

Plastics

Assessment of oxobiodegradability of polyolefinic materials in the form of films

Methodology and requirements

Plastiques — Évaluation expérimentale de l'oxobiodégradabilité
de matériaux polyoléfiniques sous forme de films —
Méthodologie et exigences

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At the publication date, there is no current European or international works on the same subject.

Preamble

This agreement is based on the methodology for the assessment of oxobiodegradability developed by CNEP and SEESIB.

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Contents

	<i>Page</i>
Preamble	2
Introduction	4
1 Scope	4
2 Reference documents	5
3 Terms, definitions and abbreviations	5
3.1 Terms and definitions	5
3.2 Abbreviations	7
4 Principle	7
4.1 General principle	7
4.2 Abiotic degradation	7
4.3 Biodegradation	9
5 Film classification	9
6 Abiotic degradation assessment method	9
6.1 General	9
6.2 Preparing and conditioning of specimens	9
6.3 Measurement of the film thickness	9
6.4 FTIR spectrophotometry analysis	10
6.5 Tensile properties of films	10
6.6 Test 1 — Thermo-oxidation	10
6.7 Test 2 — Thermo-oxidation	10
6.8 Test 3 — Thermo-oxidation of previously photo-oxidized film	12
6.9 Requirements	12
7 Acquired biodegradability assessment method	14
7.1 General	14
7.2 Precautions to be taken when conducting the tests	14
7.3 Testing	15
7.4 Requirements	19
8 Test report	20
Annex A (informative) Information on ATP determination	21
Annex B (informative) Examples of changes in ATP concentration versus time	22
Bibliography	24

Introduction

WARNING The publication of this agreement shall under no circumstances be construed as promoting dispersal of films or other plastic products in the environment. Its aim is to provide access to an assessment methodology developed by teams of scientists whose research has been recognized by the scientific community, through publication [1,2] in particular. This methodology sheds new light on the assessment of mechanisms of degradation and oxobiodegradation of polymer materials.

Twenty years ago, the primary aim was to prevent visible pollution and especially macrotoxicity caused by films in marine and continental environments. The solution to such problems was to cause film disintegration by oxidizing the macroscopic systems. The issue now is to avoid the accumulation in natural environments of oxidized particles resulting from this disintegration. Bioassimilation of these particles must therefore begin within a fairly short, planned time period, and the particles must be sufficiently oxidized to be bioassimilated.

This methodology extends the study of the combined effects of abiotic degradation and biodegradation of polyolefinic materials in the form of films when they are subjected to environmental physicochemical stresses. Physicochemical stresses (light, heat, atmospheric oxygen, humidity, etc.) can be considered to have been applied without any discontinuity other than the alternation between day and night, whereas environmental biological stresses are to be seen as more random; they are variable in nature according to the activity and concentration of microorganisms, as well as the physicochemical characteristics of environmental compartments. These combined effects are necessarily complex, and the assessment of these phenomena under laboratory conditions is therefore necessarily simplified.

This methodology first assesses the consequences of applying physicochemical abiotic stress to polyolefinic material and in a second step the consequences of the activity of microorganisms.

The aim of this methodology is to predict the fate of polyolefinic films that are accidentally dispersed in the environment.

NOTE The principles underlying this methodology are also applicable to polymers other than polyolefins, as well as to products other than films. Preliminary laboratory testing is, however, necessary to validate test conditions and parameters, as well as validation criteria, before extending the scope of this agreement can be considered. This methodology can also be applied to films dispersed in the environment in small quantities and that cannot feasibly be collected.

1 Scope

This agreement provides an experimental assessment methodology for the oxobiodegradability of polymer materials in the form of films. It also specifies requirements to be complied with in order for these materials to be designated as oxobiodegradable.

It applies to polyolefinic films (polyethylene, polypropylene) that are no thicker than 250 μm and are likely to be accidentally dispersed in the environment after use.

This agreement defines two types of oxobiodegradable materials (4.2.2) and four film classes according to their storage duration and temperature and place of use (Clause 5).

This agreement deals with the microtoxicity of polymer materials and their degradation products in relation to the *Rhodococcus rhodochrous* ATCC ® 29672^{TM 1)} bacterial strain.

NOTE 1 The *Rhodococcus* genus is found in soil and marine environments [3 to 5].

NOTE 2 Standardized methods apply to the ecotoxicity assessment of the oxobiodegradable materials covered by this agreement.

2 Reference documents

The following reference documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

NF T 51-195-5, *Plastics — Method of exposure to laboratory light sources — Part 5: Medium-pressure mercury-vapour lamps*

NF EN ISO 527-1, *Plastics — Determination of tensile properties — Part 1: General principles* (classification index: T 51-034-1)

NF EN ISO 527-3, *Plastics — Determination of tensile properties — Part 3: Test conditions for films and sheets* (classification index: T 51-034-3)

NF ISO 10640:2011, *Plastics — Methodology for assessing polymer photoageing by FTIR and UV/visible spectroscopy* (classification index: T 51-182)

ISO 4591, *Plastics — Film and sheeting — Determination of average thickness of a sample, and average thickness and yield of a roll, by gravimetric techniques (gravimetric thickness)*

ISO 4593, *Plastics — Film and sheeting — Determination of thickness by mechanical scanning*

3 Terms, definitions and abbreviations

3.1 Terms and definitions

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

3.1.1

biodegradation

degradation of a polymeric system as a result of cell-mediated phenomena

3.1.2

biodegradability

propensity of being biodegradable

[FD CEN/TR 15351]

1) American Type Culture Collection reference.

