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**Plastics — Determination of aerobic  
biodegradation of non-floating plastic  
materials in a seawater/sediment  
interface — Method by analysis of  
evolved carbon dioxide**

*Plastiques — Détermination de la biodégradation aérobie des  
matières plastiques non-flottantes à l'interface eau de mer/sédiments  
— Méthode par analyse du dioxyde de carbone libéré*



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# Contents

|                                                                                                       | Page      |
|-------------------------------------------------------------------------------------------------------|-----------|
| Foreword.....                                                                                         | iv        |
| Introduction.....                                                                                     | v         |
| <b>1 Scope.....</b>                                                                                   | <b>1</b>  |
| <b>2 Normative references.....</b>                                                                    | <b>1</b>  |
| <b>3 Terms and definitions.....</b>                                                                   | <b>1</b>  |
| <b>4 Principle.....</b>                                                                               | <b>2</b>  |
| <b>5 Test environment.....</b>                                                                        | <b>2</b>  |
| <b>6 Reagents.....</b>                                                                                | <b>2</b>  |
| <b>7 Apparatus.....</b>                                                                               | <b>3</b>  |
| <b>8 Procedure.....</b>                                                                               | <b>3</b>  |
| 8.1 Test material.....                                                                                | 3         |
| 8.2 Reference material.....                                                                           | 4         |
| 8.3 Preparation of the sediment.....                                                                  | 4         |
| 8.4 Test setup.....                                                                                   | 4         |
| 8.5 Pre-conditioning phase.....                                                                       | 4         |
| 8.6 Start of the test.....                                                                            | 5         |
| 8.7 Carbon dioxide measurement.....                                                                   | 5         |
| 8.8 End of the test.....                                                                              | 6         |
| <b>9 Calculation and expression of results.....</b>                                                   | <b>6</b>  |
| 9.1 Calculation.....                                                                                  | 6         |
| 9.1.1 Amount of CO <sub>2</sub> produced.....                                                         | 6         |
| 9.1.2 Percentage of biodegradation.....                                                               | 8         |
| 9.2 Visual inspection.....                                                                            | 8         |
| 9.3 Expression and interpretation of results.....                                                     | 8         |
| <b>10 Validity of results.....</b>                                                                    | <b>9</b>  |
| <b>11 Test report.....</b>                                                                            | <b>9</b>  |
| <b>Annex A (informative) Example of respirometric system based on CO<sub>2</sub> measurement.....</b> | <b>10</b> |
| <b>Bibliography.....</b>                                                                              | <b>11</b> |

## Foreword

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

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](http://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](http://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 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](http://www.iso.org/iso/foreword.html).

The committee responsible for this document is ISO/TC 61, *Plastics*, Subcommittee SC 5, *Physical-chemical properties*.

## Introduction

Products made with biodegradable plastics are designed to be recovered by means of organic recycling in composting plants or in anaerobic digesters. The uncontrolled dispersion of biodegradable plastics in natural environments is not desirable. The biodegradability of products cannot be considered as an excuse to spread wastes that should be recovered and recycled. However, test methods to measure rate and level of biodegradation in natural environments (such as soil or the marine environment) are of interest in order to better characterize the behaviour of plastics in these very particular environments. As a matter of fact, some plastics are used in products that are applied in the sea (e.g. fishing gear) and sometimes they can get lost or put willingly in marine environment. The characterization of biodegradable plastic materials can be enlarged by applying specific test methods that enable the quantitative assessment of biodegradation of plastics exposed to marine sediment and seawater. Plastic products are directly littered or arrive with fresh waters in the pelagic zone (free water). From there, and depending on density, tides, currents, and marine fouling plastics may sink to the sublittoral, and reach the seafloor surface. Many biodegradable plastics have a density higher than 1 and therefore tend to sink. The sediment passes from aerobic to anoxic and finally anaerobic conditions going from the surface (the interface with seawater) into deeper layers, displaying a very steep oxygen gradient.

