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Plastics — Evaluation of the action of microorganisms

Plastiques — Évaluation de l'action des micro-organismes

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Foreword

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

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

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

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

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

This third edition cancels and replaces the second edition (ISO 846:1997), which has been technically revised. The main changes compared to the previous edition are as follows.

— The size of the test specimens has been defined as (50 mm ± 1 mm) × (50 mm ± 1 mm). A fixed size allows the determination of any edge effects associated with the area 5 mm from the outer edge (see new Annex C). In this way, the evaluation of growth on the test specimens is harmonized.

— New Annexes B and C have been added and the old annexes have been renumbered.

— The former Annex C has been updated and renumbered as Annex D.

— Test A only:

  Stainless steel coupons acting as negative control specimens have been introduced to provide a reference for where fungal growth occurs in the Petri dish, even though no nutrients have been added to the test design.

  The test design does not use an agar-medium any more to provide the source of moisture to allow 95 % ± 5 % relative humidity to be achieved. Instead the test specimens are stored in closed containers that include a water reservoir to provide a relative humidity 95 % ± 5 % around the test specimens during incubation;

  A grid has been introduced for use during the evaluation of the area of growth observed on the surface of the test specimens. The use of the grid provides and objective mechanisms for assessing growth and is explained in the new Annex C.

— Test B has been deleted.

— Positive control specimens (test specimens that allow fungal growth) have been introduced to allow the determination of basic fungistatic effects of samples that contain biocides.

— The fungal inoculum has been revised to be consistent with other referenced test standards and changes to the names of fungal strains have been incorporated.

— The media used in the test have been revised based on the experience of various laboratories.

— A staining method has been proposed to aim assessment.

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

Introduction

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

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

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

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 microorganisms, e.g. discolouration or further deterioration.

This document deals with both processes as well as their combined action.

Changes to the method are based on discussions among laboratories that have performed the test for at least 5 years. On an international level, discussions have taken place within the Plastic Group of the International Biodeterioration Research Group (IBRG) between scientists with extensive experience with this document as well as the testing of the interaction between microorganisms and plastics.

Plastics — Evaluation of the action of microorganisms

WARNING — Handling and manipulation of microorganisms which are potentially hazardous requires a high degree of technical competence. Only personnel trained in microbiological techniques should carry out such tests. Codes of practice for disinfection, sterilization and personal hygiene shall be strictly observed. It is recommended that workers consult IEC 60068-2-10 and ISO 7218.

1 Scope

This document 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 or the deterioration of natural fibre composites.

The type and extent of deterioration can 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 plastics that have an even surface and that can thus be easily cleaned. The exceptions are porous materials, such as plastic foams.

This document uses the same test fungi as IEC 60068-2-10. 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 depend on the application envisaged for the plastic.

2 Normative references

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

ISO 13934-1:2013, *Textiles — Tensile properties of fabrics — Part 1: Determination of maximum force and elongation at maximum force using the strip method*

EN 10088-1, *Stainless steels — Part 1: List of stainless steels*

EN 10088-2, *Stainless steels — Part 2: Technical delivery conditions for sheet/plate and strip corrosion resisting steels for general purposes*

EN 13697:2015, *Chemical disinfectants and antiseptics — Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas — Test method and requirements without mechanical action (phase 2, step 2)*

IEC 60068-2-10, *Environmental testing — Part 2-10: Tests — Test J and guidance: Mould growth*

3 Terms and definitions

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

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

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
biodeterioration
undesired change in the properties, such as colour, strength, mass, of a material due to the action of a microorganism

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

4 Principle

4.1 General

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 microorganisms (test series I) are compared with those obtained from retained reference specimens (test series 0) or sterile specimens (test series S) kept under the same conditions.

In the case of testing fungistatic properties, a visual assessment is made between test specimens free of biocides and with those containing biocides to demonstrate the effect of a biocide in a qualitative manner.

Short descriptions of the test methods used to determine the resistance of plastics to fungi (method A) or the fungistatic effects (method B), resistance to bacteria (method C) and resistance to soil microorganisms (method D) are given in 4.2 to 4.4.