3.1.3**biodegradable**

status of a polymeric item that can be biodegraded

[FD CEN/TR 15351]

3.1.4**acquired biodegradability**

propensity of a non-biodegradable polymer to become biodegradable after an induced or naturally occurring chemical transformation

3.1.5**oxobiodegradation**

degradation identified as resulting from oxidative and cell-mediated phenomena, either simultaneously or successively

[FD CEN/TR 15351]

3.1.6**oxobiodegradability**

propensity of being oxobiodegradable

3.1.7**disintegration**

fragmentation to particles of an acceptable size (depending on the application)

[FD CEN/TR 15351]

3.1.8**abiotic degradation****non-biological degradation**

degradation of a substance by chemical or physical processes, for example hydrolysis, photolysis, reduction and oxidation

[FD ISO 6107-6]

3.1.9**adenosine triphosphate****ATP**

molecule that provides the energy necessary for metabolic chemical reactions through hydrolysis within the biochemical processes of all known living organisms

NOTE Within the context of this method, ATP is used to measure active biomass quantities in water and on the surfaces of the material's particles.

3.1.10**film thickness**

e

mean film thickness

NOTE It is expressed in microns.

3.2 Abbreviations

AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
FTIR	Fourier-transform infrared spectroscopy

4 Principle

4.1 General principle

The effects of heat (thermo-oxidation) and/or light (photothermal oxidation) enable the material to reach an oxidation level that is sufficient for biodegradation to start. This is an acquired biodegradability. Then, biodegradability assessment tests enable verification of the fitness to biodegradation and microtoxicity of the oxidized polymer material.

4.2 Abiotic degradation

4.2.1 General

In order to predict the long-term fate of a polymer material dispersed in the environment, it is necessary to establish accelerated laboratory conditions. Only chemical changes, which are really the basis of the physical changes of the polymer material, such as mechanical degradation in particular, can be accelerated in laboratory conditions. An acceleration factor can only be properly defined on the basis of chemical changes.

Studies of polyolefin time-course mechanisms have demonstrated that the phenomena governing evolution in a natural environment are photothermal oxidations (i.e. under the combined effect of light and heat) and thermo-oxidations. The progression of these oxidations is determined through the accumulation of carboxylic acid groups that can be detected using FTIR spectrophotometry. The increase in absorbance at $1\ 714\ \text{cm}^{-1}$, which determines the relative acid groups content, may be expressed based on the thickness, e , of the film. This assessment method for polymer ageing is based on the methodology described in NF ISO 10640.

Abiotic degradation assessment tests and the corresponding requirements are specified in Clause 6.

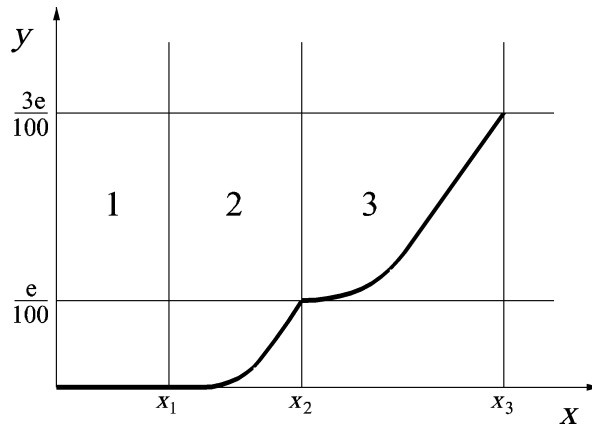
4.2.2 Material types

For the purposes of the present agreement, a distinction is made between the two following types of oxobiodegradable materials:

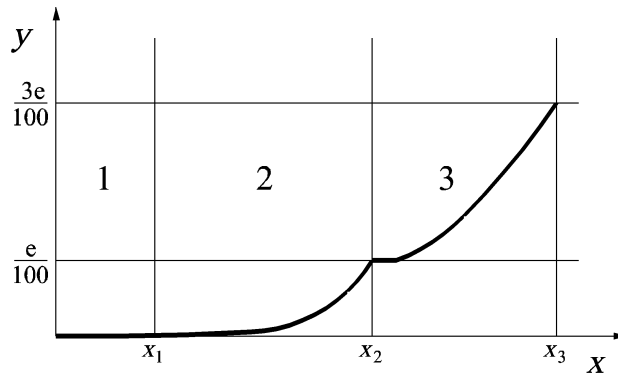
- Type I: oxobiodegradable materials with a thermostability that is controlled for storage and that can undergo short exposure to light during use;
- Type II: oxobiodegradable materials with stabilities that are controlled for storage and use and that can undergo prolonged exposure to light during use.

Figures 1a) and 1b) show the curves of the absorbance increase at $1\ 714\ \text{cm}^{-1}$ versus time for acid groups (expressed as film thickness), for Type I and Type II materials, respectively.

NOTE By convention, the curves represented in Figure 1 are those of materials having characteristics which correspond to the minimal thresholds ($e/100$, $3e/100$) specified in Clause 6.



a) Type I



b) Type II

Phase 1	Phase 2	Phase 3
Storage in the dark and under controlled temperature conditions	Use	End-of-life
No oxidation	Initiation of photothermal oxidation ^{a)} and fragmentation	Continuation of thermal oxidation to reach a sufficient oxidation state allowing the initiation of the bioassimilation
<i>a) For Type II, the initiation of photothermal oxidation is delayed compared to Type I in order to preserve product functionality.</i>		

Key

- X Time expressed in hours
- Y Absorbance increase at $1\ 714\ \text{cm}^{-1}$, expressed as film thickness e (in microns)
- 1 Phase 1
- 2 Phase 2
- 3 Phase 3

Figure 1 — Absorbance increase versus time

4.3 Biodegradation

Adenosine triphosphate (ATP) is the energy transfer molecule for all living organisms on earth. ATP "transports" the energy necessary for all biological functions, such as maintaining cellular integrity and adapting it to environmental conditions, using the substrate that is necessary for growth, or the division function. ATP is thus a molecule that is indispensable for microbial life and its quantity is directly related to the quantity of active cells.