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# Plastics — Determination of aerobic biodegradation of non-floating plastic materials in a seawater/sediment interface — Method by analysis of evolved carbon dioxide

## 1 Scope

This International Standard specifies a test method to determine the degree and rate of aerobic biodegradation of plastic materials when settled on marine sandy sediment at the interface between seawater and the seafloor, by measuring the evolved carbon dioxide.

This test method is a simulation under laboratory conditions of the habitat found in different seawater/sediment-areas in the sea, e.g. in a benthic zone where sunlight reaches the ocean floor (photic zone) that, in marine science, is called sublittoral zone

The determination of biodegradation of plastic materials buried in marine sediment is outside the scope of this International Standard.

Measurement of aerobic biodegradation can also be obtained by monitoring the oxygen consumption, as described in ISO 18830.

The conditions described in this International Standard may not always correspond to the optimum conditions for the maximum degree of biodegradation to occur.

## 2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 14852:1999, *Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium — Method by analysis of evolved carbon dioxide*

ISO 8245, *Water quality — Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC)*

## 3 Terms and definitions

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

### 3.1 theoretical amount of evolved carbon dioxide

**ThCO<sub>2</sub>**

maximum theoretical amount of carbon dioxide evolved after completely oxidising a chemical compound, calculated from the molecular formula or from determination of total organic carbon (TOC)

Note 1 to entry: It is expressed as milligrams of carbon dioxide evolved per milligram or gram of test compound.

### 3.2 total organic carbon

**TOC**

amount of carbon bound in an organic compound

Note 1 to entry: Total organic carbon is expressed as milligrams of carbon per 100 mg of the compound.

### 3.3 dissolved organic carbon DOC

that part of the organic carbon in water which cannot be removed by specified phase separation methods, for example by centrifugation at  $40\,000\text{ ms}^{-2}$  for 15 min or by membranes with pores of  $0,2\text{ }\mu\text{m}$  to  $0,45\text{ }\mu\text{m}$  diameter

### 3.4 pre-conditioning phase

pre-incubation of an inoculum under the conditions of the subsequent test in the absence of test material, with the aim to consume potential organic matter present in excess that could disturb biodegradation measurement and to improve the acclimatization of the microorganisms to the test conditions

## 4 Principle

This test method is based on the determination of evolved carbon dioxide and derives from ISO 14852. The testing medium is based on a solid phase and a liquid phase. The solid phase is a sandy marine sediment laid in the bottom of a closed flask; the liquid phase is a column of natural or artificial sea water, poured on the sediment. The test material is preferably in the form of a film to be laid down on top of the sediment, at the interface between the solid phase and the liquid phase. This is a simulation of an object that has sunk and finally reached the sea floor. The system is contained in a closed flask.

The carbon dioxide evolved during the microbial degradation is determined by a suitable analytical method. The level of biodegradation is determined by comparing the amount of carbon dioxide evolved with the theoretical amount ( $\text{ThCO}_2$ ) and expressed in percentage. The test result is the maximum level of biodegradation, determined from the plateau phase of the biodegradation curve. The principle of a system for measuring evolved carbon dioxide is given in ISO 14852:1999, Annex A.

The details of interlaboratory testing based on the test method specified in this International Standard are available in Reference [5].

## 5 Test environment

Incubation shall take place in the dark or in diffuse light in an enclosure which is free from vapours inhibitory to microorganisms and which is maintained at a constant temperature, preferably between  $15\text{ }^{\circ}\text{C}$  to  $25\text{ }^{\circ}\text{C}$ , but not exceeding  $28\text{ }^{\circ}\text{C}$ , to an accuracy of  $\pm 2\text{ }^{\circ}\text{C}$ . Any change in temperature shall be justified and clearly indicated in the test report.

NOTE Test results are obtained for temperature that may be different from real conditions in marine environment.

