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Plastics — Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved

Plastiques — Détermination de la biodégradabilité aérobie ultime des matériaux plastiques dans le sol par mesure de la demande en oxygène dans un respiromètre ou de la teneur en dioxyde de carbone libéré

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Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

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.

ISO 17556 was prepared by Technical Committee ISO/TC 61, *Plastics*, Subcommittee SC 5, *Physical-chemical properties*.

This second edition cancels and replaces the first edition (ISO 17556:2003), which has been technically revised. The main changes are as follows:

a) the introduction has been revised;

b) a definition of the term “total organic carbon” has been added (see 3.14);

c) the temperature of the test environment has been changed (see Clause 5);

d) the specifications for the analytical instrument for determining the amount of carbon dioxide evolved have been revised (see 7.2.3);

e) Subclause 8.1 describing the preparation of the test material has been revised;

f) Subclause 8.3.1 describing the collection and sieving of soil has been revised;

g) the use of a standard soil is now permitted as an alternative to natural soil (see 8.3.2);

h) Subclause 8.4 describing the start-up and execution of the test has been revised;

i) the test report has been extended (see Clause 11);

j) a new annex (Annex F) giving examples of long-term tests has been added;

k) a new annex (Annex G) giving the results of round-robin testing has been added.

Introduction

A number of plastic materials and products have been designed for applications ending up in or on soil. They have been developed for applications where biodegradation is beneficial from a technical, environmental, social or economic standpoint. Examples can be found in agriculture (e.g. mulching film), horticulture (e.g. twines and clips, flower pots, pins), funeral items (e.g. body bags), recreation (e.g. plastic “clay” pigeons for shooting, hunting cartridges), etc. In many cases, recovery and/or recycling of these plastic items is either difficult or not economically viable. Various types of biodegradable plastics have been developed which have been designed to biodegrade and disappear *in situ* at the end of their useful life. Several International Standards specify test methods for determining the ultimate aerobic or anaerobic biodegradation of plastic materials in aqueous or compost conditions. Considering the use and disposal of biodegradable plastics, it is important to establish a test method to determine the ultimate aerobic biodegradation of such plastic materials in soil.

Plastics — Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved

WARNING — Appropriate precautions should be taken when handling soil because it might contain potentially pathogenic organisms. Toxic test compounds and those whose properties are unknown should be handled with care.

1 Scope

This International Standard specifies a method for determining the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a closed respirometer or the amount of carbon dioxide evolved. The method is designed to yield an optimum degree of biodegradation by adjusting the humidity of the test soil.

If a non-adapted soil is used as an inoculum, the test simulates the biodegradation processes which take place in a natural environment; if a pre-exposed soil is used, the method can be used to investigate the potential biodegradability of a test material.

This method applies to the following materials:

— natural and/or synthetic polymers, copolymers or mixtures of these;

— plastic materials which contain additives such as plasticizers or colorants;

— water-soluble polymers.

It does not necessarily apply to materials which, under the test conditions, inhibit the activity of the microorganisms present in the soil. Inhibitory effects can be measured using an inhibition control or by another suitable method. If the test material inhibits the microorganisms in the soil, a lower test material concentration, another type of soil or a pre-exposed soil can be used.

2 Normative references

The following referenced documents are indispensable for the application 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 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

ISO 10634, *Water quality — Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

ISO 11274, *Soil quality — Determination of the water-retention characteristic — Laboratory methods*

3 Terms and definitions

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

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

NOTE It is expressed as milligrams of oxygen uptake per milligram or gram of test compound.

3.3
dissolved organic carbon
DOC
that part of the organic carbon in water which cannot be removed by specified phase separation

NOTE 1 It is expressed as milligrams of carbon per 100 milligrams of test compound.

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

3.4
theoretical oxygen demand
ThOD
maximum theoretical amount of oxygen required to oxidize a chemical compound completely, calculated from the molecular formula

NOTE It is expressed as milligrams of oxygen uptake per milligram or gram of test compound.

3.5
theoretical amount of evolved carbon dioxide
$ThCO_2$
maximum theoretical amount of carbon dioxide evolved after completely oxidizing a chemical compound, calculated from the molecular formula

NOTE It is expressed as milligrams of carbon dioxide evolved per milligram or gram of test compound.

3.6
lag phase
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

3.7
biodegradation phase
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

3.8
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

3.9
plateau phase
time from the end of the biodegradation phase until the end of the test

NOTE It is measured in days.

3.10
pre-conditioning
pre-incubation of soil 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 performance of the test by acclimatization of the microorganisms to the test conditions

3.11
pre-exposure
pre-incubation of soil in the presence of the chemical compound or organic matter under test, with the aim of enhancing the ability of the soil to biodegrade the test material by adaptation and/or selection of the microorganisms

3.12
water content
mass of water which evaporates from the soil when the soil is dried to constant mass at 105 °C, divided by the dry mass of the soil

NOTE This is simply the ratio between the mass of the water and that of the soil particles in a soil sample.

3.13
total water-holding capacity
mass of water which evaporates from soil saturated with water when the soil is dried to constant mass at 105 °C, divided by the dry mass of the soil

3.14
total organic carbon
TOC
amount of carbon bound in an organic compound

NOTE It is expressed as milligrams of carbon per 100 milligrams of the compound.

4 Principle

This method is designed to yield the optimum rate of biodegradation of a plastic material in a test soil by controlling the humidity of the soil, and to determine the ultimate biodegradability of the material.

The plastic material, which is the sole source of carbon and energy, is mixed with the soil. The mixture is allowed to stand in a flask over a period of time during which the amount of oxygen consumed (BOD) or the amount of carbon dioxide evolved is determined. Provided the CO_2 evolved is absorbed, the BOD can be determined, for example, by measuring the amount of oxygen required to maintain a constant gas volume in a respirometer flask, or by measuring either automatically or manually the change in volume or pressure (or a combination of the two). An example of a suitable respirometer is shown in Annex A. The amount of carbon dioxide evolved is measured at intervals dependent on the biodegradation kinetics of the test substance by passing carbon-dioxide-free air over the soil and then determining the carbon dioxide content of the air by a suitable method. Examples of suitable methods are given in Annexes B and C.

The level of biodegradation, expressed as a percentage, is determined by comparing the BOD with the theoretical oxygen demand (ThOD) or by comparing the amount of carbon dioxide evolved with the theoretical amount ($ThCO_2$). The influence of possible nitrification processes on the BOD has to be considered. The test is terminated when a constant level of biodegradation has been attained or, at the latest, after six months.

Unlike ISO 11266, which is used for a variety of organic compounds, this International Standard is specially designed to determine the biodegradability of plastic materials.

5 Test environment

Incubation shall take place in the dark or in diffused light in an enclosure which is free from vapours toxic to microorganisms and is maintained at a temperature constant to within ±2 °C in the range between 20 °C and 28 °C, preferably 25 °C.

6 Materials

6.1 Distilled water, containing less than 2 mg of DOC per litre.

6.2 Carbon dioxide absorber, preferably soda lime pellets.

7 Apparatus

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

7.1 Closed respirometer, including test flasks and all other necessary equipment, located in a constant-temperature enclosure or in a thermostatically controlled apparatus (e.g. a water-bath). An example is described in Annex A.