This test method makes it possible to determine the total ATP quantity of the cells in suspension in the culture medium, as well as those attached to the polymer material fragment or flask surfaces on the one hand, and the ratio of the concentration of adenosine diphosphate (ADP) to the concentration of ATP on the other.

Acquired biodegradation assessment tests and the corresponding requirements are specified in Clause 7.

5 Film classification

For the purposes of the present agreement, a distinction is made between the classes of films made from oxobiodegradable materials as defined in Table 1.

Table 1 — Classes of films made from oxobiodegradable material

Class	Inside storage and use durations	Storage temperature and place of use
	months	°C
A	12	20
B	24	20
C	12	30
D	24	30

6 Abiotic degradation assessment method

6.1 General

To predict the behaviour of polymer materials in the form of films when subjected to light and temperature stresses, Tests 1, 2 and 3, specified in 6.6, 6.7 and 6.8, respectively, shall be conducted, and the requirements of 6.9 shall be complied with.

6.2 Preparing and conditioning of specimens

Select specimens of polymer material in the form of film randomly in the product batch to be characterized; there shall be an amount of specimens sufficient for tests to be conducted.

6.3 Measurement of the film thickness

Determine the thickness of the tested film in accordance with ISO 4591 or ISO 4593.

6.4 FTIR spectrophotometry analysis

Determine the absorbance increase at $1\ 714\ \text{cm}^{-1}$ using FTIR spectrometry in accordance with NF ISO 10640:2011, Clause 5.

6.5 Tensile properties of films

As an alternative to tracking the evolution of polymer materials using FTIR spectrophotometry for Test 1 (6.6) and Test 2 (6.7), tracking through determination of tensile properties (e.g. for production control purposes) is allowed.

Determine tensile characteristics in accordance with NF EN ISO 527-1 and NF EN ISO 527-3, using three type 2 specimens (as defined in NF EN ISO 527-3) cut in the longitudinal direction (MD) of the film, with a displacement speed of 100 mm/min.

Using other specimen types and conducting the tests at a displacement speed differing from 100 mm/min is allowed, as long as all the tensile tests (including the film in its initial state) are conducted under the same conditions.

In case of dispute, the reference method is the assessment method using FTIR spectrophotometry.

6.6 Test 1 — Thermo-oxidation

Place the samples (see 6.2) in an aerated and ventilated oven under the temperature conditions and for the duration specified in Table 2, according to the test film class specified in Clause 5.

Table 2 — Test durations and temperatures

Class	Oven temperature °C	Test durations, t_1 h
A	60 ± 2	400
B	60 ± 2	800
C	70 ± 2 ^{a)}	320
D	70 ± 2 ^{a)}	640

a) For heat-shrinkable films of Classes C and D, it is allowed to conduct the test at (60 ± 2) °C and the test durations shall be 800 h and 1 600 h respectively.

6.7 Test 2 — Thermo-oxidation

6.7.1 Exposure method

Expose the specimens as new (unused, unaged) (see 6.2) in an accelerated photoageing chamber using medium pressure mercury vapour lamps, in accordance with NF T 51-195-5, under the following test conditions:

- the temperature of the exposed specimen surfaces shall be controlled and kept at (60 ± 1) °C;
- the specimens shall not be sprayed with water while in the accelerated photoageing chamber.

NOTE The water present in the polyolefinic matrix forms in situ as a result of the decomposition of primary hydroperoxides.

6.7.2 Test conditions for Type I materials

The duration of exposure in the accelerated photoageing chamber shall be equivalent to 3 months to 5 months of exposure to outside sunlight, heat and atmospheric oxygen. It depends on the local climate where the films are used.

The specimen exposure duration shall comply with the values given in Table 3.

Table 3 — Durations of exposure

Climate	Test durations, t_2
	h
Temperate climate (central and northern Europe)	100
Mediterranean climate (southern Europe, the Middle East and North Africa)	150

6.7.3 Test conditions for Type-II materials

6.7.3.1 Films exposed to direct sunlight during the first part of their lifecycle

Exposure includes a first period that corresponds to use under direct sunlight and a second period that corresponds to dispersion in the environment.

The specimen exposure duration in the accelerated photoageing chamber shall comply with the values given in Table 4. During this test, the absorbance variation at $1\ 714\ \text{cm}^{-1}$ is measured twice, the first time after the first exposure period, t_{31} , and the second time after the total exposure period, t_{3T} , as indicated in Table 4.

Table 4 — Durations of exposure

Climate	Test durations			
	Duration of use: 6 months		Duration of use: 12 months	
	First period t_{31} h	Total period t_{3T} h	First period t_{31} h	Total period t_{3T} h
Temperate climate (central and northern Europe)	150	250	300	450
Mediterranean climate (southern Europe, the Middle East and North Africa)	220	370	450	600

6.7.3.2 Films exposed to "glass"-filtered sunlight during the first part of their lifecycle

Exposure includes a first period that corresponds with use under "glass"-filtered sunlight and a second period that corresponds with dispersion in the environment.

The specimen exposure duration in the accelerated photoageing chamber shall comply with the values given in Table 5. During this test, the absorbance variation at $1\ 714\ \text{cm}^{-1}$ is measured twice, the first time after the first exposure period, t_{41} , and the second time after the total exposure period, t_{4T} , as indicated in Table 5.