4.2 Resistance to fungi

4.2.1 Method A: Fungal-growth test

Test specimens are exposed to a mixed suspension of fungal spores in the presence of a humidity ≥95 % relative humidity. After the limited nutrients from the spore itself are depleted through formation of a germination tube, the fungi can only grow at the expense of the material of the test specimens. If the specimens contain no nutritive component, the fungi cannot develop mycelia and there will be no deterioration of the plastic.

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

4.2.2 Method B: Determination of fungistatic effects

Test specimens are exposed to a mixed suspension of fungus spores in the presence of a complete nutrient 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 by metabolizing the nutrient-agar medium.

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

In order to show a basic qualitative effect of a biocide in a plastic material, specimens free from biocide shall be included in the test. Only if these biocide-free specimens show more growth than the specimens containing biocides can a qualitative indication of fungistatic or fungicidal efficacy be determined.

4.3 Method C: Resistance to bacteria

The action of bacteria on test specimens is assessed using an incomplete medium without a carbon source[1)]. If there is no growth in the agar surrounding the specimen, then the specimen does not contain any nutritive components.

If a material to be tested claims added functionality, such as a product with hygienic effects, the plastic material should be tested according to ISO 22196 which provides guidance for measuring the basic antibacterial performance of non-porous (plastic) materials that have been treated with a biocide with the intention of introducing antibacterial/hygienic properties into that material.

4.4 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 document because many plastics are used in permanent contact with soil and exposed to high humidities.

4.5 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 shall always be made as the first stage in assessing the resistance of the plastic.

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, capable of controlling the temperature to ±1 °C at 29 °C at a relative humidity of ≥ 95 %.

5.1.2 Oven, capable of controlling the temperature at 45 °C ± 1 °C for drying test specimens and at between 103 °C and 105 °C for determining the water-holding capacity of soil.

5.1.3 Climatic chamber, 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 glass containers or glass Petri dishes and soil.

5.1.5 Analytical balance, accurate to 0,1 mg.

5.1.6 Laboratory centrifuge.

1) Agar-agar used in media needs to be very low in carbon.

5.1.7 Stereoscopic microscope, magnification × 50.

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

5.1.9 Glass containers with lid, with a volume of at least 1 l (height approximately. 16 cm; diameter approximately 11 cm), with sufficient space to allow a reservoir of water to be set-up below the Petri dishes.

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

5.1.12 Stainless steel coupons (method A).

These shall be 1.4301 (in accordance with EN 10088-1) stainless steel discs (about 2 cm in diameter) with Grade 2 B (in accordance with the requirements of EN 10088-2) finish on both sides. The surface shall be as flat as possible and the stainless steel should have a gauge of 1,2 mm or 1,5 mm.

NOTE Suitable stainless steel discs can usually be purchased from local engineering companies.

5.1.13 Buchner funnel, with a sintered filter.

5.1.14 Grid (5 mm × 5 mm), inscribed on clear carrier (e.g. glass or plastic) to be used to evaluate the colonisation of the surface of test specimens by fungi.

5.1.15 *o*-phenylphenol solution.

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.

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 shall be stated in the test report. Alternative culture collections and strain numbers of the same fungal strains are listed in Annex C.

Table 1 — Fungal strains to be used when testing plastics without electronic application

Name	Strain
Aspergillus niger	ATCC 6275
Penicillium pinophilum	ATCC 36839
Paecilomyces variotii	ATCC 18502
Trichoderma virens	ATCC 9645
Chaetomium globosum	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 performing tests on plastics intended for use in electronic components and electronic equipment, using the method specified in IEC 60068-2-10 use *Aspergillus niger*, *Penicillium pinophilum*, *Paecilomyces variotii* and *Trichoderma virens* from Table 1 and the three strains given in Table 2.

Table 2 — Fungal strains to be used when testing electronics

Name	Strain
Aspergillus niger	ATCC 6275
Penicillium pinophilum	ATCC 36839
Paecilomyces variotii	ATCC 18502
Trichoderma virens	ATCC 9645
Chaetomium globosum	ATCC 6205
Aspergillus terreus	ATCC 10690
Hormoconis resinae	DSM 1203
Scopulariopsis brevicaulis	ATCC 36840

5.2.2 Stock strains

Culture the test fungi (5.2.1) in tubes on agar slants or in Petri dishes containing malt-extract agar of the following composition:

Malt extract	30 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, 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 sub-culturing shall be reduced to a minimum by suitable measures (e.g. lyophilization of cultures, storage at +4 °C or in liquid nitrogen or on cryo-beads at –70 °C).