Any respirometer capable of determining with sufficient accuracy the biochemical oxygen demand is suitable, preferably an apparatus which measures and automatically replaces the oxygen consumed so that no oxygen deficiency and no inhibition of the microbial activity occurs during the degradation process.

7.2 Apparatus for measuring the amount of carbon dioxide evolved

7.2.1 Test flasks: glass vessels (e.g. conical flasks or bottles), fitted with tubing impermeable to carbon dioxide to allow purging with gas, and located in a constant-temperature enclosure or in a thermostatically controlled apparatus (e.g. a water-bath).

7.2.2 CO_2-free-air production system, capable of supplying CO_2-free air at a flow rate of several ml/min to each test flask, held constant to within ±10 % (see example of system, including test vessels, in Annex B). Alternatively, the incubation apparatus shown in ASTM D5988 may be used.

7.2.3 Analytical equipment for accurately determining carbon dioxide. Typical examples are a carbon dioxide IR analyser, a dissolved inorganic carbon (DIC) analyser, apparatus for titrimetric determination after complete absorption in a basic solution (see Annex C), and apparatus for the gravimetric determination of carbon dioxide in accordance with ISO 14855-2.

7.3 Analytical balance.

7.4 pH-meter.

8 Procedure

8.1 Preparation of the test material

The test material shall be of known mass and contain sufficient carbon to yield a BOD or a quantity of carbon dioxide that can be adequately measured by the analytical equipment 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 ThOD or $ThCO_2$ (see Annexes C and D).

NOTE Although elemental analysis is generally less accurate for macromolecules than for low-molecular-mass compounds, the accuracy is usually acceptable for the purposes of calculating the ThOD or $ThCO_2$.

The amount of test material shall be sufficient to outweigh any variations in the background oxygen consumption or any carbon dioxide evolved from the test soil: 100 mg to 300 mg of test material to 100 g to 300 g of soil is usually adequate. The maximum amount of test material is limited by the oxygen supply to the test system. The use of 200 mg of test material with 200 g of soil is recommended unless the soil contains an excessively large amount of organic matter.

When using test systems based on the determination of the carbon dioxide evolved, higher test material amounts can be used (e.g. 2 500 mg for 200 g of soil) in order to increase the difference between the test material CO_2 production and the blank control CO_2 production. Furthermore, a greater amount of test material will be required if a final mass balance determination is to be carried out (see Annex E).

Pre-aeration of the test material or the addition of inert material is recommended, if necessary, to reduce the respiration of the soil in the blank flasks.

The test material should preferably be used in powder form, but it may also be introduced in the form of films, fragments or shaped articles.

Test samples may be reduced in size by means of cryogenic milling.

Experiments have shown that the ultimate degree of biodegradation is almost independent of the form and shape of the test material. The speed of biodegradation, however, does depend on the form and shape of the material. Test materials of similar form and shape should therefore be used if different kinds of plastic material are to be compared in tests of the same duration. If the test material is in the form of a powder, small particles of known size distribution should be used. A particle-size distribution with its maximum at 250 µm diameter is recommended. If the test material is not in powder form, the size of the pieces of material should not be greater than 5 mm $\times$ 5 mm. Also, the size of the test equipment used might depend on the form of the test material. It should be ascertained that no undesired changes are caused in the test material due to the design of the equipment, such as grinders, used. Normally, processing of the test material will not significantly influence the degradation behaviour of the material (e.g. the use of powder in the case of composites).

Optionally, determine the hydrogen, oxygen, nitrogen, phosphorus and sulfur contents, as well as the molecular mass of the test material, using, for example, size exclusion chromatography. 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 compounds with limited solubility in water, see ISO 10634.

8.2 Preparation of the reference material

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

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

8.3 Preparation of the test soil

8.3.1 Collection and sieving of soil

Use natural soil collected from the surface layer of fields and/or forests. If the potential biodegradability of the test material is to be assessed, this soil may be pre-exposed to the test material. Sieve the soil to give particles of less than 5 mm, preferably less than 2 mm, in size and remove obvious plant material, stones and other inert materials.

It is important to remove organic solids, such as straw, as far as practicable because they can decompose during the test and influence the results.

The soil may be pre-conditioned but, normally, pre-exposed soil should not be used, especially when biodegradation behaviour in natural environments is being simulated. Depending on the purpose of the test, however, pre-exposed soil may be used, provided that this is clearly stated in the test report (e.g. percent

biodegradation = x %, using pre-exposed soil) and the method of pre-exposure detailed. Pre-exposed soil can be obtained from suitable laboratory biodegradation tests 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).

Record the sampling site, its location, the presence of plants or previous crops, the sampling date, the sampling depth and, if possible, the soil history, such as details of fertilizer and pesticide application.

8.3.2 Preparation of standard soil

As an alternative to the natural soil described in 8.3.1, a standard soil may be used. The composition of the standard soil is shown in Table 1. The use of standard soil is very useful in determining the biodegradability of plastic materials in bulky soils (loamy or clayey soils), reducing handling and aeration problems.

Table 1 — Standard-soil composition

Constituent	Remarks	Percentage expressed on dry mass basis
Industrial quartz sand	Predominantly fine sand in which the size of more than 50 % of the particles lies in the range 0,05 mm to 0,2 mm	70
Clay	Kaolinite clay (containing not less than 30 % kaolinite) or calcium bentonite	10
Natural soil	See 8.3.1	16
Mature compost	Use well-aerated compost from an aerobic composting plant. In order to stabilize the microbial activity in the standard soil, it is recommended that 1-year-matured compost be used. If this is not possible, use a compost which has matured for a minimum of 2-3 months. The compost shall be homogeneous and free from large, inert objects, such as pieces of glass, stones or pieces of metal. Remove them manually and then sieve the compost through a screen of mesh size about 2 cm to 5 cm.	4

To the soil specified in Table 1 are added the salts listed in Table 2, preferably dissolved in water and preferably at the moment of adjustment of the water content (see 8.3.4).

Table 2 — Added salts

Constituent	g/kg of soil
KH_2PO_4	0,2
$MgSO_4$	0,1
$NaNO_3$	0,4
Urea	0,2
NH_4Cl	0,4

A round-robin test was carried out to validate the standard soil (see Annex G).

8.3.3 Measurement of soil characteristics

Knowledge of the soil characteristics is essential for full interpretation of the results of the study. It is therefore recommended that at least the following tests be performed on the soil selected:

a) total water-holding capacity, in accordance with ISO 11274;

b) pH of the soil, in accordance with ISO 10390;

c) organic-matter content, in accordance with ISO 10694.

8.3.4 Adjustment of the water content and the pH of the soil

Adjust the water content of the soil to a suitable value for the test material by adding an appropriate amount of water to the soil, or by drying the soil in the air in a shaded place followed by addition of an appropriate amount of water. Adjust the pH of the soil to between 6,0 and 8,0 if it is not already within this range.

NOTE The optimum water content of the test soil is dependent on the test material. It is usually between 40 % and 60 % of the total water-holding capacity.

It is recommended that the ratio of organic carbon in the test or reference material to nitrogen in the soil (C:N ratio) be adjusted to at least 40:1, if it is not already at this level, so as to ensure good biodegradation. This may be done by adding nitrogen as an aqueous solution of ammonium chloride or by using an aqueous solution containing the salts listed in Table 2.