Table 5 — Durations of exposure

Climate	Durations of exposure							
	Duration of use: 3 months		Duration of use: 6 months		Duration of use: 12 months		Duration of use: 18 months	
	First period t_{41} h	Total period t_{4T} h	First period t_{41} h	Total period t_{4T} h	First period t_{41} h	Total period t_{4T} h	First period t_{41} h	Total period t_{4T} h
Temperate climate	20	120	40	140	70	170	110	210
Mediterranean climate	30	180	60	210	105	255	165	315

6.8 Test 3 — Thermo-oxidation of previously photo-oxidized film

Use the material specimens that have reached at least $\frac{e}{100}$ after Test 2 (6.7).

Then place the specimens in an aerated and ventilated oven during 300 h at a temperature of (60 ± 2) °C.

6.9 Requirements

6.9.1 Absorbance increase at $1\ 714\ \text{cm}^{-1}$

When tested according to Tests 1, 2 and 3 with the test durations indicated according to the material type, film class and duration of use, the film shall comply with the requirements given in Table 6.

Table 6 — Requirements

Test	Phase (Figure 1)	Type of material	Requirements		
			Test duration h	Absorbance increase at 1 714 cm ⁻¹ Thickness, <i>e</i> microns	
Test 1 Thermo-oxidation	Storage in the dark and under controlled temperature conditions	Type I, Type II	<i>t</i> ₁ (see Table 2)	$\leq \frac{e}{1\,000}$	
Test 2 (Photo-oxidation)	Use	Type I	<i>t</i> ₂ (see Table 3)	$\geq \frac{e}{100}$	
		Type II	Film exposed to direct sunlight during the first part of its lifecycle	<i>t</i> ₃₁ (see Table 4)	$\leq \frac{e}{1\,000}$
				<i>t</i> _{3T} (see Table 4)	$\geq \frac{e}{100}$
		Type II	Film exposed to "glass"-filtered sunlight during the first part of its lifecycle	<i>t</i> ₄₁ (see Table 5)	$\leq \frac{e}{1\,000}$
	<i>t</i> _{4T} (see Table 5)		$\geq \frac{e}{100}$		
Test 3 (Thermo-oxidation of previously photo-oxidized film)	End-of-life	Type I, Type II	300	$\geq \frac{3e}{100}$ ^{a)}	

a) Absorbance increase at 1 714 cm⁻¹ in relation to the initial state of the film (before exposure in accordance with 6.7).

NOTE 1 The requirement of Test 1 makes it possible to verify that after the specimens have been kept at the specified temperature for the specified time, the thermo-oxidation of the polymer material is at a very early stage.

NOTE 2 For a temperate climate, for example, the requirement of Test 2 ensures that a Type I film accidentally dispersed in the environment will disintegrate after 3 months to 5 months of ageing, depending on the season.

NOTE 3 For Type I film, the requirement of Test 3 ensures that the film that has been abiotically degraded after exposure to sunlight, heat and atmospheric oxygen for 3 months to 5 months, and then spent 2 years to 3 years in the soil, has acquired a biodegradability capacity as defined in the protocol developed by Clermont-Ferrand University. This protocol was published for the first time in 2006 [2], then results were confirmed in a republication in the same journal in 2010 [1].

6.9.2 Tensile strength and strain at failure (Test 1 and Test 2)

As an alternative to the requirements given in Table 6, when tested according to Tests 1, and 2 with the test durations indicated according to the material type, film class and duration of use, the film shall comply with the requirements given in Table 7.

NOTE After Test 3, the state of degradation of the film makes it impossible to perform the tensile tests.

Table 7 — Requirements

Test	Phase (Figure 1)	Type of material	Requirements		
			Test duration h	R ^{a)} %	
Test 1 Thermo-oxidation	Storage in the dark and under controlled temperature conditions	Type I, Type II	t ₁ (see Table 2)	> 80	
Test 2 (Photo-oxidation)	Use	Type I	t ₂ (see Table 3)	< 20	
		Type II	Film exposed to direct sunlight during the first part of its lifecycle	t ₃₁ (see Table 4)	> 80
				t _{3T} (see Table 4)	< 20
		Type II	Film exposed to "glass"-filtered sunlight during the first part of its lifecycle	t ₄₁ (see Table 5)	> 80
				t _{4T} (see Table 5)	< 20

a) $R = \frac{\varepsilon_{br}}{\varepsilon_{b0}} \times 100$
 where:
ε_{br} is the residual strain (or elongation) at break of the film, expressed as a percentage;
ε_{b0} is the strain (or elongation) at break of the film at the initial state, expressed as a percentage.

7 Acquired biodegradability assessment method

7.1 General

Acquired biodegradability assessment is carried out in accordance with 7.3 for a polymer material that has first satisfied the abiotic degradation requirements specified in Clause 6.

7.2 Precautions to be taken when conducting the tests

Sterilize all equipment used for the tests in an autoclave.

Open the flasks used for the test under a sterile hood.

Sterilize the polymer material used for the tests by dipping it into 70° ethanol and dry it under the sterile hood.

7.3 Testing

7.3.1 Preparing and conditioning specimens

Take at least 100 mg of oxidized polymer material (6.7) that has reached an absorbance increase of at least $\frac{3e}{100}$, where e is the film thickness in microns.

7.3.2 Preserving the oxidized polymer material

Preserve the particles of oxidized polymer material in sterile flasks at $(4 \pm 2) ^\circ\text{C}$ during the time interval between the end of thermo-oxidation (6.7) and the beginning of incubation (7.3.4).

7.3.3 Sieving the oxidized polymer material

If possible, pass the oxidized polymer material, which is first broken into pieces and weakened, through an approximately 1 mm cut-off size metallic sieve in order to obtain more uniformly-sized particles for the incubation tests.

