INTERNATIONAL STANDARD

ISO 846

Second edition
1997-06-15

Plastics — Evaluation of the action of microorganisms

Plastiques — Évaluation de l'action des micro-organismes

Reference number
ISO 846:1997(E)

Contents

International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet central@iso.ch
X.400 c=ch; a=400net; p=iso; o=isocs; s=central

Printed in Switzerland

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 846 was prepared by Technical Committee ISO/TC 61, *Plastics,* Subcommittee SC 6, *Ageing, chemical and environmental resistance.*

This second edition cancels and replaces the first edition (ISO 846:1978), which has been technically revised.

The Plastics Project Group of the IBRG (International Biodeterioration Research Group) carried out several interlaboratory tests between 1984 and 1990, using the 1978 edition of this standard, with the aim of checking the reproducibility of the test results. The experience gained from these tests has been incorporated in the present edition. In addition, a soil-burial test method has been included in subclause 8.5, based on a specification the Eidgenössische Materialprüfungsanstalt in St. Gallen, Switzerland.

Annex A forms an integral part of this International Standard. Annexes B and C are for information only.

Introduction

Under certain climatic and environmental conditions, microorganisms may settle on and colonize the surface of plastics or plastics products. Their presence and/or their metabolic products may not only damage the plastic itself, but may also affect the serviceability of building materials and systems containing plastic parts.

The tests and test conditions specified in this International Standard are empirical and cover most- but not all- potential applications.

For specific applications and for long-term tests, procedures should be agreed upon which reflect performance under actual conditions.

The actions of microorganisms on plastics are influenced by two different processes:

a) direct action: the deterioration of plastics which serve as a nutritive substance for the growth of the microorganisms;

b) indirect action: the influence of metabolic products of the micro-organisms, e.g. discolouration or further deterioration.

This International Standard deals with both of these two processes, as well as their combined action.

INTERNATIONAL STANDARD © ISO ISO 846:1997(E)

Plastics — Evaluation of the action of microorganisms

WARNING — Handling and manipulation of microorganisms which are potentially hazardous requires a high degree of technical competence and may be subject to current national legislation and regulations. Only personnel trained in microbiological techniques should carry out such tests. Codes of practice for disinfection, sterilization and personal hygiene must be strictly observed.

It is recommended that workers consult IEC 68-2-10:1988, appendix A "Danger to personnel", and ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

1 Scope

This International Standard specifies methods for determining the deterioration of plastics due to the action of fungi and bacteria and soil microorganisms. The aim is not to determine the biodegradability of plastics.

The type and extent of deterioration may be determined by

a) visual examination

and/or

b) changes in mass

and/or

c) changes in other physical properties.

The tests are applicable to all articles made of plastic that have an even surface and that can thus be easily cleaned. The exceptions are porous materials, such as plastic foams.

This International Standard uses the same test fungi as IEC 68-2-10. The IEC method, which uses so-called "assembled specimens", calls for inoculation of the specimens with a spore suspension, incubation of the inoculated specimens and assessment of the fungal growth as well as any physical attack on the specimens.

The volume of testing and the test strains used will depend on the application envisaged for the plastic. These parameters should therefore be agreed upon before the tests and should be stated in the test report.

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 291:—[1], *Plastics — Standard atmospheres for conditioning and testing.*

IEC 68-2-10:1988, *Basic environmental testing procedures — Part 2: Tests — Test J and guidance: Mould growth.*

3 Definitions

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

3.1 biodeterioration: A change in the chemical or physical properties of a material due to the action of a microorganism.

3.2 fungistatic effect: The antimycotic effect of an antimicrobial treatment which prevents a given material from being overgrown by fungi under moist conditions.

3.3 biodegradation: The term "*biodegradation*" is being discussed by TC 61/SC 5/WG 22, *Biodegradability*, and the official definition will be included here when it is available.

4 Principle

4.1 The test involves exposing test specimens of plastic to the action of selected test strains of fungi and bacteria (or, in the case of the soil-burial test, to microbially active soil) for specified or agreed periods of time under specified conditions of temperature and humidity.

At the end of the exposure, the test specimens are assessed before and/or after cleaning by visual examination and/or any change in mass or other physical properties is determined.

The results obtained with the specimens exposed to biological attack (batch I) are compared with those obtained from untreated specimens (batch 0) or sterile specimens (batch S) kept under the same conditions.

4.2 Short descriptions of the test methods used to determine the resistance of plastics to fungi (method A) or the fungistatic effects (methods B and B'), resistance to bacteria (method C) and resistance to soil microorganisms (method D) are given below.

4.2.1 Resistance to fungi

4.2.1.1 Method A: Fungal-growth test

Test specimens are exposed to a mixed suspension of fungus spores in the presence of an incomplete nutritive medium (without a carbon source). The fungi can only grow at the expense of the material. If the specimens contain no nutritive component, the fungi cannot develop mycelia and there is no deterioration of the plastic.

---

1) To be published. (Revision of ISO 291:1977)

Method A is suitable for the assessment of the inherent resistance of plastics to fungal attack in the absence of other organic matter.

It is recommended that, when method A is carried out, methods B and B' are also carried out to assist in the interpretation of the results.

4.2.1.2 Methods B and B': Determination of fungistatic effects

Test specimens are exposed to a mixed suspension of fungus spores in the presence of a complete medium, i.e. with a carbon source. Even if the plastic does not contain any nutritive elements, the fungi can grow over the specimens and their metabolic products can attack the material.

Any inhibition of the growth either on the plastic or in the growth medium (zone of inhibition) shows fungistatic activity of the plastic or the presence of a fungicidal treatment.

In method B', the specimens are not placed on the nutritive medium until it is completely overgrown.

Methods B and B' are used when surface contamination is expected. In order to save time, and for a better understanding of the phenomenon, it is recommended that the two methods are carried out simultaneously.

