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Plastics — Ecotoxicity testing scheme for soluble decomposition intermediates from biodegradable plastic materials and products used in the marine environment — Test methods and requirements

Plastiques — Méthodes d'essai d'écotoxicité pour les intermédiaires de décomposition solubles à partir de matériaux et produits plastiques biodégradables utilisés dans le milieu marin — Méthodes d'essai et exigences

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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 document 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).

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

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

There is a growing interest in using biodegradable materials in products used in the marine environment (e.g. farming and fishing gears, floats, buoys and other non-fishing materials or products). These products are subject to wear and tear and, therefore, tend to be sources of macro- and microplastics. Biodegradability is a factor that, in principle, mitigates the environmental impacts of fragmentation, thanks to persistence times in the environment that are shorter than that of non-biodegradable materials. Therefore, test methods to measure the level of biodegradation and disintegration of plastic materials in different marine habitats have been established by ISO/TC 61/SC 14 in recent years to better characterize the degradation of plastics in these very particular environments:

— The test standards like ISO 18830 (or ISO 19679), ISO 22404, ISO 23977-1 (or ISO 23977-2) are suited to investigate the biodegradation of plastic materials exposed to marine environmental samples (sediments and seawater).

— The ISO standard specification ISO 22403 specifies test methods and requirements to assess the intrinsic biodegradability of materials exposed to marine inocula under mesophilic aerobic laboratory conditions.

— The ISO standard ISO 22766 describes methods for the determination of the degree of disintegration of biodegradable plastic materials exposed to sublittoral and eulittoral habitats under real field conditions.

— The ISO standard ISO 23832 describes methods for the determination of the degradation rate and disintegration degree of plastic materials exposed to marine environmental matrices under laboratory conditions.

Besides data on the biodegradability of plastics materials, tests on ecotoxicological effects of potential soluble decomposition intermediates of the biodegradation process to marine organisms are necessary to enable developer and manufacturer of materials to evaluate and to exclude negative effects on marine organisms. In addition, in combination with data on biodegradability, data on ecotoxicological effects can be used for e.g. risk assessment purposes.

This document specifies test methods and requirements for assessing potential adverse effects on different marine organisms caused by soluble decomposition intermediates (degradation products) resulting from the decomposition of plastic materials that are intentionally used in marine areas.

Comprehensive ecotoxicity testing schemes and evaluation criteria are already part of ISO standard specifications like ISO 17088 and ISO 23517. The scheme and criteria given in ISO 23517 are equivalent to the requirements specified in the CEN-standard EN 17033. The CEN-document EN 17427 on carrier bags suitable for treatment in well-managed home composting installations includes an ecotoxicity testing scheme that follows the same basic principles as laid down in above mentioned ISO- and EN-standard specifications: adverse effects are assessed based on results from three tests covering organisms representing different trophic levels.

This document aims to provide a suitable ecotoxicity testing scheme for marine organisms.

Plastics — Ecotoxicity testing scheme for soluble decomposition intermediates from biodegradable plastic materials and products used in the marine environment — Test methods and requirements

1 Scope

This document specifies test methods and evaluation criteria by addressing potential ecotoxicological adverse effects on marine organisms. Adverse effects on marine species can be caused by soluble degradation products of plastic materials such as intermediates or remaining residues resulting from the biodegradation of plastic materials that are used in products for marine applications (e.g. nets for fish farming, dolly ropes, floats, buoys and other non-fishing applications) and which are used in different marine zones, e.g. eulittoral, sublittoral or pelagic zones.

The ecotoxicity testing scheme covers marine organisms from four trophic levels, primary producer, primary and secondary consumers and decomposer:

— toxicity to marine algae,

— toxicity to marine invertebrates,

— toxicity to marine fish,

— toxicity to marine microorganisms.

This document is not suitable for the assessment of adverse effects caused by solid materials of any size.

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 472, *Plastics — Vocabulary*

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

ISO 10210, *Plastics — Methods for the preparation of samples for biodegradation testing of plastic materials*

ISO 10253:2016, *Water quality — Marine algal growth inhibition test with Skeletonema sp. and Phaeodactylum tricornutum*

ISO 11348 (all parts), *Water quality — Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test)*

ISO 14669, *Water quality — Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea)*

ISO 17088, *Plastics — Organic recycling — Specifications for compostable plastics*

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*

OECD 2019), Test No. 203 *Fish, Acute Toxicity Test,* OECD Guidelines for the Testing of Chemicals, *Section 2*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 472, ISO 17088 and the following 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
biodegradable plastic
plastic material which, under controlled laboratory conditions, is intrinsically biodegradable

Note 1 to entry: The relevant controlled laboratory conditions are presented in ISO 22403.

