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Plastics — Marine biodegradation testing — Preparation of optimized intertidal seawater and sediment

Plastiques — Essais de biodégradation en milieu marin — Préparation d'eau de mer et de sédiments intertidaux optimisés

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Contents

Page

Foreword

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This document was prepared by Technical Committee ISO/TC 61, *Plastics*, Subcommittee SC 14, *Environmental aspects*.

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

The assessment of the degree of biodegradation of plastics in marine habitats is one effective measure to understand and evaluate the impact of reuse, recycling and environmental pollution of plastics. The biodegradation of plastics is the process, in which plastics are decomposed by heterotrophic microorganisms, such as bacteria and fungi, through enzymatic hydrolysis and subsequent metabolization. Marine biodegradation proceeds mainly in microbial consortia that form at the interface between seawater and plastics. This is because marine microorganisms live aerobically within biofilms at the interface between the liquid phase of seawater and solid phases such as gravel and shells.

The diversity of microbial consortia in the marine environment is high, depending on their natural environmental conditions. The species and number of microorganisms vary depending on the climate, ocean currents, tides, and topography. Considering the diverse habitats of these microorganisms, three types of biodegradation assessment methods have been developed:

— one-phase systems consisting of seawater or sediment and

— two-phase systems consisting of seawater and seafloor sediments.

However, due to the diversity of microorganisms even a biodegradable material such as cellulose, which is used as a reference material, gave biodegradation results that ranged from 0 to 100 percent in ring tests of these test methods. From the perspective of biodegradable plastic specification, it is thus necessary to optimize the preparation of natural inoculum for the biodegradation tests to avoid those fluctuations in experimental outcomes.

In order to reduce the impact of seasonal and regional variation in the marine inoculum composition, this document describes a method for preparing seawater and seafloor sediments. The prepared seawater and sediment can be used for the test methods defined in ISO 19679, ISO 18830, ISO 22404, ISO 23977-1, ISO 23977-2 and ISO 23832.

Prepared seawater for biodegradation tests is obtained by rinsing seafloor sediments with seawater. Sand and gravel mixtures with particle sizes from 250 µm to 2 mm are used as sediments to provide pore water flow, oxygen supply, seawater filtration and biofilm growth. Through the preparation of defined compositions of seawater and sediments in marine tests, the number of microorganisms and aerobic conditions are stabilized, and reproducibility and comparability of biodegradation experiments (including curves, lag time, etc.) are improved.

This document specifies methods for preparing seawater and sediments in the intertidal zone for estimating the aerobic biodegradation of plastics in pelagic to coastal marine environments.

Plastics — Marine biodegradation testing — Preparation of optimized intertidal seawater and sediment

1 Scope

This document specifies procedures for preparing seawater and sediments used in test methods to assess the biodegradation of plastic materials in the marine environment. The screened sediment and sediment-rinsed seawater are prepared to sustain aerobic testing at laboratory scale. The described method is designed to separate sediment-rinsed seawater and sand-gravel sediments from intertidal sediments by wet filtration and seawater flotation. This document does not include steps to enhance the biodegradation of plastic materials by concentrating the natural seawater, adding nutrients to the seawater, and pre-culturing the inoculum.

The methods described in this document are intended to be used in addition to issued ISO standard test methods for evaluating the biodegradation and disintegration of plastic materials. The applicable evaluation test methods are ISO 18830, ISO 19679, ISO 22404, ISO 23977-1, ISO 23977-2 and ISO 23832.

NOTE The conditions described in this document do not always correspond to the optimum conditions for maximum biodegradation. This is a method of preparing test sediments from coastal seafloor sediments, not a method of preparing sediments from deep-sea seafloors.

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 18830, *Plastics — Determination of aerobic biodegradation of non-floating plastic materials in a seawater/sandy sediment interface — Method by measuring the oxygen demand in closed respirometer*

ISO 19679, *Plastics — Determination of aerobic biodegradation of non-floating plastic materials in a seawater/sediment interface — Method by analysis of evolved carbon dioxide*

ISO 22404, *Plastics — Determination of the aerobic biodegradation of non-floating materials exposed to marine sediment — Method by analysis of evolved carbon dioxide*

ISO 23977-1, *Plastics — Determination of the aerobic biodegradation of plastic materials exposed to seawater — Part 1: Method by analysis of evolved carbon dioxide*

ISO 23977-2, *Plastics — Determination of the aerobic biodegradation of plastic materials exposed to seawater — Part 2: Method by measuring the oxygen demand in closed respirometer*

ISO 23832, *Plastics — Test methods for determination of degradation rate and disintegration degree of plastic materials exposed to marine environmental matrices under laboratory conditions*

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 https://www.electropedia.org/

3.1
intertidal zone
borderline between sea and land that extends from the high tide line, which is rarely inundated with water, to the low tide line, which is typically always covered with water

Note 1 to entry: The tidal zone is frequently a sandy area that is kept constantly damp by the lapping of the waves.

Note 2 to entry: Stony and rocky shorelines also exist.

Note 3 to entry: They are also known as eulittoral zone, midlittoral zone, mediolittoral zone, intertidal zone, foreshore.