4.2.2 Method C: Resistance to bacteria

The action of bacteria on test specimens is assessed using an incomplete medium. If there is no growth in the agar round the specimen, then the specimen does not contain any nutritive components.

4.2.3 Method D: Resistance to microbially active soil (soil-burial test)

Test specimens are completely buried in natural soil with a known water-holding capacity and a specified moisture content (see annex A).

The soil-burial test has been included in this International Standard because many plastics are used in permanent contact with soil and exposed to high humidities.

4.3 Choice of properties for assessment of biodeterioration

The choice of the properties to be determined depends on the aim of the test. A visual assessment of biological attack should preferably always be made as the first stage in assessing the resistance of the plastic.

The measurement of changes in mass is recommended, especially for those plastics that contain biologically degradable substances, such as plasticizers, lubricants and stabilizers (as in plasticized PVC, for instance). The measured loss is, in this case, often lower than the actual loss as the biologically degradable substance is only partly utilized and the metabolic products often remain in the plastic.

When, above all, the surface is affected, it is recommended that determinations be made of those properties which clearly indicate surface changes, such as surface gloss, flexural properties, impact resistance and hardness.

5 Apparatus and materials

5.1 For all tests

5.1.1 Incubators

That used for tests involving fungal and bacterial attack shall be capable of controlling the temperature to ±1 °C at any temperature from 20 °C to 35 °C at a relative humidity of 90 % or greater.

That used for soil-burial tests shall be capable of controlling the temperature to ±1 °C at 29 °C at a relative humidity of 95% or greater.

NOTE — Experience has shown that it is preferable to use two incubators: one for Petri dish tests and another for soil-burial tests.

5.1.2 Oven, capable of controlling the temperature at 45 °C for drying test specimens and at between 103 °C and 105 °C for determining the water-holding capacity of soil.

5.1.3 Desiccator, capable of maintaining standard temperature and humidity conditions (23 °C and 50 % R.H.) for the conditioning of test and control specimens.

5.1.4 Autoclave, capable of maintaining a temperature and pressure of 120 °C and 2 bar, respectively, for sterilizing Petri dishes and soil.

5.1.5 Analytical balance, accurate to 0,1 mg.

5.1.6 Laboratory centrifuge.

5.1.7 Stereoscopic microscope, magnification × 50.

5.1.8 Glass or plastic disposable Petri dishes, of suitable size for exposing test specimens.

5.1.9 Glass containers, with a volume of about 1 litre (height 16 cm; diameter 11 cm), for example preserving jars with covers.

5.1.10 Distilled or deionized water.

The water used for the preparation of all solutions and nutritive media and for all determinations shall be distilled or deionized and have a conductivity of < 1 µS/cm.

5.1.11 Microbicidal solutions:

5.1.11.1 Ethanol-water mixture, in the proportions, by mass, of 70:30.

5.1.11.2 *o*-Phenylphenol.

Dissolve 1 g of *o*-phenylphenol in 50 ml of 90 % ethanol, make up to 1 000 ml with water and adjust the pH to 3,5 by adding lactic acid drop by drop.

The microbicidal solution used shall be stated in the test report.

5.2 For tests with fungi

5.2.1 Test fungi.

The test fungi shall be obtained from national culture collections. The strains to be used are listed in table 1, and be stated in the test report.

Table 1

Name	Strain
Aspergillus niger van Tieghem	ATCC 6275
Penicillium funiculosum Thom	CMI 114933
Paecilomyces variotii Bainier	ATCC 18502
Gliocladium virens Miller *et al.*	ATCC 9645
Chaetomium globosum Kunze: Fries	ATCC 6205

If there are technical reasons, and by agreement between the interested parties, other species may be used. In this case, too, the strains used shall be stated in the test report.

When carrying out tests on plastics intended for use in electronic components and electronic equipment, using the method specified in IEC 68-2-10, use *Aspergillus niger, Penicillium funiculosum*, *Paecilomyces variotii* and *Gliocladium virens* from table 1 and the four strains given in table 2.

Table 2

Name	Strain
Aspergillus terreus Thom	QM 82j
Aureobasidium pullulans (de Bary) Arnaud	ATCC 9348
Penicillium ochrochloron Biourge	ATCC 9112
Scopulariopsis brevicaulis (Saccardo) Bainier	CMI 49528

5.2.2 Stock strains.

Culture the test fungi (5.2.1) in tubes on agar slants of the following composition:

Oatmeal	20 g
Malt extract	10 g
Agar	20 g
Water	1 000 ml

Sterilize at 120 °C ± 1 °C for 20 min in an autoclave in an atmosphere saturated with water vapour.

After incubation at 29 °C ± 1 °C or 24 °C ± 1 °C, well sporulating cultures may then be used. They shall not be stored for more than 4 weeks at this temperature.

Because of the possibility of genetic and physiological changes in the test fungi during culturing on artificial media, the intervals between subculturing shall be reduced to a minimum by suitable measures (e.g. lyophilization of cultures, storage at + 4 °C or in liquid nitrogen).

5.2.3 Solutions and nutritive media:

5.2.3.1 Stock mineral-salt solution, of the following composition (use only chemicals of analytical grade or equivalent purity):

$NaNO_3$	2,0 g
KH_2PO_4	0,7 g
K_2HPO_4	0,3 g
KCl	0,5 g
$MgSO_4 \cdot 7H_2O$	0,5 g
$FeSO_4 \cdot 7H_2O$	0,01 g
H_2O	1 000 ml

Adjust the pH to 6,0 to 6,5 with sterile 0,01 mol/l NaOH solution.

5.2.3.2 Mineral-salt/wetting-agent solution, prepared by adding to 1 litre of stock mineral-salt solution (5.2.3.1) 0,1 g of a non-toxic wetting agent such as *N*-methyltaurine or polyglycol ether and sterilizing in an autoclave at 120 °C ± 1 °C for 20 min.