4 General

The marine ecotoxicity testing scheme uses test methods with several marine organisms belonging to different taxonomic groups and trophic levels (see Table 1). The selected test methods, in combination with defined test acceptability criteria, allow the characterization of toxic properties of soluble degradation products of biodegradable plastic materials (i.e. intermediates or remaining residues) to marine organisms.

Table 1 — Ecotoxicity testing scheme for evaluation of adverse effects on marine organisms that represent different trophic levels in marine aquatic food webs

Marine taxa – species	Ecological process	Test method
Algae — *Skeletonema sp* — *Phaeodactylum tricornutum*	Primary producer	Marine algal growth inhibition test according to ISO 10253 with modifications specified in Annex A
Invertebrates — *Acartia tonsa* — *Tisbe battagliai* — *Nitocra spinipes*	Primary consumer	Acute lethal toxicity test to marine copepods according to ISO 14669 with modifications specified in Annex B

Table 1 *(continued)*

Marine taxa – species	Ecological process	Test method
Fish (optional) — *Cyprinodon variegatus* — *Dicentrarchus labrax* — *Pagrus major*	Secondary consumer	Fish acute toxicity test according to OECD 203 with modifications specified in Annex C
Micoorganisms — *Aliivibrio fischeri (old name: Vibrio fischeri)*	Decomposer	Determination of inhibitory effect on light emission of luminescent bacteria according to ISO 11348 (all parts) with modifications specified in Annex D

Ecotoxicological effects on marine organisms shall be determined by comparing marine matrices from preceding marine biodegradation tests produced with or without the addition of a test material.

5 Test methods

5.1 Preparation of marine matrices for ecotoxicity testing

5.1.1 General

Plastic materials preferably in the form of film or powder with a maximum thickness or diameter of 500 µm shall be exposed to marine inocula under mesophilic laboratory test conditions following ISO standard test method ISO 18830 (or ISO 19679), ISO 22404 or ISO 23977-1 (or ISO 23977-2), for up to two years. Powder shall be prepared in accordance with ISO 10210.

The minimum initial test item concentration in the marine biodegradation tests shall be the concentration recommended in the standard test method used to produce the matrices in order to ensure comparable initial test item concentrations and proper biodegradation. For that reason, the minimum test item concentrations shall be at least 75 mg/100 g of sediment for ISO 22404 and at least 100 mg/l of seawater plus sediment for ISO 18830 or ISO 19679 and 100 mg/l seawater for ISO 23977-1 or ISO 23977-2.

The same applies to the reference material. Use microcrystalline cellulose or ashless cellulose filters as a reference material[1)]. If possible, the form, size and the amount of carbon added in form of the polymer should be comparable to that of the test material.

In addition to marine matrices exposed to plastic materials (test sample) or to reference material (reference sample), marine matrices not exposed to plastic materials (control sample) are incubated in parallel.

The reactors for preparing the matrices for the test samples shall be run until constant biodegradation per time unit (e.g. 5 % biodegradation in one month) is reached and more than 50 % of total biodegradation is observed so that degradation products might have released into the seawater.

There are two options to test the ecotoxicity of soluble decomposition intermediates from biodegradable plastic materials:

a) Option 1 – limit test: the marine test samples for ecotoxicity testing should be used for the indicated ecotoxicity tests when the absolute mineralization level reaches 50 %.

1) Microcrystalline Cellulose "Avicel" produced by Merck or Laboratory filter paper Whatman n° 42 has been found satisfactory for this purpose and are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

b) Option 2 – time dependent test: the marine test samples for ecotoxicity testing should be used for the indicated ecotoxicity tests when absolute mineralization reaches 20 %, 40 % and a plateau (equal or greater than 60 %).

NOTE 1 Option 2 can be used in cases where an adaption of bacterial activity in biodegradation is expected, when the constituents are unevenly distributed in the test material or when the rate of biodegradation of different constituents is expected to be different.

For both options, the biodegradation should be monitored following existing ISO standard test method ISO 18830 (or ISO 19679), ISO 22404 or ISO 23977-1 (or ISO 23977-2).

WARNING — The biodegradation methods used in this document are exclusively intended to be used to generate a testing matrix for ecotoxicity testing of biodegradable plastic materials or products that already fulfil the requirements (see 3.1) to be biodegradable. They shall not be used to assess the biodegradation or claim biodegradability of any tested material or product tested in the context of this document.