8.3.5 Handling and storage of the soil

Store the soil in a sealed container at 4 °C ± 2 °C until it is used in the test. Do not handle the soil in any way that could inhibit the activity of the microorganisms in it.

It is important that ISO 10381-6 be followed to ensure that the microbial activity of the soil is not affected by sampling.

8.4 Start-up and execution of the test

Prepare the following numbers of flasks:

a) three test flasks for the test material (symbol F_T);

b) three test flasks for the blank control (symbol F_B);

c) three test flasks for checking the soil activity using a reference material (symbol F_C);

and, if required:

d) one flask for checking for possible abiotic degradation or non-biological changes in the test material (symbol F_S);

e) one flask for checking for any possible inhibiting effect of the test material (symbol F_I).

Place the soil (see 8.3) at the bottom of each flask and add test material (see 8.1) or reference material (see 8.2), as indicated in Table 3, to the soil. Record the mass of each flask containing this test mixture. When two replicates are used, this shall be stated in the test report.

It is important that the test material be homogeneously mixed with the soil, in the case of powder, and as widely spread as possible in the soil, in the case of film, to improve the contact of the test material with the microorganisms in the soil. Also, it is recommended that the surface of the test mixture be pressed with a spatula to improve the contact between the test material and the microorganisms in the soil.

If the abiotic-degradation check is carried out, details of the procedures used to inhibit microbial activity at the start of the test and maintain aseptic conditions during the test shall be provided in the test report.

Place the flasks in a constant-temperature environment (see Clause 5) and allow all the flasks to reach the desired temperature. Make all necessary connections with the respirometer or CO_2-free-air production system and start the incubation.

If measuring the oxygen consumption, take the necessary readings on the manometers (if manual) or verify that the recorder of oxygen consumption is functioning correctly (automatic respirometer) (see Annex A).

If measuring the carbon dioxide evolved, measure (at regular intervals depending on the carbon dioxide evolution rate) the amount of carbon dioxide evolved from each flask, using a suitable and sufficiently accurate method (see Annexes B and C).

Table 3 — Final distribution of test and reference materials

Flask		Test material	Reference material	Test soil
F_T	Test	+	−	+
F_T	Test	+	−	+
F_T	Test	+	−	+
F_B	Blank	−	−	+
F_B	Blank	−	−	+
F_B	Blank	−	−	+
F_C	Soil activity check	−	+	+
F_C	Soil activity check	−	+	+
F_C	Soil activity check	−	+	+
F_S	Abiotic-degradation check (optional)	+	−	−
F_I	Inhibition check (optional)	+	+	+
+ = present; − = not present.				

If the biodegradation rate is considered to have slowed down because the test soil has dried out during the test, stop the measurements and remove the flasks from the respirometer or CO_2-free-air production system. Weigh the flasks and add a suitable amount of water to the test soil to bring its water content back to its initial value. Reconnect the flasks to the system and restart measurement of the oxygen consumed or carbon dioxide evolved. These operations shall be conducted without inhibiting the activity of the soil microorganisms and without influencing the measurement of oxygen consumption or carbon dioxide evolution, and the fact that they have been carried out shall be clearly stated in the test report.

When a constant level of BOD or carbon dioxide evolution is attained (plateau phase reached) and no further biodegradation is expected, the test is considered to be completed.

The test period should typically not exceed six months. However, if significant biodegradation is still observed and the plateau phase has not been reached after this length of time, then the test may be extended, but not to longer than 2 years. If the test is run for longer than six months, check periodically for possible leaks. Any extension and any special measures taken, e.g. to ensure microbial diversity or to provide sufficient nutrients, shall be detailed in the test report.

At the end of the test, remove the flasks and weigh them to check for any decrease in the water content of the test soil. Optionally, the residual test material may be extracted from the soil with a suitable solvent (if this is possible) and weighed.

9 Calculation and expression of results

9.1 Calculation

9.1.1 Percentage biodegradation from oxygen consumption values

Read the oxygen consumption value for each flask, using the method given by the manufacturer for the type of respirometer concerned. Calculate the specific biochemical oxygen demand (BOD_S) of the test material using Equation (1):

$$BOD_S = \frac{BOD_t - BOD_{Bt}}{\rho_T} \quad (1)$$

where

BOD_S is the specific BOD, in milligrams per gram of test material;

BOD_t is the BOD of the flask F_T containing test material at time t, in milligrams per kilogram of test soil, calculated by dividing the measured oxygen consumption, in milligrams, by the amount of test soil, in kilograms;

BOD_{Bt} is the BOD of the blank control flask F_B at time t, in milligrams per kilogram of test soil;

ρ_T is the concentration of the test material in the reaction mixture of flask F_T, in milligrams per kilogram of test soil;

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):

$$D_t = \frac{BOD_S}{ThOD} \times 100 \quad (2)$$

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 and the inhibition check F_I. For calculation of the ThOD, see Annex D.

9.1.2 Percentage biodegradation from carbon dioxide evolved

9.1.2.1 Theoretical amount of carbon dioxide evolved by test material

The theoretical amount of carbon dioxide evolved by the test material ($ThCO_2$) is given, in milligrams, by Equation (3):

$$ThCO_2 = \frac{44}{12} \times m \times w_C \quad (3)$$

where

m is the mass of test material, in milligrams, introduced into the test system;

w_C is the carbon content of the test material, determined from the chemical formula or from elemental analysis, expressed as a mass fraction;

44 and 12 are the relative molecular and atomic masses of carbon dioxide and carbon, respectively.

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

9.1.2.2 Percentage biodegradation

Calculate the percentage biodegradation D_t for each test flask F_T from the amount of carbon dioxide evolved during each measurement interval using Equation (4):

$$D_t = \frac{\Sigma m_T - \Sigma m_B}{ThCO_2} \times 100 \quad (4)$$

where

Σm_T is the amount of carbon dioxide, in milligrams, evolved in the test flask F_T between the start of the test and time t;

Σm_B is the amount of carbon dioxide, in milligrams, evolved in the blank control flask F_B between the start of the test and time t;

$ThCO_2$ is the theoretical amount of carbon dioxide, in milligrams, evolved by the test material.

Calculate in the same way the percentage biodegradation of the reference material in the soil activity check flask F_C.

9.2 Expression and interpretation of results

Compile a table of the BOD values or amounts of carbon dioxide measured and the percentage biodegradation values for each point in time when measurements were made. For each flask, plot a curve of BOD or carbon dioxide evolved as a function of time and a curve of percentage biodegradation as a function of time. If comparable results are obtained for the duplicate flasks, the mean curve may be plotted.

The maximum level of biodegradation determined as the mean value of the plateau phase of the biodegradation curve characterizes the degree of biodegradation of the test material.

The wettability of the test material and the shape of the pieces of test material might influence the result obtained, and this should be taken into consideration when comparing the results obtained with different test materials.

Information on the toxicity of the test material might 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 is more than 60 % at the plateau phase or at the end of the test;

and

b) the BOD values of, or 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 these criteria are not fulfilled, repeat the test using another pre-conditioned or pre-exposed soil.