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 l 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/I. Dissolve the agar by boiling the solution while 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. 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 Casein soybean peptone agar, prepared in accordance with the manufacturer's instructions.

The medium may be obtained from commercial suppliers.

5.3.2.2 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 di-potassium phosphate in 1 l of water.

6 Test specimens

6.1 Shape and dimensions

The shape and dimensions of the specimens will depend on any tests to be performed 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 with a maximum thickness of 0,5 mm.

If changes in mass are to be measured, use square specimens of size of (50 mm ± 1 mm) × (50 mm ± 1 mm) with a maximum thickness of 2 mm. 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 test series and numbers in each test series

6.2.1 Specimen test series

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

— **test series 0:** climate control specimens, stored under standard temperature and standard *moisture conditions;*

  — minimum 2 specimens

— **test series S:** sterile specimens, stored under the same conditions as test series I.

  — minimum 2 specimens

— **test series I:** test specimens inoculated with microorganisms and incubated;

  — minimum 5 test specimens of each sub-test series a and/or b (see Table 3) according to the purpose of the test.

6.2.1.2 Sub-test series a (without biocide) and sub-test series b (with biocide).

If the test specimens contain biocides and the test is intended to show the biocidal efficacy of an active substance intended to protect the material from biodeterioration, then for test series I two sets of test specimens have to be tested: one without a biocide (sub-test series a) and one test series with the biocide (sub-test series b). Therefore, 10 test specimens in total need to be inoculated. Ideally, these test specimens should be identical except for their biocide content. This test set-up would allow the demonstration of the basic preservative efficacy for an active substance.

6.2.1.3 Sub-test series c (for Test A only): stainless steel coupons as negative controls (minimum 3 specimens).

These control specimens are comprised of non-corrodible stainless steel, which contains no nutrient source for fungi. They are inoculated, incubated and assessed (see Table 4) in the same way as the test specimens in test series I. Should growth occur on these inert surfaces this indicates that nutrients have been unintentionally transported onto the surface of the control specimen with the inoculum. Any growth on these negative control specimens shall be reported according to Table 4 as well as whether growth is more or less than on the other inoculated specimens in test series I.

6.2.2 Numbers in each test series

For visual examination, prepare a total of at least 11 specimens per plastic sample and per test method. Additionally, prepare 3 negative control specimens (stainless steel coupons), if testing according to Method A of this document. Prepare 5 specimens without biocide as positive controls, if testing for biocidal effects according to Method B. These coupons shall be cleaned according to EN 13697.

NOTE The cleaning process described in EN 13697 ensures that no fatty residues from production are left on the coupons.

For determination of mass changes, prepare at least six specimens for each test series, 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 the microbicidal solution (5.1.11) for 1 min and either dry at 45 °C for 4 h or, alternatively, for 72 hours at room temperature under sterile conditions, 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.

The metal coupons that serve as negative control specimens for method A need to be cleaned according to EN 13697:2015, 5.2.3 (see Annex B)

Do not clean specimens for methods 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 each series of test specimens used for determining change in mass in a desiccator at ambient temperature 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 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 room temperature and incubate them at 29 °C ± 1 °C.

8.2 Test methods

8.2.1 General

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		C		D	
Subclause	8.2.2		8.2.3		8.2.4		8.2.5	
Medium used	none		Complete agar medium (5.2.3.5)		Incomplete agar medium (5.2.3.4) inoculated as specified in 8.2.4.5		Soil (see 5.4)	
Test series	I	S	I	S	I	S	I	S
Sub-test series	a[a], c[b]		a[a], b[c]					
Solution sprayed on specimens[c]	Sp-S	Ms-S	Sp-S	Ms-S	None	Ms-S	None	Ms-S
Incubation conditions	29 °C ± 1 °C							
	4 weeks or more;				≥95 % relative humidity[d]			

Sp-S = spore suspension;

Ms-S = microbicidal solution.