The matrices for ecotoxicity tests derived from tests in marine sediment (see ISO 22404) shall be prepared in accordance with ISO 5667-16. Conserve the samples at low temperature (approximately 4 °C) until processing. It is recommended that the samples are used for ecotoxicity testing within one week after sampling.

NOTE 2 To extract the pore water that might contain soluble decomposition intermediates, a defined amount of artificial seawater (e.g. 1 l per 1 kg sediment) can be used to extract pore water from the sediment.

5.1.2 Enriched cultures

It is recommended to use pre-exposed inoculum to reduce the expected long exposure time due to the high initial test item concentration. To pre-adapt polymer degrading microbial communities, fresh marine sediment and fresh seawater should be mixed in a ratio of 1:4 to 1:10 (sediment: seawater), e.g. one gram of sediment and 10 ml of seawater, and shaken vigorously. After stopping the shaking, waiting for at least 1 min, in some cases up to 10 min, it is recommended for the sediment to settle down. In case of significant turbidity of the seawater phase, a filtration step can be performed using a filter with ≥ 30 µm to remove coarse particles. 1 ml of the supernatant should be taken to inoculate 50 ml artificial seawater. In addition, 0,1 g/l to 1,0 g/l peptone is added as additional C- and N-source. Test material in the form of film, for example 5 cm^2 piece, is added to the cultures and incubated under conditions with constant shaking or stirring at mesophilic test conditions. After the polymer material is disintegrated to a level where, at least, cracks and holes are observed, analysed by means of visual inspection, a 1 ml aliquot is transferred into 50 ml fresh artificial seawater medium or natural seawater, amended with above mentioned inorganic and organic nutrients, and new test material. The use of additional organic nutrients, e.g. peptone, in the fresh medium after the transfer is not recommended. After 4 to 5 transfers, a pre-adapted culture should be available able to degrade the test material in a more efficient way (complete biodegradation in a few weeks instead of months). The inoculum should be used to test according to ISO 23977.

EXAMPLE Artificial seawater can be prepared as follows: 23,5 g/l NaCl, 3,9 g/l Na2SO4, 5 g/l MgCl2, 1,5 g/l CaCl2, 664 mg/l KCl, 96 mg/l KBr, 53 mg/l NH4Cl, 26 mg/l H3BO3, 17,4 mg/l K2HPO4, 200 mg/l HEPES).

5.2 Determination of ecotoxicological effects on marine algae (mandatory)

The basis for the determination of adverse effects on marine algae is ISO 10253. Marine unicellular algae are exposed to marine matrices and the increase in cell number or in biomass of the algae is determined after 72 h.

Both, the principles of the standard test method ISO 10253 and the modifications given in Annex A shall be followed to meet the special needs for testing marine matrices exposed to biodegradable materials.

In Annex A, a description of the ecotoxicity test is given including calculation of test results, validity criteria and evaluation criteria.

5.3 Determination of ecotoxicological effects on marine invertebrates (marine copepods) (mandatory)

The basis for the determination of adverse effects on marine invertebrates (marine copepods) is ISO 14669. Marine adult copepods are exposed to marine matrices and the immobility or mortality of the copepods is determined after 48 h.

The principles of the standard test method ISO 14669 and the modifications given in Annex B, which are required to meet the special needs for testing marine matrices exposed to biodegradable materials, shall be followed.

In Annex B, a description of the ecotoxicity test is given including calculation of test results, validity criteria and evaluation criteria.

5.4 Determination of ecotoxicological effects on marine fish (optional)

The basis for the determination of adverse effects on marine fish is OECD 203. Marine fish are exposed to marine matrices and the mortality and visible abnormalities related to appearance and behaviour of the fish is determined after 96 h.

The principles of the standard test method OECD 203 and the modifications given in Annex C, which are required to meet the special needs for testing marine matrices exposed to biodegradable materials, shall be followed.

In Annex C, a description of the ecotoxicity test is given including calculation of test results, validity criteria and evaluation criteria.

5.5 Determination of ecotoxicological effects on marine microorganisms (mandatory)

The basis for the determination of adverse effects on marine microorganisms is ISO 11348 (all parts). A cell suspension of a bioluminescent bacterium is exposed to marine matrices and the inhibition of light emission (bioluminescence) is determined after 30 min.

The principles of the standard test method ISO 11348 (all parts) and the modifications given in Annex D, which are required to meet the special needs for testing marine matrices exposed to biodegradable materials, shall be followed.