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 material, including name, chemical composition and formula (if known), ThOD, $ThCO_2$ (including the method of calculation), form, particle shape, amount/concentration in the samples tested, and content of additives (if possible);

c) the history of the test material (virgin granules, converted final plastic product or aged samples), detailing the pre-treatment conditions, if pre-treatment was carried out;

d) complete information on the soil, including source, date of collection, characteristics, amount used in the test, storage conditions, handling and details of any pre-exposure;

e) the main test conditions, including the amount of test material used, the incubation temperature and the duration of incubation;

f) the analytical techniques used, including the principle of the respirometer and the method used to measure the amount of carbon dioxide evolved;

g) all other operations carried out, including any addition of water to the test mixture during the test, and the results of analyses of the test mixture, including the water content, at the end of the test;

h) all the test results obtained for the test and reference materials (in tabular and graphical form), including the measured cumulative BOD or evolved carbon dioxide, the percentage biodegradation values and the curves of these parameters against time;

i) the duration of the lag phase and degradation phase, the maximum level of biodegradation, as well as the total test duration;

and, optionally, if run or determined:

j) the residual amount of test material or the percentage biodegradation calculated from the residual amount of test material;

k) the colony-forming units (cfu/g) in the soil;

l) details of the methods used during an extended test period (> 6 months) in order to support microbial diversity or to avoid nutrient deficiency;

m) all available information on the test material and the amount used if the test has been performed at a reduced test material concentration in order to avoid toxic effects;

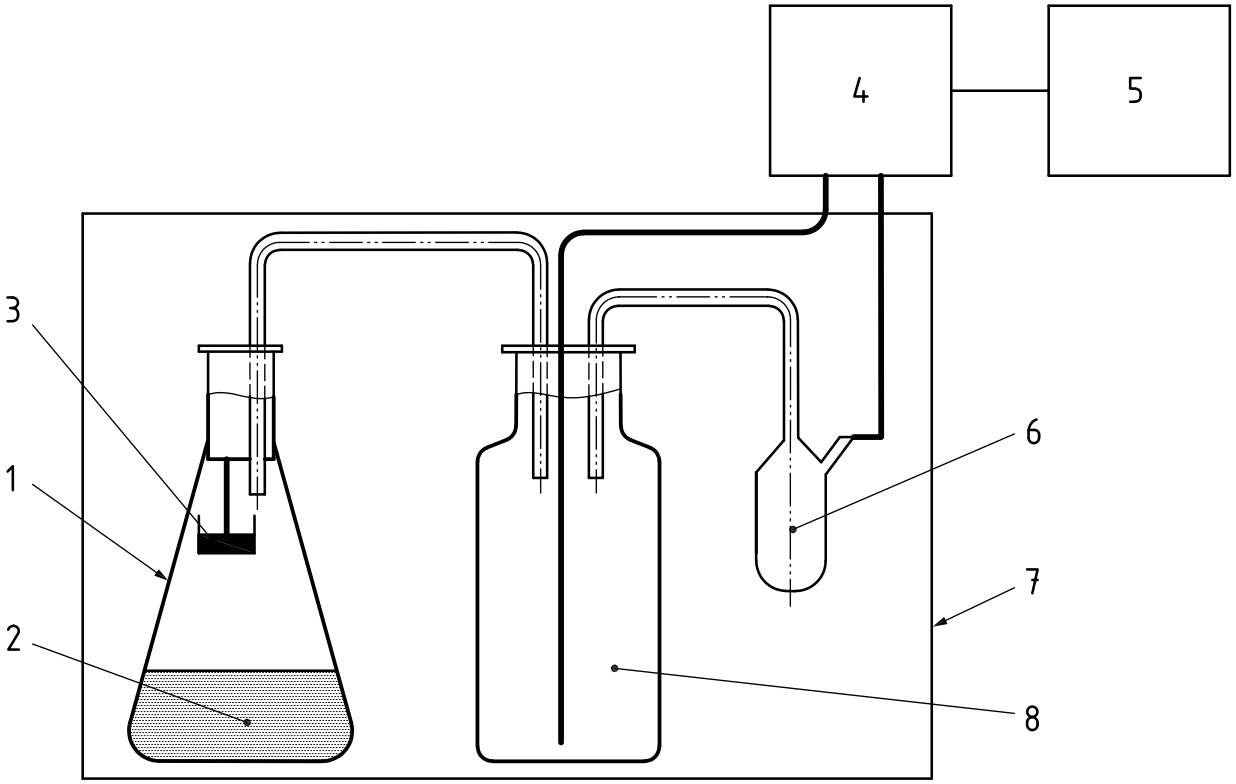
n) any other relevant data (e.g. initial molecular mass of the sample, molecular mass of the residual polymer);

o) any deviation from the test method specified.

Annex A
(informative)

Principle of a manometric respirometer (example)

The respirometer, as shown in Figure A.1, is set up in a temperature-controlled environment (e.g. a water-bath) and contains test vessels each fitted with a CO_2 absorber in the headspace, a coulometric oxygen production unit, a manometer 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. 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 a 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 used to indicate the oxygen consumption in milligrams per litre BOD on the recorder.



Key

1 test flask
2 test mixture
3 CO_2 absorber
4 monitor
5 printer, plotter or computer
6 manometer
7 thermostatically controlled enclosure
8 oxygen-generating unit

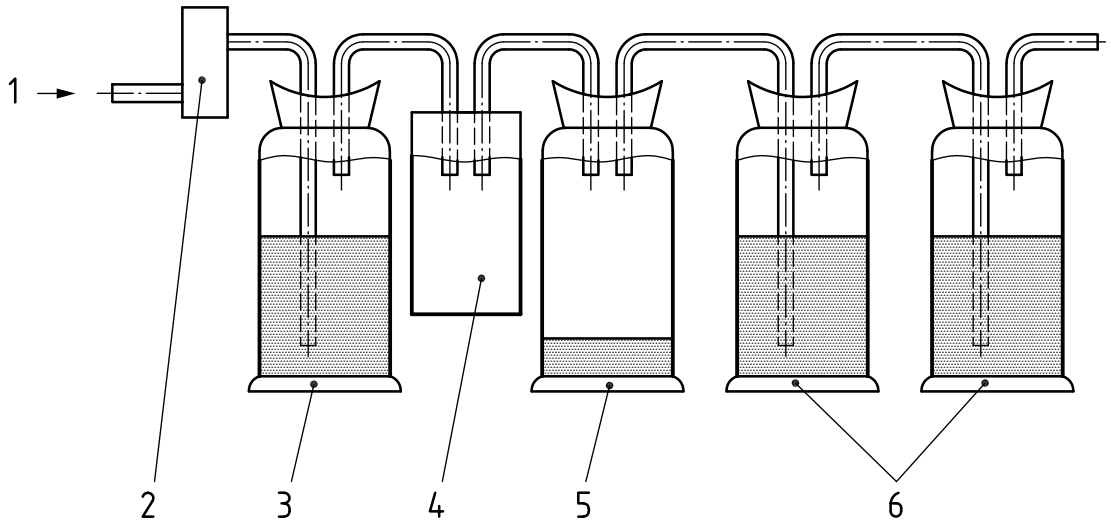
Figure A.1 — Schematic diagram of a manometric respirometer

Annex B
(informative)