## 6 Reagents

**6.1 Distilled or deionized water**, free of toxic substances (copper in particular) and containing less than  $2\text{ mg/l}$  of DOC.

### 6.2 Artificial seawater

Dissolve:

|                                                                               |       |
|-------------------------------------------------------------------------------|-------|
| Sodium chloride ( $\text{NaCl}$ )                                             | 22 g  |
| Magnesium chloride hexahydrate ( $\text{MgCl}_2 \cdot 6\text{ H}_2\text{O}$ ) | 9,7 g |
| Sodium sulfate ( $\text{Na}_2\text{SO}_4$ )                                   | 3,7 g |



|                                                |        |
|------------------------------------------------|--------|
| Calcium chloride ( $\text{CaCl}_2$ )           | 1 g    |
| Potassium chloride (KCl)                       | 0,65 g |
| Sodium hydrogen carbonate ( $\text{NaHCO}_3$ ) | 0,20 g |

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

### 6.3 Natural seawater/sediment

Take a sample of a sandy sediment and seawater with a shovel beneath the low-water line into a bucket. Transfer the wet sediment together with seawater into sealed containers for transport and fast deliver it to the laboratory. After delivery, conserve the sediment at low temperature (approximately 4 °C) until use. The seawater/sediment sample should be preferably used within 4 weeks after sampling. Record storage time and conditions.

NOTE Seawater and sediment can also be sampled from large, well-running public marine aquaria.

Measure the TOC, pH and nitrogen content of the sediment and of the natural seawater if used instead of artificial seawater. The carbon content of sediment should be in the range of 0,1 % to 2 %.

A preliminary oxidation can be applied to the sediment in order to decrease the organic matter content and the background respiration. Sediment and seawater are fluxed with air and gently stirred (max. 20 r/min to 30 r/min) in a large container for the desired period of time. Report this pre-treatment process in the test report.

## 7 Apparatus

### 7.1 Test flasks

Biometer flasks of the volume of about 250 ml are appropriate. The vessels shall be located in a constant-temperature room or in a thermostatic apparatus (e.g. water-bath). Stirring can be applied on seawater on condition that it does not disturb the sediment/seawater interface.

NOTE A suitable apparatus is shown in Figure A.1. An example of a stirred apparatus is given in OECD TG 308, Annex 4.[6]

### 7.2 Container for the $\text{CO}_2$ absorber

A glass beaker to be located in the headspace of the reactor and filled with 10 ml of  $\text{Ba}(\text{OH})_2$  0,025 N or with 3 ml of KOH 0,5 N.

### 7.3 Analytical balance

Analytical balance shall have a sensitivity of at least 0,1 mg.

### 7.4 pH meter

## 8 Procedure

### 8.1 Test material

The test material should be in film or sheet form. Cut samples of the test material in the shape of a disk. Disks shall have a smaller diameter than the glass flasks, so that the disks can be easily laid on the bottom of the glass flask.

The sample shall be of known mass and contain sufficient carbon to yield  $\text{CO}_2$  that can be adequately measured by the system used.

Use a test material concentration of at least 100 mg/l of seawater plus sediment. This mass of the sample should correspond to TOC of about 60 mg/l. The maximum mass of sample per flask is limited by the oxygen supply to the glass flask. The use of 150 mg to 300 mg of test material per litre seawater plus sediment is recommended.

Calculate the TOC from the chemical formula or determine it by a suitable analytical technique (e.g. elemental analysis or measurement in accordance with ISO 8245) and calculate the  $\text{ThCO}_2$ .

The form and shape of the test material may influence its biodegradability. Similar shapes and thicknesses should preferably be used if different kinds of plastic materials are to be compared.

NOTE 1 The test material may also be introduced as powder. However, this can be critical, as practical experience has shown that it is difficult to keep a powder settled at the sediment/seawater interface without special measures. Refer to ISO 10210 for preparation of powder from plastic materials.

