in milligrams per litre, of the dissolved-oxygen concentration in the blank bottles after incubation, at time $t$;
- $C_{t}$ is the dissolved-oxygen concentration, in milligrams per litre, in each test bottle after incubation, at time $t$;
- $C_{s}$ is the saturation value, in milligrams per litre, for dissolved oxygen.

Use as the saturation value $C_{s}$ a mean value measured after each aeration or re-aeration of the blank and test bottles. The theoretical value at standard atmospheric pressure (1 013 hPa) and 20 °C is 9.08 mg/l.

Determine, using equation (D.2), the total oxygen capacity $O_{c}$ (mg/bottle) of a bottle from the maximum oxygen content in the gas phase and the oxygen content in the aqueous phase at standard pressure and 20 °C

$$O_{c} = (0.28 \times V_{g}) + (0.009 \times V_{l})$$

where

- 0.28 is the oxygen content of normal air, in milligrams per millilitre;
- $V_{g}$ is the volume of gas in an incubation bottle, in millilitres;
- 0.009 is the oxygen content of saturated water, in milligrams per millilitre;
- $V_{l}$ is the volume of liquid in an incubation bottle, in millilitres.

Normally, $V_{l}$ will be constant in a test series, except if samples for analysis are taken, but $V_{g}$ may differ depending on the test bottles used. If the differences between the individual bottles are small, a constant $O_{c}$ may be used. If the differences are significant (e.g. > 2 ml for bottles with a volume of 200 ml), $O_{c}$ should be calculated for each bottle. If $V_{l}$ decreases according to the volume of the sample taken, $V_{g}$ increases proportionately.

Then calculate the oxygen uptake BOD (mg/bottle) from equation (D.3):

$$BOD = U_{r} \times O_{c}$$

Sum the oxygen uptake $\Sigma BOD$ (mg/bottle) for all ($n$) incubation periods using equation (D.4) to obtain the BOD at the end of the test.

$$\Sigma BOD = BOD_{1} + BOD_{2} + \ldots + BOD_{n}$$

Finally, calculate the percentage biodegradation as described in 9.1.

Use appropriate formulae to determine the abiotic elimination, as well as the biodegradation of the reference material and the inhibition control.
Annex E (informative)
Example of the determination of a carbon balance

E.1 Principle
Plastic materials are normally of more complex composition than substances with low molecular masses. The determination of CO₂ evolution or of BOD alone is often not sufficient to characterize and quantify their biodegradability. 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 CO₂ 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.

E.2 Test procedure
Determine the BOD 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 must be done carefully to obtain representative samples. Pass the samples through a membrane filter or centrifuge them at about 40 000 m·s⁻².
Determine, for each sample, the amount of biomass in the filtrate or centrifugate 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 F) 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 has to be checked in advance). Use e.g. 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.

E.3 Calculation of the carbon balance
Calculate the biochemically oxidized amount of carbon C_{BOD} (mg/l) in the test material introduced into the test system (carbon content C_{MAT}) from the percentage biodegradation D_t obtained in the respirometric test (see 9.1), using equation (E.1):

\[ C_{BOD} = \frac{C_{MAT} \times D_t}{100} \]  

(E.1)
Calculate the increase in biomass carbon in the test flasks containing test material $C_{\text{BIO}}$ (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 $C_{\text{B:start}}$ and $C_{\text{B:end}}$, as shown in equation (E.2):

$$C_{\text{BIO}} = DOC_{\text{B:end}} - DOC_{\text{B:start}}$$  \hspace{1cm} (E.2)

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

$$C_{\text{DOC}} = DOC_{\text{end}} - DOC_{\text{start}}$$  \hspace{1cm} (E.3)

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

Calculate the different amounts of transformed carbon as percentages of the introduced carbon $C_{\text{MAT}}$ and sum to obtain the calculated carbon $C_{\text{CALC}}$ (%) as shown in equation (E.4):

$$C_{\text{CALC}} = C_{\text{BIO}} + C_{\text{DOC}} + C_{\text{POL}}$$  \hspace{1cm} (E.4)

### E.4 Example: Carbon balance of poly(β-hydroxybutyrate)

Input of test material: $C_{\text{MAT}} = 600 \text{ mg/l} = 334.8 \text{ mg/l of carbon}$

Degree of biodegradation: $D_t = 78 \%$

<table>
<thead>
<tr>
<th>$C_{\text{B:start}}$</th>
<th>$C_{\text{B:end}}$</th>
<th>$C_{\text{BIO}}$</th>
<th>$DOC_{\text{start}}$</th>
<th>$DOC_{\text{end}}$</th>
<th>$DOC$</th>
<th>$C_{\text{BOD}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>61.0</td>
<td>57.8</td>
<td>2.0</td>
<td>22.0</td>
<td>20.0</td>
<td>261</td>
</tr>
<tr>
<td>% of $C_{\text{MAT}}$</td>
<td>17.2</td>
<td></td>
<td></td>
<td></td>
<td>6.0</td>
<td>78</td>
</tr>
</tbody>
</table>

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

### Annex F (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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Annex G (informative)

Bibliography


Annex ZA (normative)
Normative references to international publications with their relevant European publications

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

NOTE When an international publication has been modified by common modifications, indicated by (mod), the relevant EN/HD applies.

<table>
<thead>
<tr>
<th>IEC Publication</th>
<th>Year</th>
<th>Title</th>
<th>EN/HD</th>
<th>Year</th>
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</table>