NOTE "I" indicates **i**noculated test specimens, "S" indicates **s**terile test specimens in this table.

a Test specimens (with or without biocide).

b Negative control specimens (here: stainless steel coupon).

c Test specimens without biocide (ideally, these test specimens should be identical to a1, but without biocide).

d This humidity is achieved via a humid chamber in method A and via the agar medium in methods B and C. For method D this RH is achieved when soil moisture and temperature are according to the standard and the glass container has a lid. To confirm the RH use a calibrated data logger in the test set-up.

8.2.2 Fungal-growth test (method A)

8.2.2.1 Arrangement of test specimens in humid chamber

8.2.2.1.1 Preparation of humid chamber

A humid chamber needs to be prepared where the test specimens can be incubated at a relative humidity of 95 % ± 5 %.

NOTE This level of humidity can be generated when the Petri-dishes, holding the test specimens, are kept above a reservoir of water surface, for example, by placing them on a stainless steel mesh above the water in a closed container.

8.2.2.1.2 Placing of test specimens in humid chamber

Place the specimens separately into Petri dishes, as flat as possible onto the bottom of the Petri dish, avoiding any contact between specimens and with the walls of the Petri dishes. Close the Petri dish with a vented lid to allow the humid air into the Petri dish.

Divide the prepared Petri dishes randomly into test series as required for the test to be performed.

If it is anticipated that the specimens may lift away from the bottom of the Petri dish ballast the area of the edge (outer 5 mm) of the test specimens with inert weights (e.g. made from glass or stainless steel) to keep them as flat as possible.

8.2.2.2 Preparation of spore suspension

8.2.2.2.1 General

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

8.2.2.2.2 Harvesting the spores

Introduce into each culture tube or Petri dish (see 5.2.2) 5 ml of mineral-salt/wetting agent solution. Gently scrape the surface of the sporulating culture with a sterile tool, e.g. inoculation needle, to obtain an aqueous suspension of the spores. Gently shake the culture vessel to disperse the spores in the liquid. Repeat this procedure with the same culture vessel 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 or a Buchner funnel with a sintered glass filter to remove mycelial fragments.

8.2.2.2.3 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 number concentration of spores to about 10^6/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 can carry out preliminary tests using individual fungi or selected combinations of fungi.

8.2.2.3 Spore viability check

Fill two sterile Petri dishes with complete agar medium (5.2.3.5), following the procedure given in 8.2.2.1 and inoculate with one drop of each of the spore suspensions (before blending the spore suspension). Incubate at 29 °C ± 1 °C for 3 to 4 d (carry out the viability check at the same temperature as the actual test). In the absence of copious growth, prepare a new spore suspension from other culture tubes and repeat the test.

8.2.2.4 Inoculation or disinfection of specimens

For each specimen in test series 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 8.2.2.2. in a manner to achieve in average a minimum

number of 1 000 spores per cm^2, taking care that the spore suspension does not settle or separate during delivery.

For each specimen in test series S, pipette 3 ml of microbicidal solution (5.1.11) on to the surface.

8.2.2.5 Incubation

Incubate both the inoculated specimens and the sterile controls either 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 8.2.2.2, using washed and centrifuged spores suspended in sterile water (see 8.2.2.2.3).

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.

8.2.3 Determination of fungistatic effect (method B)

8.2.3.1 Filling the Petri dishes

After sterilization, pour complete agar medium (5.2.3.5) into sterile Petri dishes to give a depth of about 5 mm. It solidifies on cooling.

8.2.3.2 Arrangement of test specimens

Place the specimens separately into Petri dishes, as flat as possible, onto the solidified medium, avoiding any contact between specimens and with the walls of the Petri dishes. Divide the prepared Petri dishes randomly into test series as required for the test to be performed.

If it is anticipated that the specimens may lift away from the medium ballast the area of the edge (outer 5 mm) of the test specimens with inert weights (e.g. made from glass or stainless steel) to keep them as flat as possible.

8.2.3.3 Preparation of spore suspension

Follow the instructions given in 8.2.2.2, but suspending the washed residue in mineral-salt/glucose solution (5.2.3.3).

8.2.3.4 Spore viability check

Follow the instructions given in 8.2.2.3.