[SOURCE: ISO 22404:2019, 3.1]

3.2
biofilm
microbial cells and their metabolites, such as polysaccharides, proteins, lipids and nucleic acids, firmly attached to the material surface of the product in water, and stained with crystal violet

[SOURCE: ISO 4768:2023, 3.1]

3.3
biodegradation
degradation (3.4) caused by biological activity, especially by enzymatic action, leading to a significant change in the chemical structure of a material

[SOURCE: ISO 472:2013, 2.1680]

3.4
degradation
irreversible process leading to a significant change in the structure of a material, typically characterized by a change of properties (e.g. integrity, molecular mass or structure, mechanical strength) and/or by fragmentation, affected by environmental conditions, proceeding over a period of time and comprising one or more steps

[SOURCE: ISO 472:2013, 2.262]

3.5
disintegration
physical breakdown of a material into very small fragments

[SOURCE: ISO 472:2013, 2.1757]

3.6
theoretical amount of evolved carbon dioxide
$ThCO_2$
maximum carbon dioxide evolved after completely oxidising a chemical compound, calculated from the molecular formula or from determination of *total organic carbon (TOC)* (3.7)

[SOURCE: ISO 19679:2020, 3.1, modified — “theoretical amount of” removed after “maximum”.]

3.7
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.

[SOURCE: ISO 17556:2019, 3.14]

3.8
biochemical oxygen demand
BOD
mass concentration of the dissolved oxygen consumed under specified conditions by the aerobic biological oxidation of a chemical compound or organic matter in water, expressed as milligrams of oxygen uptake per milligram or gram of test compound

[SOURCE: ISO 472:2013, 2.1723]

3.9
total dry solids
amount of solids obtained by taking a known volume of test material or inoculum and drying at about 105 °C to constant mass

[SOURCE: ISO 13975:2019, 3.5]

3.10
volatile solids
amount of solids obtained by subtracting the residues of a known volume of test material or inoculum after incineration at about 550 °C from the *total dry solids* (3.9) content of the same sample

Note 1 to entry: The volatile solids content is an indication of the amount of organic matter present.

[SOURCE: ISO 17088:2021, 3.9]

4 Principle

Biodegradable plastics in seawater are primarily degraded into water and inorganic carbon dioxide, and partially assimilated into biomass by heterotrophic microorganisms in the marine food chain. In order to evaluate aerobic biodegradation on a laboratory scale, the culture conditions such as nutrients, pH and microbial species should be specified in the actual seawater and seafloor sediments used. Furthermore, marine microorganisms survive aerobically as microbial communities in biofilms that form at the interface between the liquid phase of seawater and solid phases, such as gravel, shells, and plastic films. Therefore, marine biodegradation is dependent on the marine ecological environment, and the preparation methods of seawater and sediment for marine biodegradation testing also need to be identified.

The pH of seawater is approximately 8,1, while the nutrient levels, biomass, microorganism abundance and diversity are influenced by habitat and seasonal variation.

Interlaboratory tests according to ISO 19679 and ISO 18830 were conducted in nine laboratories in seven countries, as shown in Annex I. At the end of the test, the average carbon dioxide production per gram of wet sediment was 2,1 mg, with values ranging from 0,63 mg to 4,88 mg. The biodegradation value of reference filter paper ranged from 5 % to 160 %, with an average value of 87 % and a coefficient of variation of 36 %. Similarly, an interlaboratory test was also conducted to improve the OECD 306 screening test, as the biodegradation outcome varies depending on the abundance and composition of the microbial community[8].

In particular, the number of viable microorganisms in coastal areas is thousands of times higher than in pelagic areas. On the other hand, sediments maintain aerobic conditions due to characteristics, such as oxygen-saturated water flow, fine particle filtration, and pore water circulation. Sediments also serve as a source of organic carbon, which is necessary for microbial growth, and source of calcium carbonate, which helps buffer the pH of seawater.

This method of preparation of rinsed seawater and refined sediments significantly improves these values for plastic test materials listed in Annex D and H.

In this preparation method, sediments in the sand-gravel area, including shells and corals, are selected from the subseafloor sediments in the intertidal zone of the coastal area by sorting based on the particle size of the object. Seawater for biodegradability testing is collected by washing the seafloor sediments and sand-gravel surface overgrown with biofilms using seawater.

Seafloor sediments are wet filtered using a 2 mm sieve in a container filled with seawater. This sieve is used for soil identification and classification according to ISO 14688-1, removing gravel and collecting clay, silt, and sand based on particle size. Wet filtration separates the seafloor sediments into two layers: a lower layer consisting of a sludge-like sediment including clay, silt, sand, gravel, benthic organisms, and eggs, and an upper layer comprising a seawater suspension containing floating pieces of biofilm and microorganisms. Larger aggregated floating particles are removed from the seawater using a filter paper having a pore size of about 20 µm. This is to obtain filtered seawater containing microorganisms. The sludge-like sediments are refined into sand-gravel sediments by flotation with seawater. By repeating flotation, as shown in Annex A and B, more than 5 times, sediments with a particle size of 250 µm or more are prepared and can be used for biodegradation testing. This sediment preparation method produces larger sediment particles than plastic powder samples prepared according to ISO 10210.

Compared to unprepared pelagic seawater, this preparation method provides seawater with potentially higher microbial diversity and cell count, which can lead to increased biodegradation rates. The prepared seawater and sediment are effective in emulating the biodegradation of plastic materials in marine environments based on BOD and carbon dioxide evolved in laboratory-scale testing.

