Designation: D6691 – 09

Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in the Marine Environment by a Defined Microbial Consortium or Natural Sea Water Inoculum[1]

This standard is issued under the fixed designation D6691; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method is used to determine the degree and rate of aerobic biodegradation of plastic materials (including formulation additives) exposed to pre-grown population of at least ten aerobic marine microorganisms of known genera or the indigenous population existing in natural seawater. The test method is conducted under controlled laboratory conditions.

1.2 This test method is designed to index polymer materials that are possibly biodegradable, relative to a positive reference material, in an aerobic environment.

1.3 This test method is applicable to all polymer materials containing at least 20 % carbon that are not inhibitory to the microorganisms present in a marine environment.

1.4 The values stated in SI units are to be regarded as the standard.

1.5 There is no similar or equivalent ISO standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D618 Practice for Conditioning Plastics for Testing

D883 Terminology Relating to Plastics

D1193 Specification for Reagent Water

D2593 Test Method for Butadiene Purity and Hydrocarbon Impurities by Gas Chromatography

D4129 Test Method for Total and Organic Carbon in Water by High Temperature Oxidation and by Coulometric Detection

3. Terminology

3.1 *Definitions of Terms Specific to This Standard*—Definitions of terms applying to this test method appear in Terminology D883.

4. Summary of Test Method

4.1 This test method consists of the following:

4.1.1 Selecting and characterizing (carbon content, molecular weight) plastic materials for testing,

4.1.2 Preparing a uniform inoculum of various isolated marine microorganisms, or obtaining a natural sea water sample (with added inorganic nutrients) for the test relying on the microbes present as the inoculum.

4.1.3 Exposing the test materials to the inoculum,

4.1.4 Using a respirometer to measure the total biogas (CO_2) produced as a function of time, and

4.1.5 Assessing the degree of biodegradability.

4.2 Biodegradability is assessed by determining the proportion of polymer-C converted to biogas-C. The percent of theoretical gas production, expressed as a fraction of the measured or theoretical carbon content of the test material, is reported as a function of time.

5. Significance and Use

5.1 The use of plastics aboard ships is on the rise and the use of the sea as a trash dumping site is no longer a possibility; consequently, the disposal of plastic materials while at sea remains a major issue. It is possible that biodegradable plastics will help to allay public concern by allowing for the safe disposal of plastic materials at sea. This test method has been developed to assess the rate and degree of aerobic biodegradation of plastics exposed to marine microorganisms. Aerobic biodegradation is determined by measuring the amount of biogas (carbon dioxide) produced during such an exposure.

5.2 It is acceptable to use the degree and rate of aerobic biodegradability of a plastic under the conditions of this test

[1] This test method is under the jurisdiction of ASTM Committee D20 on Plastics and is the direct responsibility of Subcommittee D20.96 on Environmentally Degradable Plastics and Biobased Products.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

method to estimate the persistence of that plastic in biologically active marine environments, for example, seashore and open-ocean. However, it shall be recognized that predicting long-term environmental fate and effects from the results of short-term exposure to a simulated marine environment is difficult. Thus, caution shall be exercised when extrapolating the results obtained from this or any other controlled-environment test to disposal in the natural environment.

6. Apparatus

6.1 *Aerobic Digestion and Gas Measuring Apparatus:*

6.1.1 Biogas production can be monitored through the use of any number of respirometry systems. The respirometry system must be able to detect low levels of carbon dioxide production. A carbon dioxide sensor consisting of a single beam, nondispersive infrared device with a maximum measurement capability of 1 % carbon dioxide is recommended.

6.1.2 *Sample Bottles*—125-mL autoclave bottles with plastic, screw-on lids. The lids shall contain three entry ports for biogas collection as well as a tetrafluorethylene seal ring. These flasks as well as their lids are supplied by the various respirometry companies.

6.1.3 All components of the gas-volume measuring and collection system must be of sufficient quality to prevent gas diffusion between the system and the surrounding atmosphere.

6.2 *Water Bath or Controlled-Environment Shaker/Incubator,* capable of maintaining the temperature of the digestion flasks at 30 ± 2°C.

6.3 *Analytical Balance,* (±0.1 mg), to weigh the test materials.

7. Reagents and Materials

7.1 All chemicals shall be of American Chemical Society (ACS) reagent-grade quality.

7.2 Type IV distilled water shall be prepared in accordance with Specification D1193.

7.3 Marine agar per litre consists of the following:

Bacto tryptone	5.0 ± 0.1 g
Bacto yeast extract	2.5 ± 0.1 g
Bacto dextrose (glucose)	1.0 ± 0.1 g
Bacto agar	15.0 ± 0.1 g

7.4 Marine broth per litre consists of the following:

Peptone	5.0 ± 0.1 g
Yeast extract	1.0 ± 0.1 g
Ferric citrate	0.1 ± 0.1 g
Sodium chloride	19.4 ± 0.1 g
Magnesium chloride, dried	5.9 ± 0.1 g
Sodium sulfate	3.24 ± 0.1 g
Calcium chloride	1.8 ± 0.1 g
Potassium bromide	0.08 ± 0.1 g
Strontium chloride	34.0 ± 0.1 mg
Boric acid	4.0 ± 0.1 mg
Sodium silicate	4.0 ± 0.1 mg
Sodium fluoride	2.4 ± 0.1 mg
Ammonium nitrate	1.6 ± 0.1 mg
Disodium phosphate	8.0 ± 0.1 mg

7.5 *Marine Solution*—Shall be either 7.5.1 or 7.5.2.