7.3.4 Incubation

7.3.4.1 Culture medium for incubation

Carry out incubation with microorganisms in an aqueous solution to which the trace elements that microorganisms need for growth have been added; the composition of this solution is given in Table 8.

Only use recognized analytical grade reagents.

Table 8 — Composition of the incubation culture medium

Compounds	Concentration g·l ⁻¹
Disodium phosphate dodecahydrate (Na ₂ HPO ₄ , 12H ₂ O)	3,8
Dehydrated potassiumdihydrogen phosphate (KH ₂ PO ₄)	1,8
Magnesium sulphate heptahydrate (MgSO ₄ , 7H ₂ O)	0,02
Ferrous ammonium sulphate hexahydrate (Fe(NH ₄).(SO ₄) ₂ , 6H ₂ O)	0,03
Calcium chloride dihydrate (CaCl ₂ , 2H ₂ O)	0,01
Sodium chloride (NaCl)	0,5
Ammonium chloride (NH ₄ Cl)	0,3
Trace minerals (1 ml for 100 ml of water in the medium)	
Manganese sulphate (MnSO ₄)	4.10 ⁻⁴
Boric acid (H ₃ BO ₃)	5,8.10 ⁻⁵
Zinc sulphate heptahydrate (ZnSO ₄ .7H ₂ O)	4,4.10 ⁻⁵
Sodium molybdate (Na ₂ MoO ₄)	2.10 ⁻³
Cobalt nitrate Co(NO ₃) ₂	Traces
Copper(II) sulphate (CuSO ₄)	Traces

7.3.4.2 **Bacterial strain**

The *Rhodococcus rhodochrous* ATCC ® 29672TM bacteria strain is to be used for the incubation tests.

7.3.4.3 **Procedure**

To prepare to measure ATP concentrations, carry out incubation in 4 ml glass flasks that are hermetically sealed in order to prevent evaporation of the culture medium.

Use a volume of 0,4 ml of culture medium in each flask.

NOTE The volume of air contained in the flasks is sufficient to oxygenate the bacterial culture for several weeks.

Open the flasks every week in order to renew the volume of air.

The concentration of polymer material fragments in the culture medium is set at $5 \text{ mg}\cdot\text{ml}^{-1} \pm 5 \%$, while bacterial concentration is set at approximately 10^4 cells per millilitre of culture medium.

Incubate at $(27 \pm 0,1) \text{ }^\circ\text{C}$, keeping the stirring speed at 120 rpm at all times in order to avoid polymer particle sedimentation and oxygenate the culture medium.

Plan nine samples in order to track changes in ATP concentration in the culture media. Samples shall be taken at the start of the test (day 0) then after 4 days, 8 days, 12 days, 30 days, 60 days, 90 days, 120 days and 180 days.

For each sample, set aside a control flask without polymer material to serve as a reference.

7.3.5 **Determination of ATP concentration**

Measure ATP concentration of the first eight samples using the protocol indicated in the kit used.

For example, for the BioThema "ATP Biomass Kit HS", reference 266 311²⁾, follow the protocol below:

- a) use 0,4 ml of culture medium;
- b) add 0,4 ml of extractant (called "extractant B/S" in the kit), that serves to lyse the cells, liberate cell content and fix the culture medium by neutralizing enzyme activity.
- c) add 100 μl of this mixture to the luminometer measurement tube;
- d) add 400 μl of ATP Reagent HS;
- e) measure the concentration, I_{smp} ;
- f) add 10 μl of ATP standard (1 picomole);
- g) measure the concentration, $I_{\text{smp+std}}$.

For each sample, measure the ATP concentration of three flasks.

2) Kit sold by BioThema AB, Handens Stationsväg 17, 136 40 HANINGE (Sweden)

Calculate ATP concentration using Equation (1):

$$ATP_{\text{smp}} = \frac{l_{\text{smp}}}{l_{\text{smp} + \text{std}} - l_{\text{smp}}} X \quad \dots (1)$$

where:

- ATP_{smp} is the ATP concentration, in picomoles per millilitre;
- l_{smp} is the concentration measured in step e) of the protocol, in picomoles per millilitre;
- $l_{\text{smp} + \text{std}}$ is the concentration measured in step g) of the protocol, in picomoles per millilitre;
- X is the dilution factor of the initial sample.

7.3.6 Viability and ADP concentration determination tests after 180 days

7.3.6.1 General

After 180 days of incubation, conduct the following two tests for each flask containing polymer material as well as for each control flask without polymer material:

- spread 100 μl of culture medium over nutrient agar (TS) in order to verify the viability of the bacterial cells, i.e. their capacity to multiply in a favourable medium after six months in the culture medium. The viability test shall be considered positive if *Rhodococcus rhodochrous* colonies are visible after a few days of oven incubation at $(27 \pm 1) ^\circ\text{C}$;
- measure ADP concentration in order to calculate the ADP/ATP ratio. This ratio makes it possible to evaluate the energy charge of bacterial cells, i.e. their "depletion state".

NOTE In order to function, a biological cell needs to convert its nutrients, such as molecules containing carbon chains for example, into a form of usable energy, ATP (7.3.5). The necessary energy for the various chemical reactions that take place within the cell is in fact released when ATP is reduced to less energy-rich molecules, such as ADP (adenosine diphosphate) and AMP (adenosine monophosphate). These molecules are then phosphorylated (recycled) into ATP when fresh nutrients are supplied. This process is the central metabolic pathway of bacterial cells.

Also measure the ATP concentrations of the flasks that contain no polymer material.

Measure ATP concentrations in three flasks after 180 days. The viability tests are also conducted on three flasks.