Example of a system for measuring the amount of carbon dioxide evolved

Set up the flasks in series as shown in Figure B.1 and connect them with gas-impermeable tubing. Aerate the test system with several millilitres per minute of CO_2-free air at a constant low pressure (preferably at least a volume of air equivalent to the volume of three flasks per hour when using a high test material concentration, for example 2 500 mg of test material for 200 g of soil). Count air bubbles or use a suitable air-flow controller (2) to check the air-flow rate. Use synthetic CO_2-free air or compressed air. In the latter case, remove CO_2 by passing the air through a bottle (3) containing dry soda lime or through at least two gas-washing bottles containing e.g. 500 ml of a 10 mol/l aqueous potassium hydroxide solution. An additional flask containing e.g. 100 ml of 0,012 5 mol/l barium hydroxide solution and an empty flask can be used to indicate the presence of any CO_2 in the air by turbidity and to prevent carry-over of liquid to the test flask. If necessary, a humidifier (4) may be inserted before the test flask (5) to humidify the air so as to avoid evaporation of moisture from the test soil. This can be done for example by bubbling the air through a constant-humidity solution such as a saturated aqueous solution of sodium phosphate. If biodegradation takes place, CO_2 is produced in the test flask and absorbed in the subsequent absorption bottles (6) as described in Annex C. In order to maintain the test soil (flask 5) wet and the conditions aerobic, adjust the flow rate at the air inlet.

To ensure complete aeration and avoid the formation of gas concentration gradients within the test flask(s), the air inlet tubing should preferably be extended into the soil, in order to ensure that air passes through the soil mass.



Key

1 air in
2 air-flow controller
3 CO_2 absorber
4 humidifier
5 test flask
6 CO_2-absorption bottles

Figure B.1 — Schematic diagram of a system for measuring the amount of carbon dioxide evolved

Annex C
(informative)

Examples of methods for the determination of evolved carbon dioxide

C.1 CO_2 determination by DIC measurement

The carbon dioxide evolved is absorbed in sodium hydroxide (NaOH) solution and determined as dissolved inorganic carbon (DIC) using, for example, 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_2 production. Connect in series with the test flask two absorption bottles, each containing 100 ml of the NaOH solution. Close the outlet of the last bottle with a small syphon to prevent CO_2 from the air from entering the NaOH solution. On the days when the CO_2 is determined, remove the absorption 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_2 produced using Equation (C.1):

$$(CO_2)_T = \frac{(DIC_T - DIC_B) \times 3,67}{10} \quad \text{(C.1)}$$

where

$(CO_2)_T$ is the mass of CO_2 evolved, in milligrams;

DIC_T is the measured DIC, in milligrams;

DIC_B is the blank DIC measured for the NaOH solution, in milligrams;

3,67 is the ratio of the molecular mass of CO_2 (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.

C.2 Titrimetric method using a barium hydroxide solution

The CO_2 produced reacts with the barium hydroxide $Ba(OH)_2$ and is precipitated as barium carbonate ($BaCO_3$) [see reaction (C.2)]. The amount of CO_2 evolved is determined by titrating the remaining $Ba(OH)_2$ with hydrochloric acid (HCl) [see reaction (C.3)].

$$CO_2 + Ba(OH)_2 \rightarrow BaCO_3 + H_2O \quad \text{(C.2)}$$

$$Ba(OH)_2 + 2HCl \rightarrow BaCl_2 + 2H_2O \quad \text{(C.3)}$$

Dissolve 4,0 g of $Ba(OH)_2 \cdot 8H_2O$ 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 litres, is 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_2 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 100 ml of $Ba(OH)_2$ solution into each of three absorption 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 $BaCO_3$ can be observed in the second bottle. At the beginning of the test, titration might be required every other day, and then every fifth day when the plateau phase is reached. After removing the absorption bottle, immediately seal it with a plug to avoid CO_2 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 $Ba(OH)_2$ 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 $Ba(OH)_2$ solution with the HCl solution. Note the volumes of the HCl solution needed for neutralization.

Calculate the mass of CO_2 trapped in the absorption bottle using Equation (C.4):

$$m = \left(\frac{2c_{\mathrm{B}} \times V_{\mathrm{B0}}}{c_{\mathrm{A}}} - V_{\mathrm{A}} \times \frac{V_{\mathrm{B}t}}{V_{\mathrm{BZ}}} \right) \times c_{\mathrm{A}} \times 22 \qquad \text{(C.4)}$$

where

m is the mass of CO_2 trapped in the absorption 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 $Ba(OH)_2$ solution, in moles per litre;

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

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

V_{BZ} is the volume of the aliquots of $Ba(OH)_2$ 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_2.

When the following conditions apply:

— the volume of the $Ba(OH)_2$ solution before and after absorption is exactly 100 ml;

— the complete solution is used for titration ($V_{B0} = V_{Bt} = V_{BZ}$);

— the concentration c_B of the $Ba(OH)_2$ solution is exactly 0,012 5 mol/l;

— the concentration c_A of the HCl solution is exactly 0,05 mol/l;

use Equation (C.5):

$$m = 1{,}1 \times (50 - V_{\mathrm{A}}) \qquad \text{(C.5)}$$

Annex D
(informative)

Theoretical oxygen demand (ThOD)

D.1 Calculation of ThOD

The theoretical oxygen demand (ThOD) of the 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 Equation (D.1):

$$\text{ThOD} = \frac{16\left[2c + 0{,}5\left(h - cl - 3n\right) + 3s + 2{,}5p + 0{,}5na - o\right]}{M_r} \quad \text{(D.1)}$$

This calculation assumes that carbon is converted to CO_2, hydrogen to H_2O, phosphorus to P_2O_5 and sulfur to an oxidation state of $+6$ and that halogens are 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. Express the ThOD in milligrams per gram of substance or in milligrams per milligram of substance.

D.2 Example: poly(β-hydroxybutyric acid) (PHB)

Summary formula[1)]: $C_4H_6O_2$, $c = 4$, $h = 6$, $o = 2$; relative molecular mass $M_r = 86$.

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

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

D.3 Example: blend of polyethylene/starch/glycerol

Component	Formula	ThOD	Amount of component		ThOD
		mg/g	%	mg/flask	mg/flask
Polyethylene	$(C_2H_4)_n$	3 400	50	500	1 700
Starch	$(C_6H_{10}O_5)_n$	1 190	40	400	476
Glycerol	$C_3H_8O_3$	1 200	10	100	120
Total blend			100	1 000	2 296

1) PHB is a polymer 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_2O, which is eliminated in the chemical reaction.

Annex E
(informative)

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

It might be helpful to use a procedure for measuring the amount and the molecular mass of polymer 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 separate 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 eluent.

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 polymer 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.

Annex F
(informative)

Examples of long-term tests

F.1 Evolution of the biodegradation of cellulose, wheat gluten, flax fibres and broom fibres in soil

NOTE Data taken from Reference [6].