5 Apparatus

5.1 Sieves, with 2 mm~3 mm opening and 250 µm or 300 µm mesh for filtering sand-gravel by wet filtration method.

5.2 Bowls, two or more 15 l~20 l bowls (e.g. stainless steel) for the kitchen to prepare the sediments by flotation and wet filtration of seawater and sediments.

5.3 Shovel, for collecting top sediment (the layer from surface till about 20 cm depth).

NOTE The type is a pointed digging shovel or gardening shovel about 1 m long.

5.4 Weight scale, capable of weighing 20 kg of seawater or sediment.

5.5 pH Meter, used for measurement of the pH of the marine test mixture. It shall be accurate to 0,1 pH-units or better.

6 Procedure

6.1 General

In the intertidal zone, microorganisms form biofilms at the interface between sediments and seawater. These biofilms exist in aerobic conditions. To collect biofilm-covered sediment and seawater rich in microorganisms, the collected seafloor sediment is passed through a sieve with a pore size of 2 mm in the seawater to remove gravel and seaweed (wet filtration). The filtrate is separated into a suspension and a sludge-like sediment. The suspension separated by decantation is suction-filtered using a filter paper with a pore size of 20 µm to prepare seawater for testing. The sludge-like sediment is washed away by seawater flotation and becomes sand-gravel sediments covered with biofilm. These processes include the removal of benthic organic matter.

Purified seawater and sediment shall be pre-incubated or stored according to the biodegradation or disintegration test methods specified in ISO 18830, ISO 19679, ISO 22404, ISO 23977-1, ISO 23977-2, and ISO 23832.

The purification and sieving steps can be performed outdoors at the sampling point or indoors in the laboratory after transporting the samples taken at sea. In this case, artificial seawater can be used. Artificial seawater formulation shall be in accordance with ISO 18830 or ISO 19679.

Collect top sediment layers from the surface to a depth of about 20 cm, suitable for lab-scale biodegradation tests.

6.2 Collection of sediment with less than 2 mm particle size and preparation of rinsed seawater

Prepare kitchen-use stainless steel mixing bowls (⌀ 45 cm) and sieves (2 mm, ⌀ 35 cm). Place the sieve in the middle of the bowl containing about 10 l of seawater. Add the shovelled seafloor sediment to the sieve. Shake the sieve to loosen and rinse the biofilm on the gravel surface. The biomass containing the biofilm disperses into the seawater in the bowl to form a brown seawater suspension. Clays, silts, sands, gravel and benthic organisms in the seafloor sediments are filtered and precipitated as a sludge-like sediment in the lower layers of the seawater suspension. Remove the residual sediment in the sieve, add fresh sediment and repeat this wet filtration 5 more times, moving the collection point.

If necessary, the collected mixture of suspended seawater and sludge-like sediment is further agitated by hand, to rinse the biofilm from the sand-gravel surface. However, the collected seawater will be in an anaerobic state, so be careful not to collect too much biomass.

6.3 Preparation of refined sediment

The sludge-like sediment prepared in 6.2 should undergo further purification through flotation with seawater to eliminate clay, silt, organic particles, and eggs. Add five times as much seawater as the sediment to the bowl and stir the sediments by hand about ten times. After one minute, remove suspended particles along with the supernatant. Repeating this flotation process around five times will remove most of the particles smaller than 250 µm. The resulting purified sediment is suitable for biodegradation testing. If necessary, use a sieve with a smaller pore size (250 µm or 300 µm), to remove particles of desired dimensions. Mix the prepared sediment with an equal volume of seawater and store at 4 °C for four months or aerobically at room temperature for approximately 1 month. It is preferable to measure the pH of the mixed sediment-seawater system under aerobic conditions where the sediment layer is 3 cm or less.

NOTE As shown in Annex D, 60 g of wet sediment in the test vessel contains 0,72 g of volatile solids (VS). Assuming an algae with an organic carbon content of 50 %,[14] the VS contains 0,31 g of TOC, which corresponds to 1,32 g of $ThCO_2$. However, at the end of the 180-day test, the amount of carbon dioxide evolved from the sediment was 14 mg, and the biodegradation rate of organic matter in the sediment was 1 %. On the other hand, the filter paper containing 73 mg of $ThCO_2$ is 74 % biodegraded, and the carbon dioxide evolved is 54 mg. Furthermore, from the viable cell count results in Annex G, the viable cell counts per ml of rinsed seawater decreased from 10^5 to 10^4 before and after the test. However, the viable cell counts at the end of the test was the same as the viable cell counts (10^4) in the seawater collected in May. On the other hand, the number of viable cell per gram of sand-gravel sediment was 10^6, and there was no change between before and after the test. Test results using two-phase system consisting of rinsed seawater and sand-gravel sediments (carbon dioxide evolved, number of viable cell before and after the test, biodegradation measurement of plastic materials, etc.) are shown in Annexes C to H. The optimized two-phase system maintains high biodegradability and high reproducibility even in long-term studies.