5.2.3.3 Mineral-salt/glucose solution, prepared by adding to stock mineral-salt solution (5.2.3.1) sufficient glucose to give a concentration of 30 g/l ± 1 g/l and sterilizing in an autoclave at 115 °C ± 1 °C for 30 min.

5.2.3.4 Incomplete agar medium, prepared by adding to stock mineral-salt solution (5.2.3.1) sufficient agar to give a concentration of 20 g/l. Dissolve the agar by boiling the solution whilst stirring. Sterilize in an autoclave at 120 °C ± 1 °C for 20 min. Adjust the pH to 6,0 to 6,5 with sterile 0,01 mol/l NaOH solution.

5.2.3.5 Complete agar medium, prepared by adding to the incomplete agar medium (5.2.3.4) sufficient glucose to give a concentration of 30 g/l ± 1 g/l. Sterilize in an autoclave at 115 °C ± 1 °C for 30 min. After sterilization, adjust the pH to between 6,0 and 6,5 at 20 °C with sterile 0,01 mol/l NaOH solution.

5.3 For tests with bacteria

5.3.1 Test bacterium *Pseudomonas aeruginosa*, strain NCTC 8060 or ATCC 13388.

A well-defined strain of the test bacterium shall be obtained from a national culture collection. Cultivate the test strain on brain-heart infusion agar (5.3.2.1).

If, by agreement, additional test bacteria are used, they shall be mentioned in the test report.

5.3.2 Nutritive media and solutions

5.3.2.1 Brain-heart infusion agar.

Casein soybean peptone agar may be used as an alternative.

The medium may be obtained from commercial suppliers and shall be prepared in accordance with the manufacturer's instructions.

5.3.2.2 Brain-heart infusion broth.

Casein soybean peptone broth may be used as an alternative.

The medium may be obtained from commercial suppliers and shall be prepared in accordance with the manufacturer's instructions.

5.3.2.3 Mineral-salt agar, prepared by making up a solution of the following composition:

KH_2PO_4	0,7 g
K_2HPO_4	0,7 g
$MgSO_4 \cdot 7H_2O$	0,7 g
NH_4NO_3	1 g
NaCl	0,005 g
$FeSO_4 \cdot 7H_2O$	0,002 g
$ZnSO_4 \cdot 7H_2O$	0,002 g
$MnSO_4 \cdot 7H_2O$	0,001 g
H_2O	1 000 ml

and adding 20 g of agar to the solution. Sterilize in an autoclave at 120 °C ± 1 °C for 20 min. Adjust the pH to 7,0 at 20 °C with 0,01 mol/l NaOH solution.

5.3.2.4 Sterile buffer solution, pH 7,0 at 20 °C.

Prepare the following two solutions separately:

KH_2PO_4	9,1 g/l	(solution A)
Na_2HPO_4	11,9 g/l	(solution B)

Mix 600 ml of solution A with 400 ml of solution B. Sterilize in an autoclave at 120 °C ± 1 °C for 20 min. Adjust the pH to 7,0 at 20 °C by adding 0,01 mol/l NaOH solution.

5.4 For soil-burial tests

Use an activated soil with a moisture content of (60 ± 5) % of the water-holding capacity of the soil (see annex A).

The water-holding capacity is the water content of a soil when it is saturated with water.

The pH of an aqueous soil extract (1 g of soil in 20 g of water) shall be between 4,0 and 7,0.

Determine the moisture content and water-holding capacity of the soil in accordance with annex A. If the moisture content of the soil exceeds the above figure, spread it out in a thin layer under ambient laboratory conditions. Do not heat the soil or allow it to dry out as this may affect the soil microflora. If the moisture content needs to be raised, use an aqueous solution of 1 g of ammonium nitrate and 0,2 g of dipotassium phosphate in 1 litre of water.

6 Test specimens

6.1 Shape and dimensions

The shape and dimensions of the specimens will depend on any tests to be carried out following exposure to the fungi, bacteria or soil.

If it is necessary to measure changes in the thickness of the specimens, use specimens taken from the original material. If the material is to be moulded before use, use specimens of maximum thickness 0,5 mm.

If changes in mass are to be measured, use square specimens of side 30 mm to 60 mm with a maximum thickness of 2 mm. When visual examination is used to assess changes in appearance, specimen dimensions are not as critical. However, a thickness of 0,5 mm to 2 mm is recommended.

Since the microorganisms may attack the surface of the plastic tested, only results using specimens of the same dimensions may be compared.

6.2 Specimen batches and numbers in each batch

6.2.1 Specimen batches

For each sample and each test method, prepare three batches of specimens:

batch 0: control specimens, stored under standard temperature and moisture conditions;

batch I: specimens inoculated with microorganisms and incubated;

batch S: sterile specimens, stored under the same conditions as batch I.

6.2.2 Numbers in each batch

For visual examination, prepare at least five specimens for each batch, i.e. a total of at least 15 specimens per sample and per test method.

For determination of mass changes, prepare at least six specimens for each batch, i.e. a total of at least 18 specimens per sample and per test method.

For other assessment procedures, use the number of specimens specified in the referring standard.

The test procedure for each assessment shall be carried out separately.

However, specimens for determining changes in mass or other physical properties may also be used for visual examination.

7 Preparation of specimens

7.1 Cleaning

Dip specimens for methods A and C into an ethanol-water mixture (5.1.11.1) for 1 min and dry at 45 °C for 4 h, unless they are adversely affected by ethanol. In the latter case, store the specimens in a sterile container, handling them with sterile forceps. Carry out all subsequent handling of the specimens using forceps to avoid contamination by extraneous organic matter.

Do not clean specimens for methods B, B' or D.