8.2.3.5 Inoculation or disinfection of specimens

For each specimen in test series I, spray or pipette a suitable amount of the spore suspension prepared in 8.2.3.3 on to the specimen surface and agar surface. For each specimen in test series S, pipette a suitable amount of microbicidal solution (5.1.11) on to the surface.

8.2.3.6 Incubation

Follow the instructions given in 8.2.2.5.

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).

8.2.4 Procedure with bacteria (method C)

8.2.4.1 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.

8.2.4.2 Preparation of mineral-salt agar medium

Prepare a sufficient amount of the medium, following the instructions given in 5.2.3.4.

Allow to cool to 45 °C and proceed as described in 8.2.4.5.

8.2.4.3 Preparation of bacterial cell suspension

From the bacterial stock culture on agar medium prepared in 5.3.1, inoculate casein soybean peptone broth (5.3.2.1) 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 (5.3.2.2). 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 turbidimetry). Use the cell suspension within 1 h.

Do not introduce any additional nutrients into the cell suspension.

8.2.4.4 Viability check

Add three drops of the cell suspension prepared in (8.2.4.3) to each of two Petri dishes with 10 ml of sterile casein soybean peptone 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.

8.2.4.5 Inoculation of mineral-salt agar medium

Inoculate the molten agar prepared in 8.2.4.2 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.

8.2.4.6 Filling the Petri dishes and arrangement of test specimens

8.2.4.6.1 Test series I (inoculated specimens for incubation)

Pour a sufficient amount of the inoculated agar prepared in 8.2.4.5 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 about 1 mm.

8.2.4.6.2 Test series S (sterile controls)

Pour into sterile Petri dishes uninoculated mineral-salt agar prepared as described in 8.2.4.2. Disinfect up to six specimens by cleaning them with the microbicidal solution (5.1.11) and placing them on the solidified agar. Cover the specimens with an uninoculated agar layer.

8.2.4.7 Incubation

Incubate both test series (I and S) at 29 °C ± 1 °C and 95 % ± 5 % 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.

8.2.5 Soil-burial test (method D)

8.2.5.1 Biological activity of the soil

Bury strips (25 mm ± 3° mm × 100 mm ± 10° mm) of bleached or non-bleached, untreated cotton fabric (mass per square metre, 250 g ± 25 g) in the soil (see 5.4) and incubate for 7 d. 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.

Determine the tensile strength of the cotton strips before and after incubation following ISO 13934-1.

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

8.2.5.2 Procedure

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

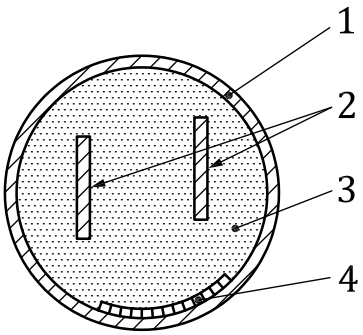
8.2.5.2.2 For the determination itself, bury specimens in at least two jars, using a spatulum and forceps, as shown in Figure 1, for each incubation period. Introduce into each jar a control cotton strip to check the activity of the soil (see 8.2.5.1). In order to ensure the circulation of oxygen, do 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.

8.2.5.2.3 For the control test under sterile conditions, sterilize the soil in tightly closed 1 l glass jars (at least two jars for each incubation period) together with a cotton strip (see 8.2.5.1) 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 (5.1.15). Finally, pour 3 ml of this solution over the soil.

WARNING — In order to prevent the tightly sealed glass jars 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 can also be sterilized using gamma radiation (25 KGy).



Key

1 glass jar
2 test specimens
3 soil
4 cotton strip

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

8.2.5.2.4 Incubate the prepared test jars for 4 weeks or longer in an incubator at 29 °C ± 1 °C and 95 % ± 5 % relative humidity.

NOTE The jars are incubated at high humidity 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 (test series I and S) with the naked eye and then, if necessary, with a stereoscopic microscope (at a magnification of × 50).