Annex C presents the cumulative amount of carbon dioxide evolved in one-phase systems of rinsed seawater and two-phase systems of rinsed seawater and sand-gravel sediments prepared in the intertidal zone. Cumulative evolved carbon dioxide results showed that the usable lifespan of each test system depended on the number of viable bacteria and nutrient biomass content. The active period of the seawater one-phase system is less than one month. For biodegradation measurements over 180 days, a one-phase test system with nutrients or a two-phase test system with sediment is recommended.

Annexes D to H demonstrate the optimality of this preparation method by demonstrating the biodegradation of the test materials in a two-phase system consisting of rinsed seawater and sand-gravel sediments. The validity of this evaluation was verified from the cumulative amount of carbon dioxide evolved from the reference material and the blank, the dispersion, and the degree of biodegradation of the reference material. After 180 days of testing, the number of viable bacteria decreased in seawater but not in sediments. The absolute biodegradation of the test materials was over 70 % and the relative biodegradation was over 90 %. The coefficient of variation is about 5 %, which is less than 20 %.

6.4 Purification of seawater for biodegradation test from suspended seawater

Leave the suspended seawater prepared in 6.2 at room temperature for about one hour to remove fine particles and filter the supernatant through an air conditioner filter or collect it by decantation. Furthermore, the supernatant is purified by suction filtration, using filter paper with a pore size of approximately 20 µm to

separate microorganisms and contaminants. However, it is recommended to select the filter paper according to the biodegradability test conditions. Purified seawater is stored at 4 °C for 4 months or aerobically store at room temperature for about one month. Sediment and seawater should be stored in containers with forced aeration systems used for sediment/seawater interface degradation testing according to ISO 23832.

Storage increases the risk of bacterial inactivation because bacterial biodiversity changes with storage temperature. Change storage temperature suitable for bacteria and report storage conditions.

If necessary, measure the TOC, volatile solid and nitrogen content of the sediment and of the seawater.

7 Validity of preparation

7.1 Sediment for disintegration testing

The preparation is considered as valid, if

a) the organic carbon content or volatile solid of sediment is in the range of 0,1 % to 1 % or 0,2 % to 2 %;

b) no offensive odor due to putrefaction;

c) pH is in the range of 7,0 to 8,5 with mixture of sediment and seawater at 25 °C.

Refer to ISO 23832 for determination of degradation rate and disintegration degree of plastic materials under marine environmental matrices.

Estimation of organic carbon in natural organic matter can be based on elemental analysis or volatile solids. Calculate the TOC using a volatile solids content of 50 % according to the estimation example given in the Note in 6.3[14]. Volatile solids should be measured per 100 g of wet sediment.

7.2 Refined sediment for biodegradation testing

The preparation is considered as valid, if

a) the organic carbon content or volatile solid of sediment is in the range of 0,1 % to 1 % or 0,2 % to 2 %;

b) the inoculum in the mixture of refined sediment and seawater has produced more than 0,05 mg but less than 0,25 mg of carbon dioxide per gram of wet-sediment (mean values) after 10 days of incubation at 25 °C.

The ISO 19679 test method states that after 6 months, the cumulative amount of carbon dioxide evolved from the wet sediment should not exceed 3,5 mg per gram. The $ThCO_2$ evolved from the reference materials listed in Annex D is approximately 70 mg, and the cumulative amount of carbon dioxide evolved from the 60 g of wet sediment used is preferably less than $ThCO_2$. The test conditions correspond to a cumulative amount of carbon dioxide produced after 6 months of 1,2 mg per gram of wet sediment. The average carbon dioxide accumulation curve for the wet sediment shows a cumulative value of 1,2 mg after 180 days and 0,25 mg after 10 days. Verification of sediment activity by evolved carbon dioxide should be done before biodegradation test of plastic material.

c) no offensive odour due to putrefaction;

d) pH is in the range of 7,0 to 8,5 with mixture of sediment and seawater at 25 °C.

If these criteria are not fulfilled, fresh sediment and seawater shall be prepared in accordance with the procedures described above.

8 Preparation report of seawater and sediments

The preparation report of seawater and sediments shall provide all relevant information, particularly the following:

a) a reference to this document, i.e. ISO 16623:2024;

b) recorded photographs of wet filtration and flotation work at the sampling site and types of sieves used;

c) the location and date of collection, amount, and characteristics of the marine matrices, including pH, ash content, TOC, nitrogen content, storage conditions, handling, and potential;

and, optionally, if run or determined

d) the colony forming units by culture method or total and viable count by flow cytometry or microscopy in seawater and sediment.

Annex A
(informative)

Diagram of seawater and sediment preparation — Seawater and sediment preparation by wet filtration and flotation

Wet filtration divides the seafloor sediment into a sludge-like sediment of clay, silt, sand-gravel on the bottom, and suspended seawater containing biofilms and microorganisms rinsed from the gravel surface on the upper layer. This suspension is suction-filtered through a filter paper with a pore size of about 20 µm to prepare seawater for testing. Sand-gravel sediments for testing are also prepared from sludge-like sediments by flotation to remove clays, silts, and benthic organisms with particle sizes less than 250 µm. A diagram of preparation with sediments and seawater is shown in Figure A.1.