7.2 Labelling and storage

Store the cleaned and labelled (or marked) specimens in Petri dishes (5.1.8) at ambient temperature.

Labelling or marking may result in surface reactions by the plastic during the test. In such cases, store the specimens separately in suitable containers (e.g. Petri dishes) and mark the Petri dishes, not the specimens, to avoid surface reactions. In all other cases, the specimens may be labelled directly using a suitable marker.

7.3 Conditioning and weighing

Store batches of specimens used for determining change in mass at ambient temperature in a desiccator (5.1.3) until the mass of each specimen (m_1, m_2, m_3, etc.) is constant to the nearest 0,1 mg (usually after 48 h). Record the mass of each specimen. Unless otherwise agreed, specimens for visual examination and/or for determination of changes in physical properties other than mass do not need conditioning at this stage.

It may be agreed between the interested parties to store the specimens in a desiccator (5.1.3) at 45 °C. In this case, cool over silica gel to room temperature before use and store until constant mass is reached at 20 °C ± 1 °C and (65 ± 3) % R.H. If this procedure is followed, it shall be mentioned in the test report.

8 Procedures

8.1 Test temperature

Prepare and assess specimens at standard conditions (see ISO 291) of 23 °C ± 1 °C and (50 ± 3) % R.H.; incubate them at 24 °C ± 1 °C or 29 °C ± 1 °C.

8.2 Test methods

A general scheme of the test methods described is shown in table 3.

The choice of method and of the properties to be measured depends on the material under test and the conditions of use envisaged to it.

Table 3 — Summary of test methods

	Tests with fungi							Tests with bacteria		Tests with soil	
Method	A		B		B'			C		D	
Subclause	8.2.1		8.2.2		8.2.2.7			8.2.3		8.2.4	
Medium used	Incomplete agar medium (5.2.3.4)		Complete agar medium (5.2.3.5)		None	Complete agar medium (5.2.3.5)		Mineral-salt agar (5.3.2.3) inoculated as specified in 8.2.3.5		Soil (see 5.4)	
Batch	I	S	I	S	I	I	S	I	S	I	S
Solution sprayed on specimen[1)]	Sp-S	Ms-S	Sp-S	Ms-S	Sp-S	Sp-S	Ms-S	None	Ms-S	None	Ms-S
Incubation conditions	24 °C ± 1 °C or 29 °C ± 1 °C							29 °C ± 1 °C			
	4 weeks or more; > 95 % relative humidity[2)]										

1) Sp-S = spore suspension; Ms-S = microbicidal solution.

2) This humidity is reached by the agar medium in methods A, B, B' and C. For method D, the incubator shall have a controlled relative humidity of at least 95 %.

8.2.1 Fungal-growth test (method A)

8.2.1.1 Filling the Petri dishes

After sterilization, pour incomplete agar medium (5.2.3.4) into sterile Petri dishes to give a depth of about 5 mm. It solidifies on cooling.

8.2.1.2 Arrangement of test specimens

Place the specimens separately, as flat as possible, on the solidified medium, avoiding any contact between specimens or with the walls of the Petri dishes. Divide the prepared Petri dishes randomly into two equal batches, one labelled I, the other S.

If it is anticipated that the specimens may lift away from the medium, ballast them with weights.

8.2.1.3 Preparation of spore suspension

Produce a spore suspension from well sporulated cultures, using mineral-salt/wetting-agent solution (5.2.3.2), as follows:

8.2.1.3.1 Harvesting the spores

Introduce into each culture tube (see 5.2.2) 5 ml of mineral-salt/wetting agent solution. Gently scrape the surface of the sporulating culture with a sterile inoculation needle to obtain an aqueous suspension of the spores. Gently shake the culture tube to disperse the spores in the liquid. Repeat this procedure with the same culture tube three times. Then shake the spore suspension of each fungal culture with sterile glass beads and filter through a thin layer of sterile cotton or glass wool to remove mycelial fragments.

8.2.1.3.2 Washing the spores by centrifugation, and preparation of working suspensions

Aseptically centrifuge the filtered spore suspension and discard the supernatant liquid. Re-suspend the residue in 25 ml of mineral-salt solution (5.2.3.1) and centrifuge again. Suspend the washed residue in 50 ml of stock mineral-salt solution. This repeated washing of the spore suspensions is intended to guarantee that all surface-active substances are removed which might cause stress cracking in some plastics.

Adjust the concentration to about 10^6 spores/ml (determined using a counting chamber or by turbimetry).

Repeat these operations with each test fungus. Blend equal volumes of five suspensions containing the same number of spores to obtain the final mixed spore suspension ready for inoculation. Use the spore suspension within 6 h of preparation.

NOTE — When new plastics formulations are tested, the investigator may wish to carry out preliminary tests using individual fungi or selected combinations of fungi.

8.2.1.4 Spore viability check

Fill two sterile Petri dishes with complete agar medium (5.2.3.5), following the procedure given in 8.2.1.1 and inoculate with one drop of each of the spore suspensions (before blending the spore suspension). Incubate at 24 °C ± 1 °C or 29 °C ± 1 °C for 3 to 4 days (carry out the variability check at the same temperature as the actual determination). In the absence of copious growth, prepare a new spore suspension from other culture tubes and repeat the test.

8.2.1.5 Inoculation or disinfection of specimens

For each specimen in batch I, spray or pipette evenly on to the surface of the specimen and of the agar 0,1 ml of the spore suspension prepared in 8.2.1.3. For each specimen in batch S, pipette 3 ml of microbicidal solution (5.1.11) on to the surface.