Inoculum: agricultural soil (500 g, < 2 mm)

Materials: cellulose (5 g), wheat gluten (5 g), flax fibres (2 g), broom fibres (2 g)

Duration: cellulose: 2 years

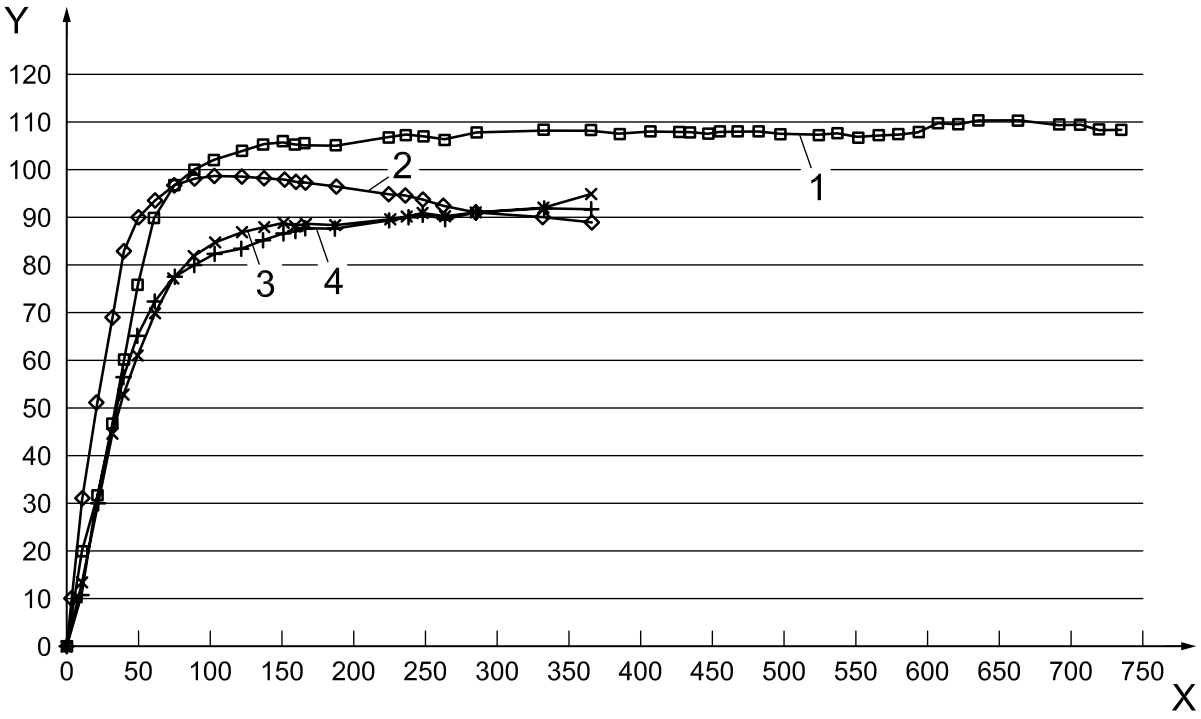
wheat gluten, flax fibres, broom fibres: 1 year

Temperature: 20 °C $\pm$ 2 °C

Results (2 replicates): see Table F.1 and Figure F.1

Table F.1 — Percentage biodegradation (first example)

Test material	Percentage biodegradation %		
	1 year		2 years
	Average	Standard deviation	1 replicate
Cellulose	108,3	2,8	108,5
Wheat gluten	89,2	2,6	—
Flax fibres	94,8	6,8	—
Broom fibres	91,8	5,6	—



Key

X time (days)
Y percentage biodegradation
1 cellulose
2 wheat gluten
3 flax fibres
4 broom fibres

Figure F.1 — Evolution of the biodegradation of cellulose, wheat gluten, flax fibres and broom fibres in soil

F.2 Evolution of the biodegradation of cellulose, birch leaves, oak leaves and pine needles in soil

NOTE Data taken from Reference [7].

Inoculum: agricultural soil (500 g, < 2 mm)

Materials: cellulose (1 g), birch leaves (1 g), oak leaves (1 g), pine needles (1 g)

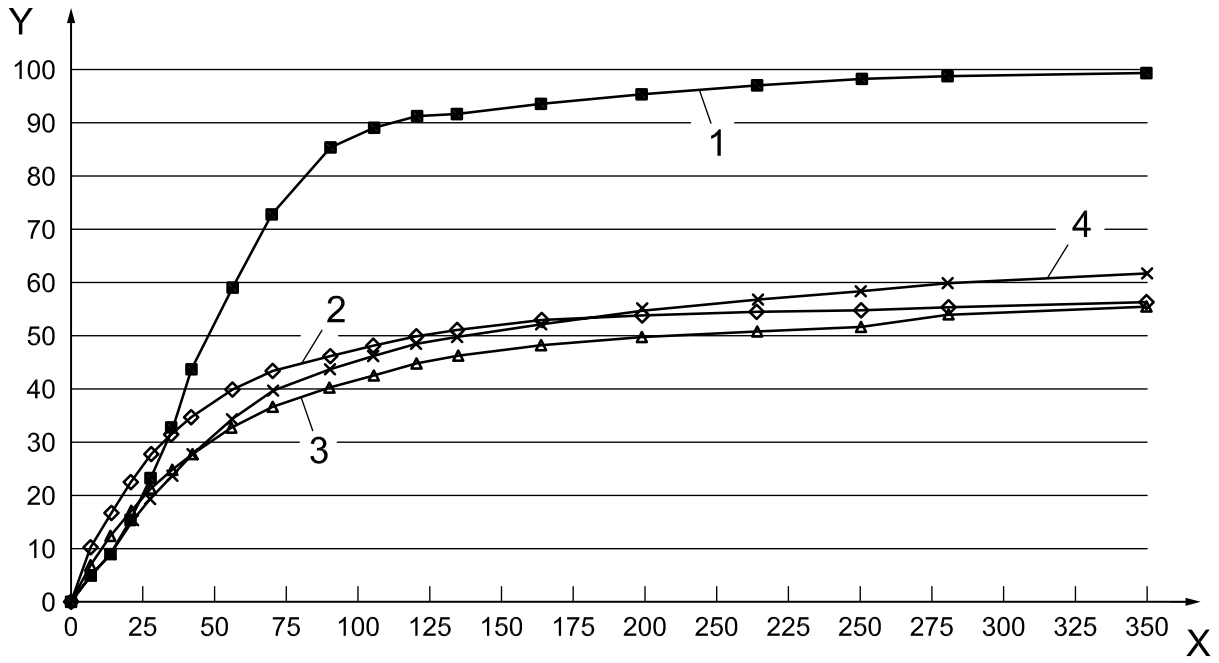
Duration: 1 year

Temperature: 20 °C ± 2°C

Results: see Table F.2 and Figure F.2.