The ATP and ADP concentration measurement protocol for this final sample is not the same as that indicated in the BioThema "ATP Biomass Kit HS" (see 7.3.6.3 and 7.3.6.4). To determine ADP concentrations, all the ADP contained in the bacteria and the culture medium shall be converted into ATP.

Run a first ATP concentration measurement, then convert the ADP into ATP for a second ATP measurement. Then, determine the ADP concentration by calculating the difference between the two measurements, followed by the ADP/ATP ratio.

7.3.6.2 Test solutions

NOTE In what follows, the compositions of the four aqueous solutions needed to analyze ADP concentrations are specified, except for diluent B, which has a confidential composition.

- 7.3.6.2.1 Solution 1: Diluent B+K+Mg**, composed of 10 ml of diluent B (from the ATP kit), 200 μl 1M KCl and 10 μl 1M MgSO_4 . The KCl et MgSO_4 solutions are made with sterile, ultrapure water.

7.3.6.2.2 Solution 2: PEP (phosphoenolpyruvate) composed of 120 mg of PEP and 5 ml 0,05 M TRIS-HCl buffer solution (with a 7,2 pH) made with sterile, ultrapure water. The pH is adjusted using a concentrated KOH solution.

7.3.6.2.3 Solution 3: PEP+PK (phosphoenolpyruvate kinase), composed of 5 mg of PK dissolved in 1 ml of PEP solution; separate into 200 µl aliquots and store at -40 °C.

7.3.6.2.4 Solution 4: ATP reagent, ATP reagent HS (freeze-dried) reconstituted with 2,5 ml of sterile, ultrapure water containing no ATP, instead of using diluent B supplied in the BioThema "ATP Biomass Kit HS".

7.3.6.3 Determination of ATP concentration

Measure ATP concentrations following the protocol below:

- a) mix a volume of the sample with the same volume of extractant B/S;
- b) add to the luminometer tube:
 - 1) 30 µl of the sample/extractant B/S mixture;
 - 2) 240 µl of Diluent B+K+Mg (solution 1);
 - 3) 20 µl of sterile, ultrapure H₂O, containing no ATP.
- c) cover the tubes with parafilm;
- d) incubate the mixture for 10 min at 37 °C;
- e) add 60 µl of ATP reagent;
- f) measure the concentration, $l_{\text{smp},0}$;
- g) add 10 µl of ATP standard (1 picomole);
- h) measure the concentration, $l_{\text{smp+std},0}$.

Calculate ATP concentration after 180 days, $ATP_{\text{smp},0}$, using Equation (2):

$$ATP_{\text{smp},0} = \frac{l_{\text{smp},0}}{l_{\text{smp+std},0} - l_{\text{smp},0}} X_0 \quad \dots (2)$$

where:

- $ATP_{\text{smp},0}$ is the ATP concentration of the culture medium, in picomoles per millilitre;
- $l_{\text{smp},0}$ is the concentration measured in step e) of the protocol, in picomoles per millilitre;
- $l_{\text{smp+std},0}$ is the concentration measured in step g) of the protocol, in picomoles per millilitre;
- X_0 is the dilution factor of the initial sample.

7.3.6.4 Determination of ATP + ADP concentration

Measure ATP + ADP concentrations following the protocol below:

- a) mix a volume of the sample with the same volume of extractant B/S;
- b) add to the luminometer tube:
 - 1) 30 µl of the sample/extractant B/S mixture;
 - 2) 240 µl of Diluent B+K+Mg (solution 1);
 - 3) 10 µl of PEP+PK (solution 3);
 - 4) 10 µl of sterile, ultrapure H₂O, containing no ATP.
- c) cover the tubes with parafilm;
- d) incubate the mixture for 10 min at 37 °C;

- e) add 60 μl of ATP reagent;
- f) measure the concentration, l_{smp} ;
- g) add 10 μl of ATP standard (1 picomole);
- h) measure the concentration, $l_{\text{smp+std}}$.

Calculate ATP concentration after 180 days and conversion of ADP into ATP, $ATP_{\text{smp},1}$, using Equation (3):

$$ATP_{\text{smp},1} = \frac{l_{\text{smp},1}}{l_{\text{smp} + \text{std},1} - l_{\text{smp},1}} X_1 \quad \dots (3)$$

where:

- $ATP_{\text{smp},1}$ is the ATP concentration, in picomoles per millilitre, after conversion of ADP into ATP;
- $l_{\text{smp},1}$ is the concentration measured in step e) of the protocol, in picomoles per millilitre;
- $l_{\text{smp+std},1}$ is the concentration measured in step g) of the protocol, in picomoles per millilitre;
- X_1 is the dilution factor of the initial sample.

7.3.6.5 Calculation of ADP concentration and of the ADP/ATP ratio

Calculate the ADP concentration, ADP_0 , after 180 days and the ADP/ATP ratio, R , after 180 days using Equations (4) and (5):

$$ADP_0 = ATP_{\text{smp},1} - ATP_{\text{smp},0} \quad \dots (4)$$

$$R = \frac{ATP_{\text{smp},1} - ATP_{\text{smp},0}}{ATP_{\text{smp},0}} \quad \dots (5)$$

where:

- R is the ADP/ATP ratio of the culture medium, dimensionless value;
- ADP_0 is the ADP concentration of the culture medium, in picomoles per millilitre;
- $ATP_{\text{smp},1}$ is the ATP concentration of the culture medium, in picomoles per millilitre, after conversion of ADP into ATP;
- $ATP_{\text{smp},0}$ is the ATP concentration of the culture medium, in picomoles per millilitre;

7.4 Requirements

The tested polymer material is declared to have acquired biodegradability when the following three conditions are satisfied:

- a) the ATP concentrations in the flasks containing the polymer material shall stabilize at a value 3 times higher than the value observed for the control flasks containing no polymer material during the test period between the first and sixth month of the experiment;

NOTE 1 This requirement reflects the fact that the bacteria use the oxidized polymer as a nutrient.