8.2.1.6 Incubation

Incubate both the inoculated specimens and the sterile controls either at 24 °C ± 1 °C or at 29 °C ± 1 °C for 4 weeks, or longer by agreement between the interested parties. Take precautions to prevent condensed water dropping onto the surface of the specimens. If the test lasts more than 4 weeks, re-inoculate the specimens every 4 weeks in accordance with 8.2.1.5, using washed and centrifuged spores suspended in sterile water (see 8.2.1.3.2).

If a visual examination is required, the test may be terminated if fungal growth is visible to the naked eye during the 4 week incubation period.

If the result is not positive, the test period shall be extended. If the specimens are transferred to a freshly prepared agar substrate and re-inoculated at 4-week intervals, this will give better results than only repeated re-inoculation of the specimens.

8.2.2 Determination of fungistatic effect (method B)

8.2.2.1 Filling the Petri dishes

Follow the instructions given in 8.2.1.1 but using the complete agar medium (5.2.3.5).

8.2.2.2 Arrangement of test specimens

Follow the instructions given in 8.2.1.2. Do not clean the specimens with ethanol-water mixture unless there is a storage time of at least 72 h between cleaning and the start of the determination.

8.2.2.3 Preparation of spore suspension

Follow the instructions given in 8.2.1.3, but suspending the washed residue in mineral-salt/glucose solution (5.2.3.3).

8.2.2.4 Spore viability check

Follow the instructions given in 8.2.1.4.

8.2.2.5 Inoculation or disinfection of specimens

For each specimen in batch I, spray or pipette a suitable amount of the spore suspension prepared in 8.2.2.3 on to the specimen surface and agar surface. For each specimen in batch S, pipette a suitable amount of microbicidal solution (5.1.11) on to the surface.

8.2.2.6 Incubation

Follow the instructions given in 8.2.1.6.

If it is agreed that the incubation period should last longer than 4 weeks, spray or pipette the specimens at 4-week intervals with a small amount of mineral-salt/glucose solution (5.2.3.3).

8.2.2.7 Method B'

Variant B' of method B involves waiting until the medium is fully overgrown, but no sporulation is visible, before placing the specimens on the medium. In most cases, this method results in more severe attack than method B.

For method B', follow the instructions given in 8.2.2.1 and 8.2.2.2 but place specimens in only half of the Petri dishes. These constitute batch S. The Petri dishes with agar but without specimens constitute batch I. Place the other half of the specimens in unfilled Petri dishes (batch I_2). Thus the following batches are obtained:

batch S — Petri dishes with agar and specimens;

batch I — Petri dishes with agar but without specimens;

unfilled dishes — Petri dishes without agar but with specimens.

Then follow the instructions given in 8.2.2.3, 8.2.2.4 and 8.2.2.5, inoculating batch I and disinfecting batch S.

Incubate all three batches at 24 °C ± 1 °C or 29 °C ± 1 °C.

As soon as fungal growth is observed in batch I, but before any sporulation is visible (in general after 2 to 3 days), remove the specimens from the unfilled Petri dishes and place them on the cultures in batch I.

Re-incubate, following the instructions given in 8.2.2.6.

8.2.3 Procedure with bacteria (method C)

8.2.3.1 Cleaning the specimens

See 7.1.

Store the specimens in a sterile container, handling them with sterile forceps, handle them thereafter only with sterile forceps.

8.2.3.2 Preparation of mineral-salt agar medium

Prepare a sufficient amount of the medium, following the instructions given in 5.3.2.3.

Allow to cool to 45 °C and proceed as described in 8.2.3.5.

8.2.3.3 Preparation of bacterial cell suspension

From the bacterial stock culture on agar medium prepared in 5.3.1, inoculate brain-heart infusion broth (5.3.2.2) and incubate for 24 h at 29 °C ± 1 °C. Use a sterile platinum, nichrome or plastic loop to transfer the 24 h culture to 10 ml of sterile buffer solution (5.3.2.4). Dilute this suspension with sterile buffer solution to obtain a cell suspension containing about 10^6 cells per millilitre (determined e.g. using a counting chamber or by turbimetry). Use the cell suspension within 1 h.

Do not introduce any additional nutrients into the cell suspension.

8.2.3.4 Viability check

Add three drops of the cell suspension prepared in (8.2.3.3) to each of two Petri dishes with 10 ml of sterile brain-heart infusion agar (5.3.2.1) and incubate for 24 h to 48 h at 29 °C ± 1 °C. If the bacteria are not well established in both Petri dishes, repeat the determination with a new cell suspension.

8.2.3.5 Inoculation of mineral-salt agar medium

Inoculate the molten agar prepared in 8.2.3.2 with a sufficient amount of bacterial cell suspension to obtain a concentration of about 50 000 cells per millilitre of agar. Mix the agar and the cell suspension and pour the plates without delay.

8.2.3.6 Filling the Petri dishes and arrangement of test specimens

8.2.3.6.1 Batch I (inoculated specimens for incubation)

Pour a sufficient amount of the inoculated agar prepared in 8.2.3.5 into sterile Petri dishes to provide an agar layer about 5 mm deep.

After the agar has solidified, place one specimen on the surface of each agar layer and pour on to the specimen sufficient inoculated agar to cover it. Allow to gel.

The resultant layer shall cover the specimen to a depth of 1 mm.

8.2.3.6.2 Batch S (sterile controls)

Pour into sterile Petri dishes uninoculated mineral-salt agar prepared as described in 8.2.3.2. Disinfect up to six specimens by dipping them into *o*-phenylphenol solution (5.1.11.2) and placing them on the solidified agar. Disinfect the agar with the same solution. Cover the specimens with an uninoculated agar layer.

8.2.3.7 Incubation

Incubate both batches (I and S) at 29 °C ± 1 °C and 90 % relative humidity for 4 weeks, or longer by agreement between the interested parties.

If bacterial growth becomes clearly visible to the naked eye during the 4 week incubation period, the test may be considered as completed if only a visual examination is required.