Table F.2 — Percentage biodegradation (second example)

Test material	Percentage biodegradation %	
	Absolute	Relative to cellulose
Cellulose	99,7	100,0
Birch leaves	56,2	56,4
Oak leaves	55,8	56,0
Pine needles	61,9	62,1



Key

X time (days)
Y percentage biodegradation
1 cellulose
2 birch leaves
3 oak leaves
4 pine needles

Figure F.2 — Evolution of the biodegradation of cellulose, birch leaves, oak leaves and pine needles in soil

F.3 Evolution of the biodegradation of cellulose and straw

Inoculum: mixture of 1 part agricultural soil and 2 parts forest soil (500 g, < 2 mm)

Materials: cellulose (1 g), straw (1 g)

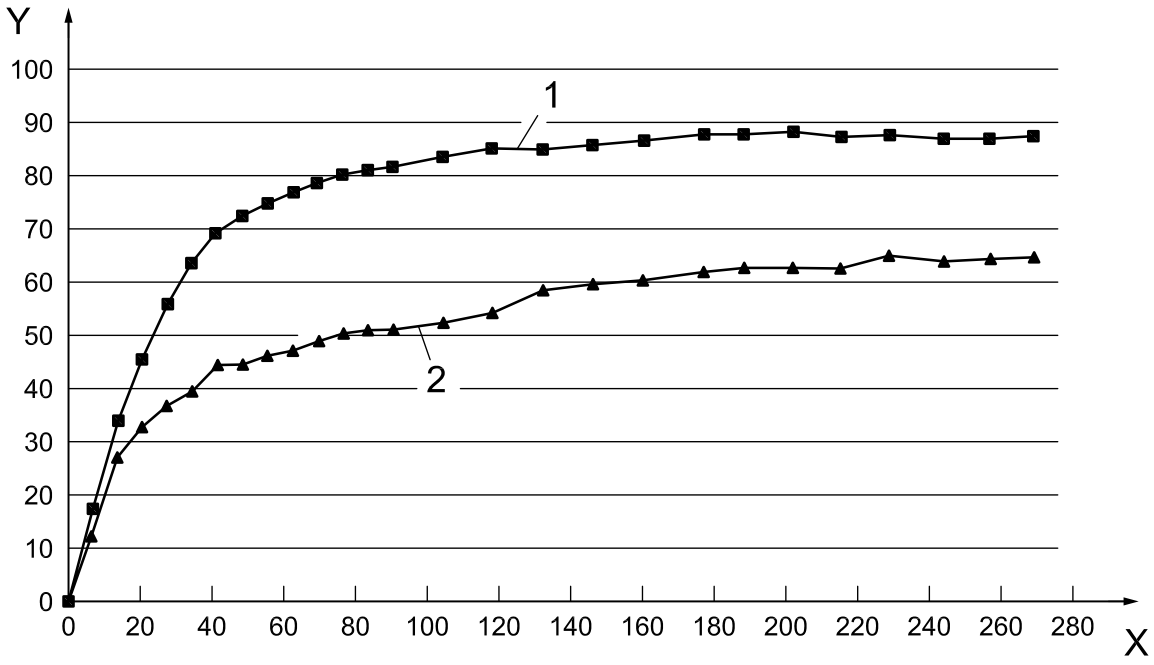
Duration: 270 days

Temperature: 20 °C ± 2 °C

Results (2 replicates): see Table F.3 and Figure F.3.

Table F.3 —Percentage biodegradation (third example)

Test material	Percentage biodegradation %		
	Absolute		Relative to cellulose
	Average	Standard deviation	
Cellulose	89,8	1,9	100,0
Straw	66,4	0,0	74,0



Key

X time (days)

Y percentage biodegradation

1 cellulose

2 straw

Figure F.3 — Evolution of the biodegradation of cellulose and straw

Annex G
(informative)

Round-robin testing

A round-robin test was carried out in 2009 to validate the use of standard soil instead of a natural soil. The composition of the standard soil is given in 8.3.2. The sand and clay in this standard soil give the soil its texture, the natural soil provides microorganisms and the mature compost provides organic matter and additional microorganisms. Six different laboratories were involved in the round-robin test. Each laboratory independently collected a soil for use as the “natural soil”, i.e. as the inoculum for the “standard soil”. The test materials were microcrystalline cellulose reference material (RM) and a starch/poly(butylene adipate-co-butylene terephthalate) blend test material (TM). For the purposes of the round-robin test, participants were allowed to use a commercial soil, a natural soil or a mixture of different soils (e.g. a mixture of forest soil, one pasture soil and one garden soil). When using the materials in high concentration (10 g in 800 g of wet soil), the natural soil was fertilized using the mixture of salts listed in Table 2.

All the participants determined the biodegradation in soil measuring the amount of evolved carbon dioxide using either: an IR analyser, titration and, in one case, the gravimetric determination of CO_2 in accordance with ISO 14855-2. The temperature was between 20 °C and 28 °C. Two participants used a mix of two or three kinds of soil, two participants used a soil collected from a field and the other two used a commercial soil.

The final percentage biodegradation values are given in Table G.1.

Table G.1 — Results of round-robin test

Partici-pant	Inoculum	Apparatus	Time days	Biodegra-dation % TM	Standard deviation	Biodegra-dation % RM	Standard deviation	Relative biodegradation of TM[a]
1	Natural soil	CO_2-free-air flow system	120	33,0[b]	2,16	70,3	0,31	47,0
1	Standard soil	CO_2-free-air flow system	120	11,8	1,17	59,0	0,5	20,1
2	Natural soil	ASTM D5988-03, 7.2.2	120	16,9[b]	2,9	63,8[b]	1,4	26,5
2	Standard soil	ASTM D5988-03, 7.2.2	120	23,8[b]	0,9	70,4[b]	3,4	33,8
2	Natural soil	CO_2-free-air flow system	120	21,9[b]	0,9	69,9[b]	1,6	31,3
2	Natural soil	ASTM D5988-03, 7.2.2	120	18,8[b]	0,2	61,1[b]	0,8	30,8
2	Natural soil	ASTM D5988-03, 7.2.2	120	19,9[b]	1,1	81,6[b]	2,8	24,4
3	Natural soil	CO_2-free-air flow system	182	61	1	72[c]	3,5	83,6
3	Standard soil	CO_2-free-air flow system	182	26	4	46	4,5	56,5
4	Natural soil	CO_2-free-air flow system	134	59,3	2,3	60,9	1,9	97,3
4	Standard soil	CO_2-free-air flow system	134	70,8	1,78	70,1	0,0	101,0
4	Natural soil	CO_2-free-air flow system	115	31,1	4,5	56,6	4,3	54,9
4	Standard soil	CO_2-free-air flow system	115	37,2	6,9	64,5	5,1	57,7
5	Natural soil	CO_2-free-air flow system	70	92,6	3,2	86,5	9,0	107,1
5	Standard soil	CO_2-free-air flow system	60	74,1[b]	0,5	77,0[b]	6,6	96,2
6	Natural soil	CO_2-free-air flow system	118	21,5[b]	4,9	104[b]	2,6	20,7
6	Standard soil	CO_2-free-air flow system	118	26[b]	4,2	107[b]	1,4	24,3

[a] Relative biodegradation of TM = (% biodegradation of TM/% biodegradation of RM) × 100.

[b] Had not yet reached plateau phase.