- b) the ADP/ATP ratio shall be ≤ 3 after a duration of 180 days;

NOTE 2 This requirement reflects the fact that the energetic level of the bacteria is satisfactory.

- c) the results of the viability test carried out on the polymer material after an incubation period of 180 days shall be positive.

NOTE 3 This requirement reflects the fact that the polymer material does not contain any elements that are toxic for the *Rhodococcus rhodochromus* ATCC @ 29672™ strain.

8 Test report

The test report shall include the following information:

- a) reference to this document, i.e. AC T 51-808;
- b) all the information necessary to identify and describe the tested film, including the material, thickness, origin and product reference number;
- c) oxobiodegradable material type (4.2.2);
- d) film class (Clause 5);
- e) abiotic degradation assessment method: absorbance variation or tensile properties;
- f) photoageing chamber characteristics and test conditions;
- g) results of the abiotic degradation assessment test, including:
 - 1) absorbance increase after the thermo-oxidation test (6.6);
 - 2) absorbance increase after the photo-oxidation test (6.7);
 - 3) absorbance increase after the thermo-oxidation test on pre-oxidized film (6.8),
and/or where relevant, the conditions and results of the tensile property assessment tests, including:
 - 4) ratio of the residual strain at break after the thermo-oxidation test (6.6) to the strain at break at the initial state;
 - 5) ratio of the residual strain at break after the photo-oxidation test (6.7) to the strain at break at the initial state;
- h) results of the acquired biodegradability assessment test, including:
 - 1) ATP concentration, ATP_{smp} , for all eight samples (7.3.5) (individual and mean values);
 - 2) ATP concentration, $ATP_{smp,0}$, for the samples after 180 days (7.3.6.3) (individual and mean values);
 - 3) ADP/ATP ratio, R , after 180 days (7.3.6.5) (individual and mean values);
 - 4) nature and result of the viability test (7.3.6.1) (individual results).
- i) any factor that may have affected results, such as any incident or operational detail not indicated in this document;
- j) test date.

Annex A
(informative)
Information on ATP determination

Energy for a living cell is obtained by the hydrolysis of ATP to adenosine diphosphate (ADP) and/or to adenosine monophosphate (AMP). The chemical formula of ATP is $C_{10}H_{16}N_5O_{13}P_3$; the structure of the molecule is given in Figure A.1.

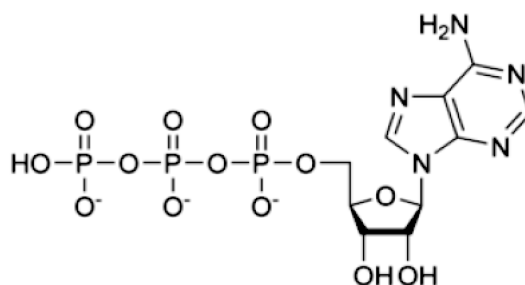
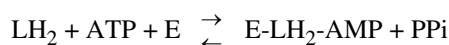


Figure 1 — Molecular structure of ATP

At any one time, the amount of ATP in a cell will depend on its metabolic activity. Measurement of ATP is a recognised technique used to estimate the amount of active microbial biomass and hence the amount of growth that has taken place or the level of active biomass that is maintained.

ATP measurement is based on the reaction of luciferin with luciferase (originating from fireflies) which occurs in the presence of free ATP. During this process, light is produced.

Under optimal conditions, 1 photon of light is produced per molecule of ATP. The light generated is measured using a sensitive photometer (or luminometer) and expressed in Relative Light Units (RLUs). The ATP content of the sample is then calculated with the aid of a conversion factor.



where:

LH_2 luciferin

ATP adenosine triphosphate

E luciferase

AMP adenosine monophosphate

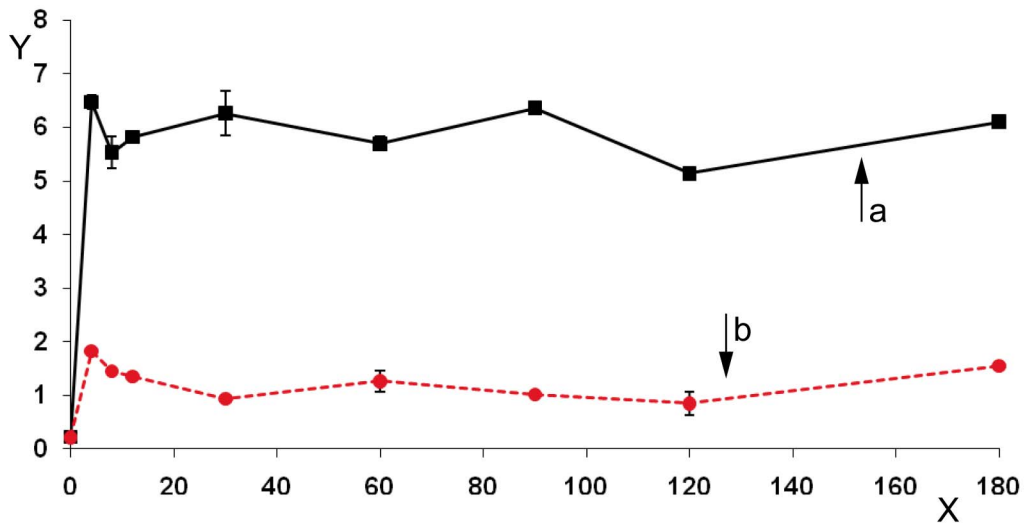
PPi pyrophosphate

L oxyluciferin

H_2O water

Annex B
(informative)