8.2.4 Soil-burial test (method D)

8.2.4.1 Biological activity of the soil

Bury strips (2,5 cm × 10 cm) of bleached, untreated cotton fabric (mass per square metre, 250 g) in the soil (see 5.4) and incubate for 7 days. The strips shall retain less than 25 % of their original tensile strength at the end of this period. When the soil shows this level of cellulolytic activity, the activity of the entire flora will normally also be sufficient.

NOTE — It is recommended that a cotton control strip is always buried together with the specimens to check the biological activity of the soil.

8.2.4.2 Procedure

8.2.4.2.1 Fill a sufficient number of 1 litre preserving jars with test soil having a moisture content equal to (60 ± 5) % of the water-holding capacity of the soil (see 5.4).

8.2.4.2.2 For the determination itself, bury specimens in at least two jars, using a spatula and forceps, as shown in figure 1, for each incubation period. Introduce into each jar a control cotton strip for to check the activity of the soil (see 8.2.4.1). In order to ensure the circulation of oxygen, do not close the jars tightly but place a loop of approximately 1 mm wire between the cover and jar.

Do not compact the soil in the jars. The depth of the layer covering the specimens shall not exceed 12,5 cm. Square specimens for mass-loss determinations may be buried vertically and specimens for tensile tests horizontally in bigger jars.

8.2.4.2.3 For the control test under sterile conditions, sterilize the soil in tightly closed 1 litre preserving jars (at least two jars for each incubation period) together with a cotton strip (see 8.2.4.1) in an autoclave at 120 °C (pressure 2 bar) for 30 min on three successive days. Place two plastic specimens in each jar, having first dipped the specimens into *o*-phenylphenol solution (5.1.11.2). Finally, pour 3 ml of this solution over the soil.

WARNING — In order to prevent the tightly sealed containers from collapsing during cooling after sterilization, use an autoclave with a pressure overlay. When older autoclaves without such overlays are used, allow the jars to cool down to about 65 °C before the autoclave is opened.

NOTE — The soil and the specimens may also be sterilized using gamma radiation.

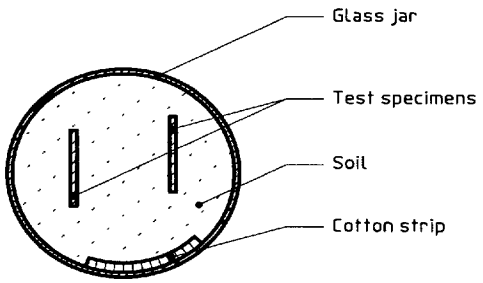

Figure 1 — Arrangement of test specimens in the soil in soil-burial tests (cross-section)

8.2.4.2.4 Incubate the prepared test jars for 4 weeks or longer in an incubator at 29 °C ± 1 °C and (97 ± 2) % relative humidity.

NOTE — The jars are incubated at (97 ± 2) % R.H. to prevent the soil from drying out.

In long-term soil-burial tests, check the moisture content of the soil at intervals by weighing the jars at the beginning of the determination and checking their mass at suitable intervals, adding 1 g/l ammonium nitrate solution if necessary.

A period of 4 weeks for soil-burial tests is only sufficient for plastics which deteriorate easily. In order to be able to differentiate between different plastics, the test period shall be extended to 6 months. To assess the long-term behaviour of plastics for damp courses for buildings, landfill liner materials, etc., carry out several determinations in parallel, removing specimens after 6 months and 12 months and, if necessary, depending on the decay rate, after 18 months and 24 months or after 24 months and 48 months.

9 Assessment

9.1 Assessment of fungal growth on the specimens by visual examination (methods A, B and D)

First examine the exposed specimens (batches I and S) with the naked eye and then, if necessary, with a stereoscopic microscope (at a magnification of × 50). Assess the fungal growth in accordance with the scale given in table 4. In the case of method A, also give the intensity of growth on the medium surrounding the specimens.

NOTE — In order to increase the accuracy of the assessment, it is suggested that a grid be placed over the specimen to enable percentage surface growth to be assessed.

If the results of the visual examination of the specimens in one batch vary by more than two scale ratings, repeat the determination with fresh specimens.

The examination of the cleaned specimens (see 9.2.1) may supply further information. Colour photography is a useful aid in recording the results of the visual examination.

Table 4 — Assessment of fungal growth

Intensity of growth	Evaluation
0	No growth apparent under the microscope.
1	No growth visible to the naked eye, but clearly visible under the microscope.
2	Growth visible to the naked eye, covering up to 25 % of the test surface.
3	Growth visible to the naked eye, covering up to 50 % of the test surface.
4	Considerable growth, covering more than 50 % of the test surface.
5	Heavy growth, covering the entire test surface.

9.2 Evaluation of the test specimens for the determination of changes in mass and/or in other physical properties

9.2.1 Cleaning

Remove the test specimens from the agar, dip them for 5 min into an ethanol-water mixture (5.1.11.1), rinse under running water, wipe with filter paper and allow to dry overnight at room temperature.

Sterilize jars with visual growth using either gas (e.g. ethylene oxide) or vapour (in an autoclave) at the end of the determination.

9.2.2 Change in mass

For the determination of the change in mass, place the cleaned specimens in a desiccator and weight them regularly to the nearest 0,1 mg until constant mass is attained (in general 48 h). Record the final masses as m_1', m_2', ...

NOTE — It is recommended that the procedure described in 7.3 is followed.

Determine, for each specimen, the difference in mass Δm before and after exposure, e.g. $\Delta m_1 = m_1' - m_1$. This difference is generally negative, corresponding to a loss in mass.

The inoculated specimens and the sterile control specimens shall have the same dimensions when mass changes are measured.