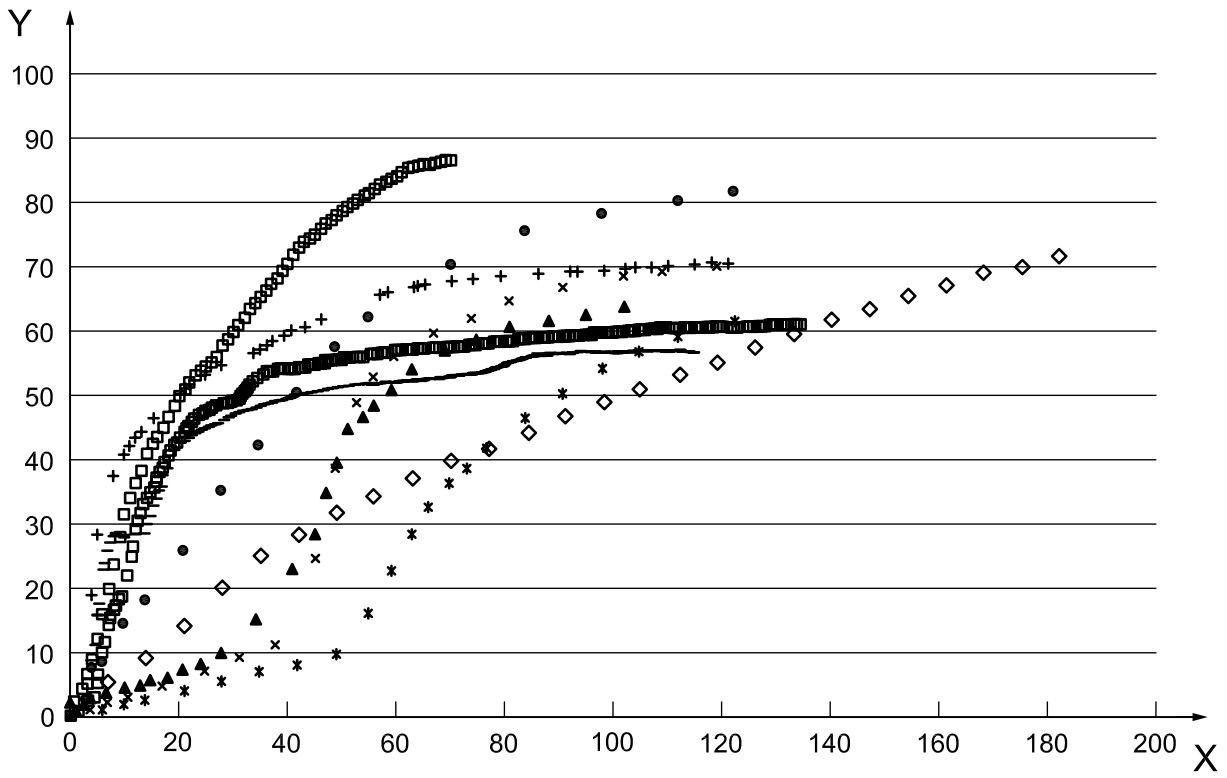
[c] Still in a high CO_2 evolution phase.

Averages of the biodegradation values obtained in the different laboratories for natural soil and for standard soil are shown in Table G.2. Values from participant No. 6 were not considered because the biodegradation of the reference material exceeded 100 % in both cases and a plateau phase was not reached.

Table G.2 — Average percentage biodegradation in natural soil and in standard soil

Material	Natural soil	Standard soil
Test material	39,39 ± 26,03	40,62 ± 25,96
Reference material	69,19 ± 9,91	64,50 ± 10,91

Figures G.1 and G.2 show the biodegradation curves for the various participants for the reference material in natural soil and in standard soil, respectively.

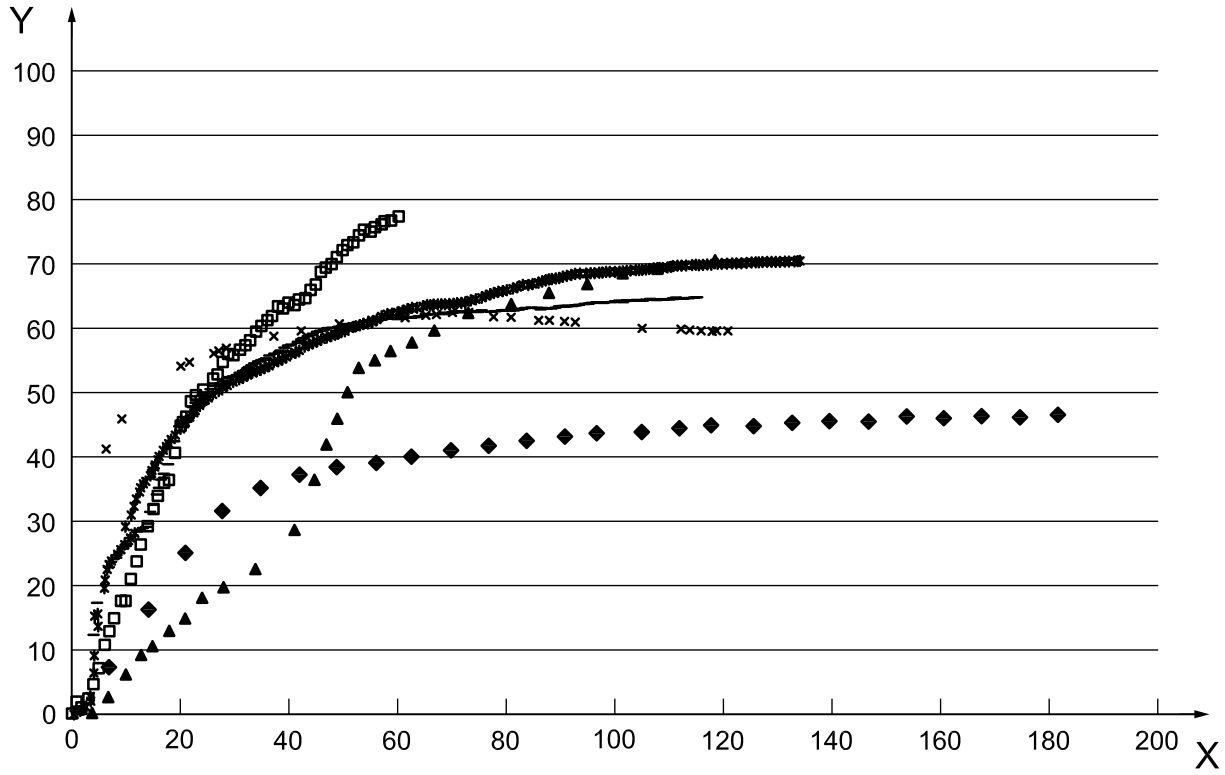


Key

X time (days)

Y percentage biodegradation

Figure G.1 — Evolution of the biodegradation of microcrystalline cellulose in natural soil



Key

X time (days)

Y percentage biodegradation

Figure G.2 — Evolution of the biodegradation of microcrystalline cellulose in standard soil

The variability of the results at the interlaboratory level for natural soils is not high when testing cellulose. Cellulose seems to be a material whose biodegradation is not significantly affected by the test conditions. Biodegradation of the test material, on the other hand, appears to be more sensitive to the test conditions, resulting in greater variation in the test results. It is difficult to know whether this is the consequence of using different soils or whether other variables dominate, such as laboratories using different test conditions (i.e. different temperatures, aeration rates and material concentrations).

The preparation of a standard soil inoculated with natural soil does not seem to affect the interlaboratory variability, as can be seen from the standard deviation (see Table G.2).

The average values for the standard soil do not substantially differ from those for the natural soil. This reinforces the idea that the biological factor (i.e. the microbial population) of the natural soil is a relevant factor which is also important when the soil is used as an ingredient in the standard soil.

In order to make it possible to retrieve compounds at the end of the test, for the purpose of carrying out a final mass balance determination as described in Annex E, it seems necessary to start with a large amount of test material.

Generally speaking, standard soil can help in standardizing the test procedure, as it makes use of a standard matrix, with a standard texture and particle size. In particular, this seems to be helpful when using bulky soils.

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