6 Test report

The test report shall provide all relevant information, including:

a) all information necessary to identify the product or material tested;

b) a description of requirements of this document and a statement, for each requirement, as to whether the test result is in agreement with the requirement to:

   — 5.2 and Annex A regarding adverse effects on marine algae;

   — 5.3 and Annex B regarding adverse effects on marine invertebrates (marine copepods);

   — 5.4 and Annex C regarding adverse effects on marine fish;

   — 5.5 and Annex D regarding adverse effects on marine microorganisms.

c) the documentation enabling the identification of any supplementary information (including externally sourced technical data) necessary to support the conclusions made in the assessments.

Annex A
(normative)

Determination of ecotoxicological effects on the marine algae *Skeletonema* sp. and *Phaeodactylum tricornutum*

A.1 Preparation of samples

The determination of adverse effects to the algae *Skeletonema* sp. or *Phaeodactylum tricornutum* performed on the following marine matrices:

— control sample: marine matrices not exposed to a test material;

— test sample: marine matrices exposed to the test material;

— reference sample: marine matrices exposed to a reference material.

The preparation of the marine matrices for ecotoxicity testing shall be conducted according to 5.1.

Turbid samples should be allowed to settle for 1 h or centrifuged, for example for 10 min at 5 000 *g*, or should be filtered using filter paper with 0,2 µm pore size.

A.2 Test species

Use the marine unicellular algae *Skeletonema* sp. or *Phaeodactylum tricornutum.*

These algae are important and widely distributed phytoplankton species in estuarine and coastal areas.

A.3 Performing the tests

Conduct the tests in 250 ml conical glass flasks, loosely covered with air-permeable stoppers.

The inoculum and a concentrated growth medium shall be prepared in accordance with ISO 10253.

Mix 10 ml of the inoculum (see ISO 10253:2016, 7.2) and 10 ml of a concentrated growth medium (see ISO 10253:2016, A.2) with either 80 ml of the control sample, or 80 ml of the test sample or 80 ml of the reference sample to obtain a final volume of 100 ml.

NOTE Due to mixing of the inoculum with concentrated growth medium and either control sample, test sample or reference sample, the highest control, test, or reference sample concentration in the test is 80 %.

The initial cell number of algae shall be between 2×10^3 and 1×10^4 cells per ml. The algae cells should be in an exponential growth phase.

The test shall be performed with at least four replicates for each test sample and reference sample, and six replicates for the control sample.

Before the start of the test the pH is measured in each flask and if needed, adjusted to pH 8,0 ± 0,2 (see ISO 10253:2016, 7.1).

Gently shake the flasks using a laboratory shaker at a temperature of 20 °C ± 2 °C and continuously illuminated (light intensity: approximately 4 440 lux to 8 880 lux, corresponding to 60 µE × m^{-2} × s^{-1} to 120 µE × m^{-2} × s^{-1}) in a cabinet or room for a test period of 72 h following ISO 10253.

Measure the cell density in all flasks after 72 h. Appropriate methods for measuring algae cell density are given in ISO 10253:2016, 6.2 and Annex B. In addition, the pH in each flask is determined at test end.

A.4 Calculation of percentage inhibition

Calculate the percentage inhibition following ISO 10253:2016, 9.2:

a) calculate the average specific growth rate (μ) for each individual test sample, control sample and, if needed, reference sample using ISO 10253:2016, 9.2, Formula (1);

b) calculate the mean control value of the specific growth rate for the control samples;

c) calculate the percentage inhibition for each individual test and, if needed, reference sample using ISO 10253:2016, 9.2, Formula 2.

A.5 Evaluation of results

The percentage inhibition in a test sample shall be ≤ 10 % of those from the control sample. In case, the inhibition of algal growth is >10 % then there is an indication of an impact due to exposure to the biodegradation productions from the biodegradable plastic.

If the difference in the endpoint response in the reference sample is higher than 10 % of those from the control sample, then this can be considered as an indication that a transient toxicity caused by the biodegradation of the high amount of biodegradable materials added to the marine matrices is present and affecting the test. The test is to be considered as not valid and to be repeated after a further biodegradation of the material.

A.6 Validity of the test

The test is considered valid if the criteria in ISO 10253:2016, Clause 8 are met.

Annex B
(normative)

Determination of ecotoxicological effects on marine copepods (Copepoda, Crustacea)

B.1 Preparation of samples

The preparation of samples shall be performed according to A.1.