For fine mycelium on a light background, it has been shown that it is helpful to stain the mycelium with methylene blue. For this method, prepare a solution (0,001 5 g/l) of methylene blue in water. Using forceps, take the specimen and immerse it briefly into this solution. Gently agitate the test specimen in the solution and then remove it to stand for approximately 10 s. To rinse excess stain from the specimens, allow fresh tap water to run into a small vessel until the vessel overflows. Hold the stained test specimens in the vessel, taking care not to hold them directly under the water jet, but into the gentle through-flow of water in the vessel. After any excess of stain has been rinsed away, the mycelium will appear light blue. Caution: Some plastics will also take up the stain. Try the effect of staining on a piece of plastic that is not part of the test prior to using this method on the specimens. When using this staining method for the assessment of mycelium growth, make a remark in the test report that the test specimens had been stained. When using this technique keep in mind that the mycelium would have been more easily visible to the naked eye when grown on a darker coloured test specimen.

Assess the fungal growth in accordance with the scale given in Table 4 observing the instructions in Annex C. In the case of method B, also report if a zone free of growth is visible in the medium surrounding the specimens and report how wide it is.

Edge effect: when test specimens are only slightly overgrown at the edges. This can have several causes. For example:

— the material to be tested is layered and, whereas the outer surface cannot be overgrown, the inner layers can provide a source of nutrients for fungi and have been exposed at the edges;

— the mycelia of the fungi appear to grow on the edge of the test specimen, whereas this is simply an "overhang" from mycelium growing on the surrounding medium.

Report when edge effects are seen after incubation, but disregard it during the assessment of fungal growth as long it is no wider than 5 mm from each side.

If the results of the visual examination of the specimens in one test series vary by more than two scale ratings, it is recommended to repeat the determination with fresh specimens.

The examination of the cleaned specimens (see 9.2.1) may supply further information.

Table 4 — Assessment of fungal growth on test specimens and on negative control specimens (stainless steel coupons) in test A and, if needed, on positive control specimens in test B

Intensity of growth	Evaluation
0	No growth apparent under the microscope.
1a	No growth visible to the naked eye, but clearly visible under the microscope. Covering up to 25 % of the test surface
1b	No growth visible to the naked eye, but clearly visible under the microscope. Covering up to 50 % of the test surface
1c	No growth visible to the naked eye, but clearly visible under the microscope. Covering more than 50 % of the test surface
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 the microbicidal solution (5.1.11), rinse under running water, wipe with filter paper and allow to dry overnight at room temperature.

Sterilize soil containing 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 weigh 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, etc.

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

Determine, for each specimen, the difference in mass (Δm) before (see 7.3) 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 specified 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

10.1 General

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

10.2 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 performed after cleaning (see 9.1), rate these observations (e.g. concerning discolouration, formation of bubbles) in the same way as fungal growth (see Table 4).

10.3 Change in mass

As described in 9.2.2 determine, for each specimen, the change in mass $\Delta m_{1\text{-}n}$ and calculate and record the arithmetic mean for each test series, $\overline{\Delta m_0}$, $\overline{\Delta m_I}$, and calculate, to the first decimal place, the average percentage change in mass using Formula (1):

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

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

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 test series I and the specimens in test series S have the same dimensions and therefore comparable mass before exposure.

10.4 Changes in other physical properties

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

For each property, calculate the percentage change in the inoculated specimens with respect to the sterile specimens from Formula (2):

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

For each property, calculate the percentage change in the inoculated specimens compared with the control specimens from Formula (3):

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

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 document.

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 a photographic documentation is available.

12 Test report

The test report shall include the following particulars:

a) a reference to this document, i.e. ISO 846:2019;

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

c) the dimensions of the specimens;

d) the incubation time and incubation temperature used;

e) the method(s) used (A, B, C or D) and the number determinations performed in each case;

f) the fungi, bacteria and soil used;

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

h) the physical properties measured;

i) the methods used to measure the physical properties;

j) the results obtained:

   1) the fungal-growth rating for each specimen (see 10.2);

   2) the absolute change in mass, in grams, for each specimen, the mean for each test series and the average percentage change (see 10.3);

3) the mean change in each other property measured for each test series, plus the percentage changes with respect to the sterile specimens and with respect to the control specimens (see 10.4);

k) any special observations, such as infections by fungi and bacteria other than the test organisms, unusual growth characteristics, factors influencing sporulation, any discoloration;

l) any deviations from this document;

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

n) the date(s) of the determinations;

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

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 (8.2.5.1) 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 weightings 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 weighing 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.