NOTE 2 When the test material in form of film is laid down on the surface of the sediment, it could limit the gas exchange between the water body and the sediment, promoting the formation of anaerobic zones under the test material. In order to reduce this effect, it is possible to perforate the film sample homogeneously over the entire surface.

## 8.2 Reference material

Use ashless cellulose filters as a reference material<sup>1)</sup>. If possible, the TOC, form, and size should be comparable to that of the test material. As a negative control, a non-biodegradable polymer (e.g. polyethylene) in the same form as the test material can optionally be used.

## 8.3 Preparation of the sediment

Filter the sediment in a funnel with a coarse filter paper to eliminate excess seawater. Sediment is ready for testing when dripping of sea water is ended. Sediment after filtering is named "wet sediment" hereafter.

## 8.4 Test setup

Provide several flasks, so that the test includes at least the following:

- a) three flasks for the test material (symbol  $F_T$ );
- b) three flasks for the blank (symbol  $F_B$ );
- c) three flasks for reference material (symbol  $F_C$ ).

In addition, it is possible to add three more flasks for negative control (symbol  $F_N$ ), if required.

NOTE Two flasks for test material, blank, reference material, and negative control may be used instead of three for screening purposes.

## 8.5 Pre-conditioning phase

In a typical case, use a test flask with a volume of 250 ml. Lay down 30 g of the wet sediment on the bottom of the flask. Carefully pour 70 ml of natural or artificial seawater. The test should be performed with a water/sediment volume ratio between 3:1 and 5:1 and a sediment layer of about 0,3 cm to 0,5 cm, depending on the granulometry of the sediment.

NOTE When using very coarse-grained sediment, the layer may be increased up to 1,5 cm.

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1) Laboratory filter paper Whatman n° 42 has been found satisfactory for this purpose and is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Add carbon dioxide absorber to the absorber compartments of the test flask in a typical case 3 ml of KOH 0,5 N or 10 ml of Ba(OH)<sub>2</sub> 0,025 N. Place the flasks in a constant-temperature environment and allow all vessels to reach the desired temperature. Take the necessary readings and monitor the CO<sub>2</sub> evolution.

This phase is carried out in order to verify that the endogenous respiration is similar in the different vessels and also to obtain a preliminary oxidation of excess organic matter, in order to start the test with a lower endogenous respiration. The inoculum can be gently stirred in order to accelerate the biodegradation of excess organic matter.

This phase is generally protracted for a week but is possible to extend this time if a high amount of CO<sub>2</sub> evolved is measured.

In case the CO<sub>2</sub> evolution of a vessel is different, reject the diverging vessel or in case of multiple anomalies, start again using new sediment.

## 8.6 Start of the test

Dunk the plastic film sample, cut as described in 8.1, on the sediment of each vessel. Mass of samples (test and reference material) should be about 20 mg each when using a flask with a volume of 250 ml corresponding to an initial test item concentration specified in 8.1. In order to ensure a homogeneous contact between sample and sediment, it is recommended to cover the sample with a suitable cover slip. The cover slips shall be introduced in blank vessels also, for assuring similar conditions.

NOTE A suitable cover slip can be made using a common non-biodegradable vinyl-coated fibreglass mosquito net with a fibre diameter of about 280 µm and a 1,8 mm × 1,6 mm mesh.

For an example, see [Annex A](#).

Repeat the procedure for the reference material and the material for the negative control to the respective flasks. Record the mass of the sediment, the sample and the volume of seawater introduced in each vessel.

Nutrients may be supplemented as needed to support microbial diversity and to maintain the capacity to biodegrade the test material. The need and timing of additional nutrients or other appropriate measures may be judged by observation of the temporal course of the biodegradation of the reference substance cellulose. Any addition and the applied method shall be reported in the test report.