B.2 Test species

Use one of the three following marine copepod species (Copepoda, Crustacea):

a) *Acartia tonsa* Dana (Copepoda, Calanoida);

b) *Tisbe battagliai* Volkmann-Rocco (Copepoda, Harpacticoida);

c) *Nitocra spinipes* Boeck (Copepoda, Harpacticoida)

All three copepod species can be found in a large portion of the world´s estuaries and areas of upwelling where food concentrations are high. All three species can be cultured continuously in the laboratory.

The test organisms are cultured according to ISO 14669 to provide age standardized cohorts.

B.3 Performing the test

Tests are conducted in 100 ml glass beakers, each containing either 60 ml of the control sample, or 60 ml of the test sample or 60 ml of the reference sample.

Before the start of the test, determine the dissolved oxygen concentration and the pH in at least one replicate of each sample (see A.1) shall be measured.

Expose 10 copepods (see B.2) per replicate with four replicates for each sample (see B.1). Loose-fitting lids or covers are recommended to minimize evaporation of the test solutions.

During the test, keep the glass beakers at a temperature of 20 °C ± 2 °C and under a 16 h/8 h light/dark photoperiod according to ISO 14669.

After 48 h, count the surviving copepods in each glass beaker. A copepod is regarded as immobile or dead if, after a gentle stimulation, no movement is visible after 10 s.

After counting the surviving copepods at 48 h, measure the dissolved oxygen concentration and the pH in at least one replicate of each sample (see B.2).

B.4 Evaluation of results

The mortality/immobilisation in the test sample shall be ≤10 % of those from the control sample. In case, the mortality/immobilisation of copepods is >10 % then there is an indication of an impact due to exposure to the biodegradation productions from the biodegradable plastic.

If the difference in the endpoint response in the reference sample is higher than or equal to 10 % of those from the control sample, then this can be considered as an indication that a transient toxicity caused by the biodegradation of the high amount of biodegradable materials added to the marine matrices is

present and affecting the test. The test is to be considered as not valid and to be repeated after a further biodegradation of the material.

B.5 Validity of the test

The test is considered valid if:

a) the dissolved oxygen concentration at the end of the test is greater ≥ 4 mg/l;

b) the percentage mortality/immobility of the control samples is ≤10 %;

c) the percentage mortality/immobility of the reference sample is ≤10 % compared to the control sample.

Annex C
(informative)

Determination of ecotoxicological effects on marine fish

C.1 Preparation of samples

The preparation of samples shall be performed according to A.1.

C.2 Test species

Use one of the three following marine fish species:

a) Sheepshead minnow (*Cyprinodon variegatus*)

b) European sea bass (*Dicentrarchus labrax*)

c) Red sea bream (*Pagrus major*)

The three marine fish species are readily available, can be easily maintained, and were historically used in chemical safety testing. They can be bred and cultivated either in fish farms or in the laboratory under disease-free conditions. In case that species are used other than listed above or in Annex 2 of OECD 203, the rationale shall be reported together with any adaptations to the test guideline's recommendations.

The test organisms should be selected from the same source and population and should have the same age or size according to OECD 203.

C.3 Performing the test

Tests are conducted in any glass, stainless steel or other chemically inert vessel that is large enough to keep fish free from stress and complies with the loading rate criteria given in §20 of OECD 203. The volume of the test vessel shall be chosen with respect to a maximum loading rate of 0,8 g wet weight fish per litre.

NOTE Due to large experimental volume that is needed for the fish test, the volumes for the preparation of marine matrices for ecotoxicity testing according to 5.1 will be significantly larger than for all other species tests.

C.4 Evaluation of results

The mortality in the test sample shall be ≤10 % (or one fish, if fewer than 10 control fish are tested) of those from the control sample. In case, the mortality of fish is >10 % then there is an indication of an impact due to exposure to the biodegradation productions from the biodegradable plastic.

If the difference in the endpoint response in the reference sample is higher than or equal to 10 % of those from the control sample, then this could be considered as an indication that a transient toxicity caused by the biodegradation of the high amount of biodegradable materials added to the marine matrices is present and affecting the test. The test is to be considered as not valid and to be repeated after a further biodegradation of the material.

C.5 Validity of the test

The test is considered valid if:

a) in the control samples, the mortality does not exceed 10 % (or one fish, if fewer than 10 control fish are tested) at the end of the exposure time;

b) the dissolved oxygen concentration is ≥ 60 % of the air saturation value in all test vessels throughout the exposure.