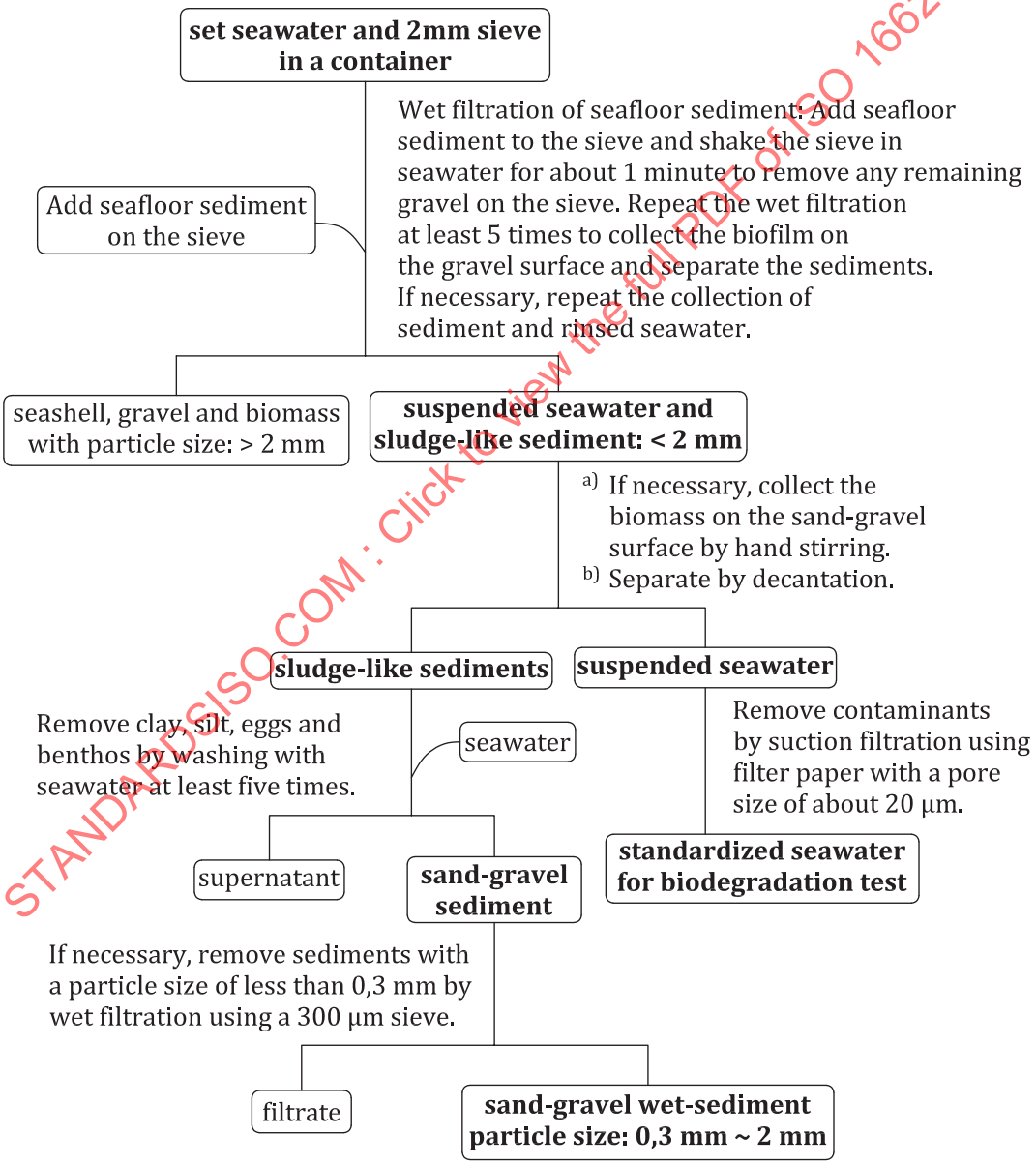


Figure A.1 — Diagram of seawater and sediment preparation by we filtration and flotation

Annex B
(informative)

Preparation of seawater and sediments from seafloor sediments: Wet filtration and flotation of sediments

An example of the collection is shown in Figure B.1.

Wet filtration of seafloor sediments. Add shovelled seafloor sediments on a 2 mm sieve submerged in seawater and remove any remaining gravel after shaking the sieve by hand. Collect fresh seafloor sediments, and repeat this wet filtration at least 5 times. See Figure B.1 a).

Wet filtration separates the sediment into residual sediment and brown rinse seawater with mud at the bottom. The brown suspension is seawater containing microorganisms and biofilms. Sludge-like sediment consisting of clay, silt, and sand-gravel settles at the bottom. The suspension separated by decantation is suction filtered using filter paper with a pore size of about 20 µm to prepare seawater for testing. See Figure B.1 b).

Sand-gravel sediment refined by flotation of sludge-like sediment with seawater. Contaminants such as clay, silt, benthic organisms and eggs are removed by five or more rounds of flotation to collect test sediments with particle sizes from 250 µm to 2 mm. See Figure B.1 c).

If necessary, remove residual particles by wet filtration using a 250 µm or 300 µm sieve. See Figure B.1 d).

NOTE Sediments and seawater were collected in the cove of Miho, Shimizu-ku, Shizuoka City on May 28, 2021. The pH and seawater temperature are 7,9 °C and 21,4 °C.