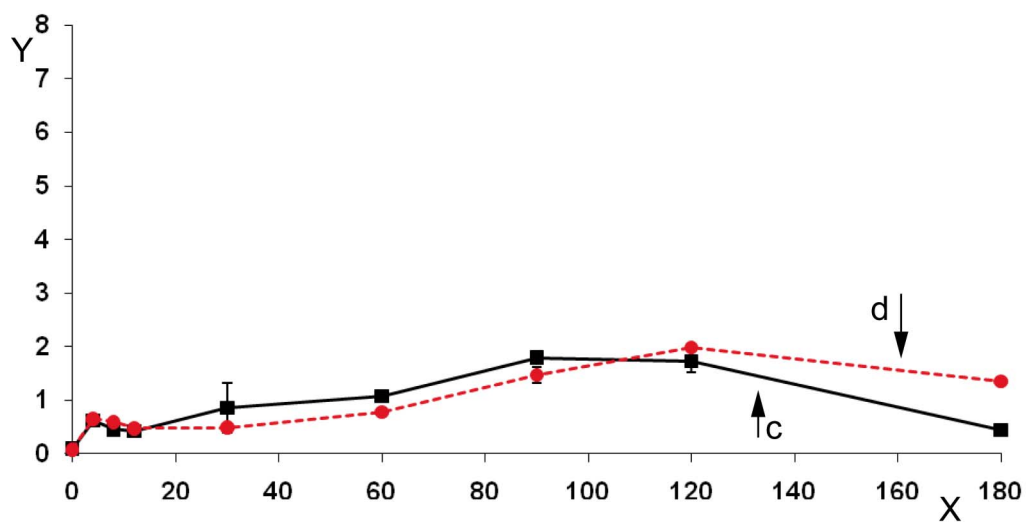
Examples of changes in ATP concentration versus time



Key

- X Test duration, expressed in days
- Y ATP concentration, expressed in pmol/ml
- a Film subjected to prior photo-oxidation and thermo-oxidation at 60 °C, culture medium with oxidized particles
- b Culture medium without oxidized particles (control flask for the experiment)

Figure B.1 — Polyethylene film considered to be potentially biodegradable

**Key**

- X Test duration, expressed in days
Y ATP concentration, expressed in pmol/ml
c Film subjected to prior photo-oxidation and thermo-oxidation at 60 °C, culture medium with oxidized particles
d Culture medium without oxidized particles (control flask for the experiment)

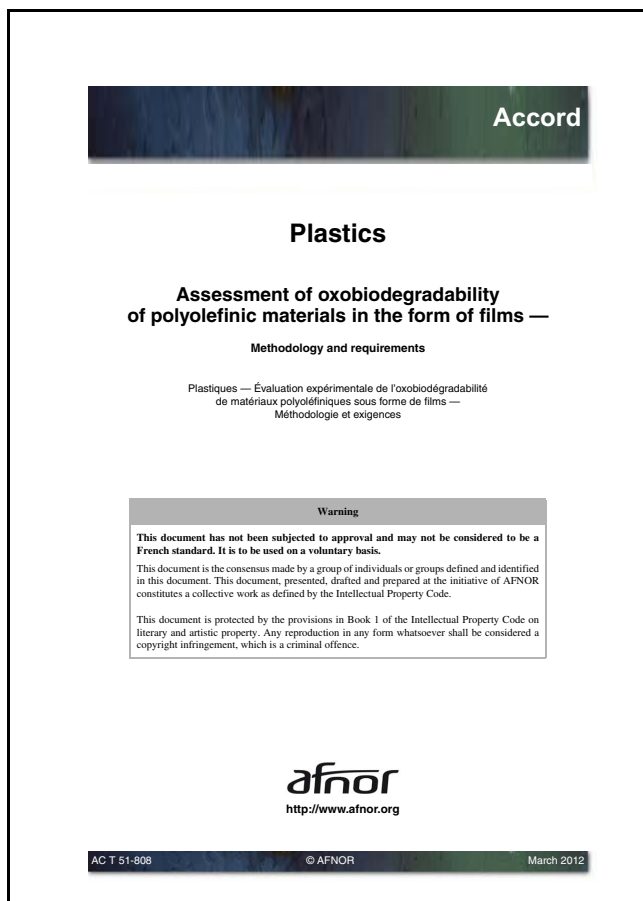
Figure B.2 — Polyethylene film considered to be potentially non-biodegradable

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- [7] FD ISO 6107-6, *Water quality — Vocabulary — Part 6* (classification index: T 90-506).

Other documents:

- [8] BS 8472:2011, *Methods for the assessment of the oxo-biodegradation of plastics and of the phyto-toxicity of the residues in controlled laboratory conditions*.
- [9] ASTM D 6954-04, *Standard Guide for Exposing and Testing Plastics that Degrade in the Environment by a Combination of Oxidation and Biodegradation*.



This agreement provides an experimental assessment methodology for the oxobiodegradability of polymer materials in the form of films. It also specifies requirements to be complied with in order for these materials to be designated as oxobiodegradable. It applies to polyolefinic films (polyethylene, polypropylene) that are no thicker than 250 µm and are likely to be accidentally dispersed in the environment after use. It defines two types of oxobiodegradable materials and four film classes according to their storage duration and temperature and place of use. It deals with the microtoxicity of polymer materials and their degradation products in relation to the *Rhodococcus rhodochrous* ATCC ® 29672TM bacterial strain.

Keywords plastics, wastes, polyolefins, classifications, biodegradability, deterioration, thickness measurement, spectrophotometric analysis, tensile properties, oxidation tests, exposure time, sunlight, absorbance, toxicity, microorganisms, environmental protection.

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