9.2.3 Determination of changes in other physical properties

Measure the properties of the exposed specimens and the unexposed control specimens — if possible simultaneously — in accordance with the respective material, product or test method standard, as follows:

Clean the specimens as in 9.2.1 and condition in accordance with the standard for the plastic moulding material concerned.

Select the property to be measured, the specimen-conditioning and test conditions and the dimensions of the specimens from the standard for the plastic moulding material concerned.

If necessary, the test conditions shall be agreed upon between the interested parties, in which case they shall be mentioned in the test report.

10 Expression of results

From the individual results, calculate the arithmetic means and standard deviations.

10.1 Visual assessment

Express the results of the visual assessment for each specimen in terms of a fungal-growth rating as given in table 4.

If an additional visual assessment of the specimens is carried out after cleaning (see 9.1), express the results obtained in the same way.

The results may be interpreted as shown in table 5.

Table 5 — Interpretation of results

Method	Intensity of growth[1)]	Assessment of test material
A	0	The material is not a nutritive medium for microorganisms (it is inert or fungistatic)
	1	The material contains nutritive substances or is contaminated to such a small degree that it permits only slight growth
	2 to 5	The material is not resistant to fungal attack and contains nutritive substances suitable for the development of microorganisms
B or B'	0	Strong fungistatic effect
	0 + inhibition zone round specimen	Strong fungistatic effect, extending into the zone surrounding the specimen by diffusion
	1	The material is not completely fungistatic
	2 to 5	Effectiveness decreasing down to complete absence of a fungistatic effect

1) See rating scale in table 4.

10.2 Change in mass

Determine, for each specimen, the change in mass Δm ($= m' - m$) and calculate and record the arithmetic mean for each batch, $\overline{\Delta m_0}$, $\overline{\Delta m_I}$ and $\overline{\Delta m_S}$.

Calculate, to the first decimal place, the average percentage change in mass using the formula

$$\frac{\overline{\Delta m_I} - \overline{\Delta m_S}}{\overline{m_e}} \times 100 \qquad \ldots (1)$$

where $\overline{m_e}$ is the mean of the original specimen masses.

By means of statistical analysis (e.g. Student's t-test), determine whether the mass of the specimens has changed significantly (confidence limit 99 %).

Formula (1) is only applicable if the specimens in batch I and the specimens in batch S have the same dimensions and therefore comparable mass before exposure.

10.3 Changes in other physical properties

Calculate, for each batch (0, I and S) the arithmetic mean values of the change in each property and record them as $\overline{V_0}$, $\overline{V_I}$ and $\overline{V_S}$.

For each property, calculate the percentage change in the inoculated specimens with respect to the sterile specimens from the formula

$$\frac{\overline{V_I}}{\overline{V_S}} \times 100 \quad \text{... (2)}$$

For each property, calculate the percentage change in the inoculated specimens compared with the control specimens from the formula

$$\frac{\overline{V_I}}{\overline{V_0}} \times 100 \quad \text{... (3)}$$

The first value characterizes the biological attack better than the second.

11 Accuracy of the measurements

All measurements are performed with the precision of the respective standard. Results and determinations are reported with statistical analysis (mean/standard deviation).

The accuracy of results for changes of mass, dimensions or other physical properties will depend on the precision of the test procedure and the variability inherent in the exposures conducted according to this International Standard.

The accuracy of results from visual evaluations can be very dependent on the person conducting the evaluation. Therefore, comparisons between materials that are based on changes in appearance should only be made when the photographic documentation is recommended in such cases.

12 Test report

The test report shall include the following particulars:

a) a reference to this international Standard;

b) all information necessary for the complete identification of the material tested;

c) the dimensions of the specimens;

d) the incubation and incubation temperature used;

e) the type of microbicidal solution (5.1.11) used;

f) the method(s) used (A, B, B', C or D) and the number determinations carried out in each case;

g) the fungi, bacteria and soil used;

h) details of the origin of the fungal and bacterial strains and the soil;

i) the physical properties measured;

j) the methods used to measure the physical properties;

k) the results obtained:

1) the fungal-growth rating for each specimen (see 10.1),

2) the absolute change in mass, in grams, for each specimen, the mean for each batch and the average percentage change (see 10.2),

3) the mean change in each other property measured for each batch, plus the percentage changes with respect to the sterile specimens and with respect to the control specimens (see 10.3);

l) any special observations, e.g. infections by fungi and bacteria other than the test organisms, unusual growth characteristics, factors influencing sporulation, any discoloration;

m) any deviations from this International Standard;

n) all details necessary for the identification of the test laboratory;

o) the date(s) of the determinations;

p) the name and signature of the head of the test laboratory.

13 Bibliography

EMPA 223/23:1982, *Prüfung der Widerstandsfähigkeit von bahnenförmigen Kunststoffen gegen Mikroorganismen im Erdeingrabungsversuch,* Eidg. Materialprüfungs- und Forschungsanstalt (EMPA), St. Gallen, Switzerland.

Annex A
(normative)

Determination of the water content and water-holding capacity of a soil

A.1 General

Any test soil with the prescribed cellulolytic activity (8.2.4.1) may be used for these determinations. Comparisons between commercial standard soils and commercial compost soils have been shown to give comparable results. Each soil shall be pre-incubated at approximately 60 % of its water-holding capacity and 25 °C to 30 °C for 2 to 3 months before starting the test. Every determination shall be carried out at this water content to optimize microbial activity. The water content specified for a soil with a water-holding capacity of 100 % is 60 %. The water-content values for soils with a 60 % and 180 % water-holding capacity are 36 % and 108 %, respectively.

A.2 Determination of water content

Spread out approximately 50 ml of soil in each of three Petri dishes. Dry the soil in the dishes to constant mass by heating in an oven at 104 °C ± 1 °C for 4-h periods and cooling in a desiccator and weighing, to the nearest 1 mg, after each heating period. Constant mass can be assumed to have been reached when two consecutive weighings differ by less than 0,1 %.