Figure B.1 — Wet filtration and flotation in sediment preparation

There is a risk of a wave accident, so take a sample with a few people, and if you have a coastal security office, get a permit in advance. Wear hip boots when scooping undersea sediments. The seafloor is covered

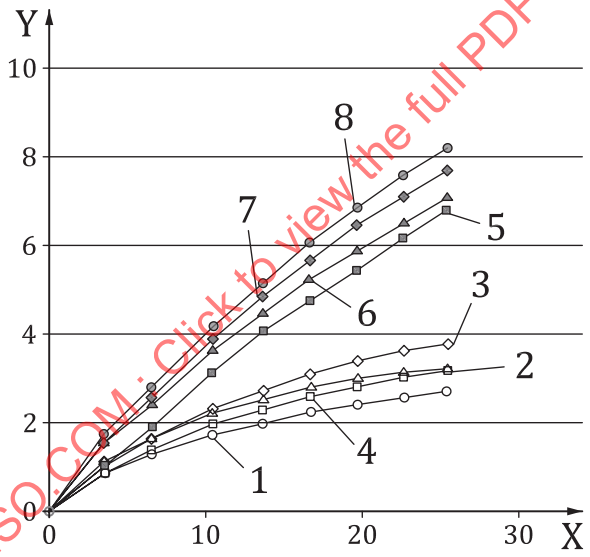
with barnacles, so be careful of falls and lacerations. The best time to collect sediment is at low tide in the spring tide, when the amount of carbon dioxide generated is high.

Annex C
(informative)

Evolved carbon dioxide of seawater and sediment prepared from seafloor sediments

C.1 Carbon dioxide evolved from rinsed seawater and sand-gravel sediment

Cumulative carbon dioxide evolution and rate are compared in seawater one-phase systems and seawater-sediment two-phase systems. The results in Figure C.1, Tables C.1, C.2 and Figure D.1 show that the two-phase system is effective for long-term marine biodegradation tests of 180 days. For two-phase systems consisting of seawater filtered with pore sizes of 11 µm to 25 µm and refined sediment, both the cumulative CO_2 evolution and rate are high, and the coefficient of variation is also low. After 25 days, the rate of CO_2 evolution in the one-phase system of seawater decreases to about one tenth of that in the two-phase system. As shown in Tables G.2 and G.3, the test stability of the two-phase system was verified from results of viable cell counts in seawater and sediments of blank test vessels after 196 days of biodegradation testing. Furthermore, the high biodegradability of the film sample was also confirmed.



Key

X	time (days)	4	rinsed seawater left overnight
Y	carbon dioxide evolved (mg)	5	two phase system of sediment and 4)
1	rinsed seawater passed through pore size 2,5 µm	6	two phase system of sediment and 2)
2	rinsed seawater passed through pore size 11 µm	7	two phase system of sediment and 3)
3	rinsed seawater passed through pore size 20-25 µm	8	two phase system of sediment and 1)

NOTE According to ISO 19679, the test mixture was prepared from 120 ml of seawater and 30 ml (60 g) of wet sediment. The test was performed under the conditions at 25 °C and a flow rate of about 0,5 ml/min. The amount of carbon dioxide evolved was determined from the volume and carbon dioxide concentration of the outlet carrier gas collected in the bag. The volume of the gas and the concentration of carbon dioxide were measured by a gas burette and an NDIR sensor. The cumulative evolved carbon dioxide shows the mean (n = 3). The value of pore size is particle retention capacity of filter paper used.

Figure C.1 — Comparison of single-phase and two-phase systems based on the carbon dioxide evolved

C.2 Marine biodegradability of plastics and test systems

Seawater containing biofilms and microorganisms was prepared by removing contaminants from suspensions rinsed from gravel and shell surfaces shown in Annex B, Photo 2. Contaminants were removed by suction filtration using 2,5 µm, 11 µm, or 20 µm to 25 µm filter papers or left overnight. Using prepared seawater and repurified sediments, carbon dioxide evolved was measured for seawater one-phase systems and seawater-sediment two-phase systems at 25 °C for 25 days. The cumulative amount and rate of carbon dioxide evolved at the end of the test are shown in Tables C.1 and C.2, respectively.

After 25 days, the cumulative carbon dioxide evolution of the seawater one-phase system is about half that of the seawater-sediment two-phase system, but the carbon dioxide evolution rate is about one-tenth that of the seawater-sediment two-phase system. One reason for this is that the number of viable cells in seawater is approximately 100 times lower than in sediments, as shown in Annex G. Beyond 25 days, the carbon dioxide evolved from the test system is determined by the amount of sediment.

If the biodegradation lag time of the plastic is expected to exceed one month, then a two-phase system of sea water and sediment should be used. If a one-phase system of seawater is used, add beneficial nutrients to the microflora.

Table C.1 — Cumulative carbon dioxide after 25 days in marine biodegradation test

Preparation method of rinsed seawater	Pore size of filter paper in suction filtration			Standing overnight
	2,5 µm	11 µm	20 µm to 25 µm	
	mg (CV)	mg (CV)	mg (CV)	mg (CV)
Seawater-sediment two-phase systems	8,21 (14 %)	7,09 (5,4 %)	7,71 (5,3 %)	6,78 (13 %)
Seawater one-phase systems	2,71 (22 %)	3,26 (15 %)	3,80 (22 %)	3,18 (2,9 %)

NOTE 1 Values are the mean of cumulative values (n = 3) after 25 days shown in Figure C.1, and CV is the coefficient of variation of the mean.