## 8.7 Carbon dioxide measurement

**8.7.1** The CO<sub>2</sub> reacts with Ba(OH)<sub>2</sub> and is precipitated as barium carbonate (BaCO<sub>3</sub>). The amount of CO<sub>2</sub> produced is determined by titrating the remaining barium hydroxide with 0,05 N hydrochloric acid to a phenolphthalein end-point or by automatic titrator. Because of the static incubation, the barium carbonate builds up on the surface of the liquid and shall be broken up periodically by shaking the container gently to ensure continued absorption of the evolved CO<sub>2</sub>. This problem can be avoided by using KOH instead of Ba(OH)<sub>2</sub>, which does not form a precipitate.

NOTE A discussion on the use of KOH in place of Ba(OH)<sub>2</sub> is reported in Reference [5].

**8.7.2** The containers for the CO<sub>2</sub> absorber shall be removed and titrated before their capacity is exceeded. The period of time will vary with sediments and test materials and increases slowly as the carbon content of the sediment is reduced (a recommended frequency of every 3 to 4 days for the first two to three weeks and every 1 to 3 weeks, thereafter). At the time of removal of the containers, the reactor should be allowed to sit open so that the air is refreshed before replacing 10 ml of fresh barium hydroxide and resealing the reactor. The reactors should remain open approximately 15 min.

**8.7.3** The carbon dioxide evolution rate may reach a plateau when all of the accessible carbon has been oxidized. The test may be terminated at this point or earlier, at the discretion of the user. If possible, the

residual test material may be extracted from the sediment with an appropriate method and quantified (optional).

NOTE The evolved CO<sub>2</sub> can be quantitatively measured also using other suitable methods such as those based on infrared CO<sub>2</sub>-analysers or those based on TOC analysers equipped with an infrared photometer or on gravimetric analysis.

## 8.8 End of the test

When a constant level of CO<sub>2</sub> evolution is attained (plateau phase reached) and no further biodegradation is expected, the test is considered to be completed. The maximum test period is 24 months. In the case of long test durations, special attention shall be paid to the technical system (e.g. tightness of the test vessels and connections). Any special measures taken e.g. to ensure microbial diversity or to provide sufficient nutrients shall be detailed in the test report. On the last day of the test, measure the pH, acidify all the bottles with 1 ml of concentrated hydrochloric acid in order to decompose the carbonates and bicarbonates, continue the test for 24 h and finally measure the amount of carbon dioxide evolved in each of the series of flasks.

## 9 Calculation and expression of results

### 9.1 Calculation

#### 9.1.1 Amount of CO<sub>2</sub> produced

The first step in calculating the amount of CO<sub>2</sub> produced is to correct the test material reactors for endogenous CO<sub>2</sub> production. The control reactor serves as a blank to correct for CO<sub>2</sub> which may be produced through endogenous respiration of the microorganisms. The amount of CO<sub>2</sub> produced by a test material is determined by the difference (in ml of titrant) between the experimental and blank containers. The next step is to convert millilitres of HCl titrated into milligrams of CO<sub>2</sub> produced.

### 9.1.1.1 Ba(OH)<sub>2</sub> used as CO<sub>2</sub> absorber

When CO<sub>2</sub> enters the absorber containers, it reacts in the following manner:



The BaCO<sub>3</sub> formed is insoluble and precipitates. The amount of Ba(OH)<sub>2</sub> remaining in the solution is determined by titration of the 10 ml with HCl according to the following chemical reaction:



From [Formulae \(1\)](#) and [\(2\)](#), it can be seen that 1 mmol of CO<sub>2</sub> is produced for every 2 mmol of HCl titrated. This means that the number of mmol of CO<sub>2</sub> produced is derived using [Formula \(3\)](#):

$$\text{mmol CO}_2 = \frac{\text{mmol HCl}}{2} \quad (3)$$

The normality of HCl used is 0,05 N. Substituting for mmol gives:

$$\text{mmol CO}_2 = \frac{(0,05 \text{ N}) \times (\text{ml of HCl})}{2} \quad (4)$$

To convert to mg CO<sub>2</sub>, the value shall be multiplied by the molecular weight of CO<sub>2</sub> which is 44:

$$\text{mg CO}_2 = \frac{[(0,05) \times \text{ml titrated}]}{2} \times 44 = 1,1 \text{ ml of HCl titrated} \quad (5)$$

Thus, to convert ml of HCl to mg CO<sub>2</sub>, the former is multiplied by 1,1.