For soils used previously, the previously determined drying time may be used without checking that constant mass has been reached.

For each Petri dish, calculate the water content as a percentage of the dry mass of the soil as shown in the example, rounding the result to the nearest 1 %. Calculate the mean of the three determinations.

EXAMPLE

Petri dish, empty	11,325 g
Dish containing moist soil	20,475 g
Moist soil only	9,150 g
Dish containing dried soil	16,600 g
Dried soil only	5,275 g
Water content (loss on drying)	3,875 g
Water content, as percentage of dried-soil mass	73 %

A.3 Determination of water-holding capacity

Fill each of three 50 ml glass filter crucibles (filter size 3, e.g. 2D3) with soil to 0,5 cm below the brim. Drop the crucible three times from a height of 1 cm on to a wood surface to compact the soil.

Then place each filter crucible in a glass beaker and fill the beaker with water until the water level in the filter crucible reaches a level 1 cm above the filter. When the upper soil appears moist as a result of capillary action, add more water till it covers the soil surface.

After 12 h to 16 h (overnight), remove the filter crucible from the beaker. Using a water-jet pump, suck off the water not retained in the soil, maintaining the suction for 10 min ± 1 min and keeping the crucible covered with a wet cloth weighted with a glass plate.

Dry the water-saturated soil in the filter crucible to constant mass by heating in an oven at 104 °C ± 1 °C for 4-h periods and cooling in a desiccator and weighing, to the nearest 1 mg, after each heating period. Constant mass can be assumed to have been reached when two consecutive weighings differ by less than 0,1 %.

For soils used previously, the previously determined drying time may be used without checking that constant mass has been reached.

For each filter crucible, calculate the water-holding capacity as a percentage of the dry mass of the soil as shown in the example, rounding the result to the nearest 1 %. Calculate the mean of the three determinations.

EXAMPLE

Filter crucible, empty	11,325 g
Filter crucible containing fresh soil	20,475 g
Fresh soil	9,150 g
Crucible containing water-saturated soil	24,105 g
Water-saturated soil only	12,780 g
Filter crucible containing dried soil	16,600 g
Dried soil only	5,275 g
Water-holding capacity, in grams	7,505 g
Water-holding capacity, as percentage of dried-soil mass	142 %

Result: The maximum quantity of water the soil can retain is 142 % of its dry mass.

Annex B
(informative)

Precision

The precision of methods A, B and C was determined in round-robin tests before the first edition of ISO 846 was published in 1978. Methods A, B, C and D were also determined in round-robin tests within the Plastics Working Group of the IBRG (International Biodeterioration Research Group).The results of these tests have been published in the following papers:

Hitz, H.R., Merz, A., and Zinkernagel, R. "Determination of the resistance of plasticised PVC to attack by fungi and bacteria by the weight loss method and evaluation of mechanical properties. A report on the cooperative test of Task Group A of ISO Technical Committee 61, Working Group 6 (biological attack)", *Material u. Organismen,* **2** (4), pp. 271-296 (1967).

Seal, K.J., and Pantke, M. "An interlaboratory investigation into the biodeterioration testing of plastics, with special reference to polyurethanes — Part 1: Petri dish test", *Material u. Organismen,* **21** (2), pp. 151-164 (1986).

Pantke, M., and Seal, K.J. "An interlaboratory investigation into the biodeterioration testing of plastics, with special reference to polyurethanes — Part 2: Soil-burial experiments", *Material u. Organismen*, **25** (2), pp. 87-98 (1990).

Seal, K.J., and Pantke, M. "An interlaboratory investigation into the biodeterioration testing of plastics, with special reference to polyurethanes — Part 3: Tests with bacteria", *Material u. Organismen*, **25** (4), pp. 241-254 (1990).

In the IBRG soil-burial experiments, two different soils were used. Additional soil-burial experiments were performed within WG 5 *Biological textile-testing methods* of SC 3 in CEN/TC 248, *Textiles and textile products,* using different soils. Experiments have shown that the soil does not need to be specified, but the water content has to be controlled at 60 % of the water-holding capacity in order to obtain reproducible results.

Annex C
(informative)

Information on test fungi

Name	Strains used in this International Standard	Identical strains
Aspergillus niger van Tieghem	ATCC 6275	CBS 131.52 CMI 45551 DSM 1957 IFO 6341 NRRL 334 QM 324 QM 458
Penicillium funiculosum Thom	CMI 114933	ATCC 36839 CBS 631.66 DSM 1944
Paecilomyces variotii Bainier	ATCC 10121 ATCC 18502	CBS 284.48 CMI 40025 DSM 1961 NRRL 1115 QM 6764
Gliocladium virens Miller *et al.*	ATCC 9645	CBS 430.54 CMI 45553 DSM 1963 IFO 6355 NRRL 2314 QM 365
Chaetomium globosum Kunze: Fries	ATCC 6205	CBS 148.51 CMI 45550 DSM 1962 NRRL 1870 QM 459
Aspergillus terreus Thom	QM 82j	ATCC 10690 CBS 377.64 CMI 45543 DSM 1958 IFO 6346 NRRL 571
Aureobasidum pullulans (de Bary) Arnaud	ATCC 9348	CMI 145194 DSM 2404 QM 3090
Penicillium ochrochloron Biourge	ATCC 9112	ATCC 9824 CBS 110.66 CMI 62271 DSM 1945 NRRL 744 QM 477
Scopulariopsis brevicaulis (Saccardo) Bainier	CMI 49528	ATCC 36840 QM 9985

ICS 07.100.99; 83. 080.01

Descriptors: plastics, plastics products, pest resistance, microorganisms, tests, pest resistance tests, physicochemical tests, estimation, deterioration, biodeterioration, specimen preparation.

Price based on 22 pages