Table C.2 — Carbon dioxide evolution rate after 25 days in marine biodegradation test

Preparation method of rinsed seawater	Pore size of filter paper in suction filtration			Standing overnight
	2,5 µm	11 µm	20 µm to 25 µm	
	mg/day (CV)	mg/day (CV)	mg/day (CV)	mg/day (CV)
Seawater-sediment two-phase systems	0,373 (4,8 %)	0,355 (2,1 %)	0,368 (2,4 %)	0,367 (6,4 %)
Seawater one-phase systems	0,046 (44 %)	0,043 (6,1 %)	0,058 (39 %)	0,056 (7,0 %)

NOTE 2 The amount of carbon dioxide evolved per day is the average value (n = 3) obtained by dividing the difference in cumulative values from 22nd to 25th by the period shown in Figure C.1. CV indicates the coefficient of variation of the average value.

Annex D
(informative)

Test validity of ISO 19679 — Validity of biodegradation test based on carbon dioxide evolved

Test validity shall be obtained by satisfying the following criteria.

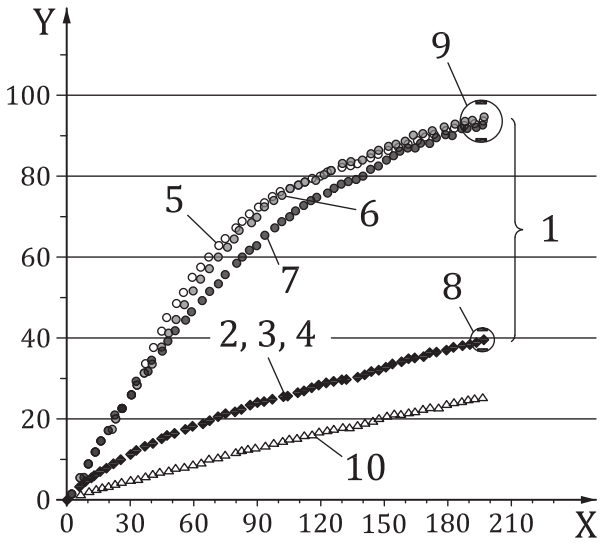
a) The degree of biodegradation of the reference material exceeds 60 % after 180 days. The evaluation and criteria values were 54 mg and 44 mg [($ThCO_2$ (73 mg) × 0,6)].

b) The evolved CO_2 of the blank vessels at the end of the test does not exceed 3,5 mg CO_2/g wet sediment after 6 months. The evaluation and criteria values were 14 mg and 210 mg (wet sediment 60 g x 3,5 mg/g)).

c) The amount of CO_2 evolved from the three blank vessels are within 20 % of the mean at the plateau phase or at the end of the test. The evaluated value was within the criteria circle (radius 2,8 mg = 14 mg × 0,2).

d) The difference between the percentage biodegradation of the reference material in the different vessels is less than 20 % of the mean at the end of the test. The evaluated value is within the criteria circle (radius 5,4 mg = 54 mg x 0,1).

e) The percentage of biodegradation of the negative control (LDPE) is below 10 % at the end of the test. These evaluated and biodegradation values are shown in Figures E.1, F.1, and Table H.1.



Key

X	time (days)	5	whatman filter paper grade 42-1
Y	evolved carbon dioxide (mg)	6	whatman filter paper grade 42-2
1	evaluated CO_2 for filter paper with 54 mg	7	whatman filter paper grade 42-3
2	blank-1	8	criteria circle of blank with ±20 % error
3	blank-2	9	criteria circle of reference with ±10 % error
4	blank-3	10	cumulative amount of CO_2 contained in supply air

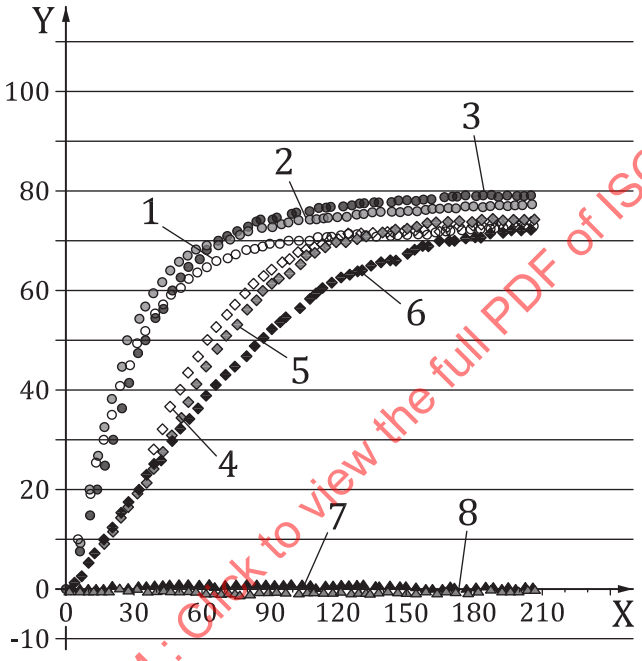
NOTE After 10 days and 180 days, the blank vessel containing 60 g of wet sediment produced 3,1 mg and 14 mg of carbon dioxide, corresponding to 0,05 mg and 0,25 mg per gram, respectively. The wet sediment contains approximately 0,31 g of TOC, which corresponds to 1,32 g of $ThCO_2$. After 180 days, approximately 1 % of the TOC in the wet sediment was biodegraded and 14 mg of carbon dioxide was produced. On the other hand, the standard material was 74 % biodegraded and produced 54 mg of carbon dioxide.