### 9.1.1.2 KOH used as CO<sub>2</sub> absorber

The evolved CO<sub>2</sub> will react with KOH in the following manner:



K<sub>2</sub>CO<sub>3</sub>, the product of [Formula \(6\)](#), is soluble and does not precipitate.

The fresh KOH solution, where no CO<sub>2</sub> has been absorbed, can be titrated with HCl as:



The KOH solutions used as CO<sub>2</sub> absorbers will have both unreacted KOH and K<sub>2</sub>CO<sub>3</sub> as per [Formula \(6\)](#).

During titration, both chemical species will react with HCl, as follows:



The pH shifts in [Formulae \(6\)](#) and [\(7\)](#) are superimposed and cannot be distinguished. Only a single end point in the range of pH between 7 and 8, corresponding to the two reactions, can be identified by using a suitable indicator.

The adsorbed CO<sub>2</sub> can be determined by subtracting from the H<sup>+</sup> equivalents needed to neutralize the original KOH solution and the H<sup>+</sup> equivalents needed to neutralize the reactions represented by [Formulae \(8\)](#) and [\(9\)](#). In practice:

$\text{mmol CO}_2 = \{\text{ml HCl consumed [Formula (7)]} - \text{ml HCl consumed in Formula (8)} + \text{Formula (9) end point}\} \times N \text{ HCl}$

where N is the normality of the HCl solution.

If an end point titrator is available, the mmol of CO<sub>2</sub> can be determined, without using an indicator, with a further reaction. A further addition of HCl makes HCl react with KHCO<sub>3</sub>, produced with reaction [Formula (9)]:



The number of equivalent consumed in Formula (10), and therefore in Formula (9), corresponds to the K<sub>2</sub>CO<sub>3</sub> produced during Formula (6), that in turn corresponds to the absorbed CO<sub>2</sub>.

Consequently, 1 mole of KHCO<sub>3</sub> corresponds to 1 mole of CO<sub>2</sub> reacted in Formula (6):

$\text{mmol CO}_2 = \text{mmol HCl consumed in Formula (10) end point}$

Therefore:

$\text{mmol CO}_2 = \text{ml HCl consumed in Formula (10)} \times N \text{ HCl}$

where N is the normality of the HCl solution.

The amount of CO<sub>2</sub> expressed in milligrams is finally obtained as follows:

$\text{mg CO}_2 = \text{mmol CO}_2 \times 44$

### 9.1.2 Percentage of biodegradation

The percentage of biodegradation is the ration between the evolved CO<sub>2</sub> and theoretical CO<sub>2</sub> (ThCO<sub>2</sub>). The ThCO<sub>2</sub> is:

$$\text{ThCO}_2 = \text{specimen (mg)} \times \text{TOC(\%)} \times \frac{44}{12} \quad (11)$$

where

TOC (%) is the TOC of the plastic material (or reference material) divided by 100;

44 is the molecular weight of CO<sub>2</sub>;

12 is the molecular weight of C.

Therefore:

$$\% \text{ Biodegradation} = \frac{\text{mg CO}_2 \text{ produced}}{\text{ThCO}_2} \times 100 \quad (12)$$

## 9.2 Visual inspection

At the end of the test, check the condition of the samples. If still present, samples can be retrieved for mass determination, other analysis, and photographs.

## 9.3 Expression and interpretation of results

Compile a table of the CO<sub>2</sub> values measured and the percentages of biodegradation for each measurement interval and each test flask. For each vessel, plot an evolved CO<sub>2</sub> cumulative curve and a biodegradation curve in percentage as a function of time.