Figure D.1 — Carbon dioxide evolved and validity of biodegradation test

Annex E
(informative)

Example of biodegradation of reference material by ISO 19679

According to ISO 19679, the test mixture was prepared from 120 ml of seawater and 30 ml (60 g) of sediment. Seawater was prepared by filtering seawater washed (see 6.4) with Whatman grade 41 (20 µm to 25 µm) to remove clay material, fine benthos and eggs. Sediments (300 µm to 2 mm particle size) were prepared from seafloor sediments by wet filtration and flotation. The size of the test film was adjusted to a thickness and TOC of 35 µm and about 20 mg, respectively. An example of reference materials is shown in Figure E.1.



Key

X	time (days)	4	filter paper-1
Y	percentage biodegradation	5	filter paper-2
1	PHBH-1	6	filter paper-3
2	PHBH-2	7	LDPE-1
3	PHBH-3	8	LDPE-2

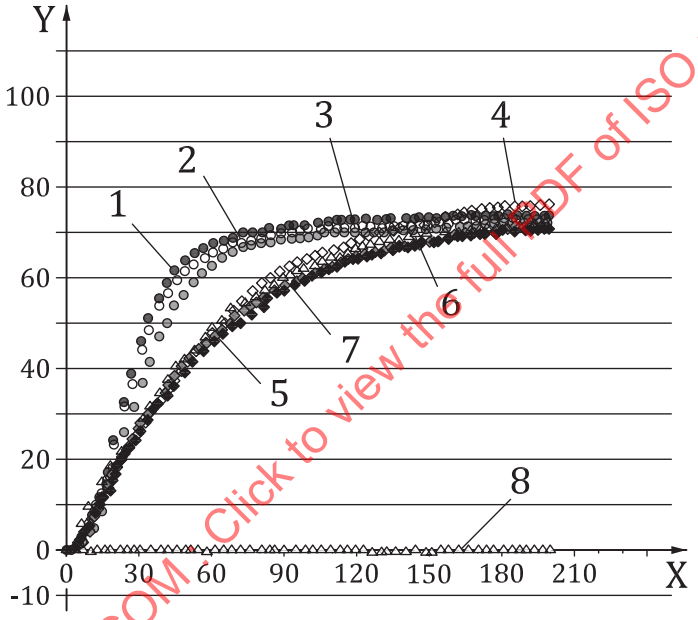
NOTE Biodegradation measurement was performed under the conditions at 25 °C and 0,5 ml/min airflow for 196 days. The amount of carbon dioxide evolved was determined from the volume and carbon dioxide concentration of the outlet carrier gas collected in the bag. The volume of the gas and the concentration of carbon dioxide were measured by a gas burette and an NDIR sensor. The pH of the mixture of sediment and seawater is 8,1. The wet sediment contains 0,72 g of volatile solids and approximately 0,31 g of TOC. Whatman grade 42 (2,5 µm) filter paper was used as reference material. To prevent oxygen loss in the sediment, test films were allowed to stand on the sediment surface without the use of a net covering the films specified in ISO 19679. However, to prevent sample from floating, a glass rod with a diameter of 3 mm and a length of 5 cm was inserted into the punched LDPE film sample.

Figure E.1 — Example of biodegradation reference materials by ISO 19679

Annex F
(informative)

Example of biodegradation of plastic films by ISO 19679 — Biodegradation of plastic films using rinsed seawater and sand gravel sediments

According to ISO 19679, the test mixture was prepared from 120 ml of seawater and 30 ml (60 g) of sediment. Seawater was prepared by filtering washed (6.4) seawater through Whatman grade 41 (20 µm to 25 µm) to remove clay material, fine benthic fauna, and eggs. Sediments (300 µm to 2 mm particle size) were prepared from seafloor sediments by wet filtration and flotation. The size of the test film was adjusted to a thickness of 35 µm and TOC of about 20 mg. An example of reference materials is shown in Figure F.1 and Table H.1.



Key

X	time (days)	4	PBSA-1
Y	percentage biodegradation	5	PBSA-2
1	PGA-1	6	PBSA-3
2	PGA-2	7	PCL-mean (n=3)
3	PGA-3	8	LDPE-mean (n=2)

NOTE Biodegradation measurement was performed under the conditions at 25 °C and 0,5 ml/min airflow for 196 days. The amount of carbon dioxide evolved was determined from the volume and carbon dioxide concentration of the outlet carrier gas collected in the bag. The volume of the gas and the concentration of carbon dioxide were measured by a gas burette and an NDIR sensor. The pH of the mixture of sediment and seawater is 8,1. The wet sediment contains 0,72 g of volatile solids and approximately 0,31 g of TOC. To prevent oxygen loss in the sediment, test films were allowed to stand on the sediment surface without the use of a net covering the films specified in ISO 19679.

Figure F.1 — Example of biodegradation of plastic films by ISO 19679