7.5.1 *Refer to* Table 1. All of the components must be mixed with 1 L of Type IV distilled water, until all of the salts have dissolved and then sterilized.

TABLE 1 Components of Minimal Marine Solution

Substance	Formula	MW, g/mol	Concentration, g/L
Ammonium chloride	NH_4Cl	53.49	2.00 ± 0.05
Synthetic sea salt	. . .	. . .	17.50 ± 0.05
Magnesium sulfate, 7-hydrate	$MgSO_47H2O$	246.48	2.0 ± 0.05
Potassium nitrate	KNO_3	101.1	0.5 ± 0.05
Potassium phosphate	K2HPO4 · 3H2O	228.2	0.1 ± 0.05

7.5.2 Natural sea water with inorganic nutrients (0.5 g/L of NH_4Cl and 0.1g/L of $KH_2(PO_4)$.

7.6 *Reference Materials*—Cellulose, chitin and Kraft paper, or all three, can act as the positive control and solitary inoculum as the negative control. Reference materials shall be provided in the same form as the test specimens, that is, powders, films, foams, and so forth. Sodium bicarbonate (100 mg) and sodium sulfite (100 mg) in an acidic water solution (100 mL) shall be tested also to ensure that the CO_2 sensors of the respirometry apparatus are functioning properly.

7.7 Microorganisms shall be selected on the basis of ability to degrade various biodegradable polymers, starches, cellulosics, and bacterial polyesters. Table 2 shows the composition of the synthetic sea salt solution.

7.8 It is important that sampling for the natural sea water be from a site not influenced by sewage outflow, chemical dumping, waste water discharge areas or oil slicks in the water. Also, do not take the samples from a river estuary having

TABLE 2 Composition of Synthetic Sea Salt Solution at Approximate Salinity of 34 ppt, Production Variance of ±5 %

Ion	Concentration, mg/L
Chloride	19251
Sodium	10757
Sulfate	2659
Magnesium	1317
Potassium	402
Calcium	398
Carbonate/bicarbonate	192
Strontium	8.6
Boron	5.6
Bromide	2.3
Fluoride	1.0
Iodide	0.22
Lithium	0.18
Copper	trace (<0.03)
Iron	trace (<0.03)
Nickel	trace (<0.04)
Zinc	trace (<0.02)
Manganese	trace (<0.01)
Molybdenum	trace (<0.01)
Cobalt	trace (<0.05)
Vanadium	trace (<0.04)
Selenium	trace
Lead	trace (<0.005)
Arsenic	trace (<0.0002)
Cadmium	trace (<0.02)
Chromium	trace (<0.0006)
Aluminum	trace (<0.04)
Tin	trace
Antimony	trace
Rubidium	trace
Barium	trace (<0.05)
Mercury	none
Nitrate	none
Phosphate	none

significant tidal flow characteristics as it is possible that this will not be representative of natural sea water.

8. Hazards

8.1 All microorganisms present the possibility of disease and shall be handled with due caution. Hands shall be washed before and after exposure. Latex gloves and safety glasses shall be used along with a mouth cover. All spills containing organisms shall be cleaned with germicidal/antibacterial agents, and all old cultures shall be autoclaved before being discarded.

8.2 This test method requires the use of hazardous chemicals. Avoid contact with chemicals and follow the manufacturer's instructions and Material Safety Data Sheets.

8.3 All purchased media also can be hazardous. Read all safety instructions.

9. Inoculum Test Organisms

9.1 The inoculum shall consist of the compositions found in 9.1.1 or 9.1.2.

9.1.1 A minimum of ten test organisms. The microorganisms were identified by using bacterial identification tests (that is, Biolog system, gram stains). Their identifications to at least genus are the following: *Alteromonas haloplanktis*, *Xanthomonas campestri*, *Vibrio alginolyticus*, *Vibrio proteolyticus*, *Actinomycete sp.*, *Bacillus megaterium*, *Bacillus sp.*, *Zooster sp.* and *Pseudomonas sp. Pseudomonas sp* has multiple species.

9.1.2 Natural sea water collected in the local area that does not have any hydrocarbon residue present on the surface, and includes the addition of inorganic nutrients (.5 g/L of NH_4Cl and .1 g/L of $KH_2(PO_4)$ and maintains aeration during the respirometry.

10. Test Specimen

10.1 Weigh test samples to the nearest 0.1 mg and have sufficient carbon content (minimum 20 %) to yield carbon dioxide volumes that can be measured accurately by the respirometer. A method for determining carbon content of the test material is determined by calculation or elemental analysis in accordance with Test Method D4129.

10.2 Acceptable forms in which it is possible for the test specimen to be included are powders, films, pieces, fragments, formed articles, or aqueous solutions. The test materials shall be conditioned in accordance with Practice D618. Test specimens in the form of powders shall be characterized as to particle size distribution. Mean particle size less than 25 mm is recommended.

10.3 If the specimens are solids, grinding is recommended to obtain a powder form of the sample to maximize surface area. Cryogenically milling with liquid nitrogen by means of impact is a recommended method to obtain powders.

10.4 *Optional*—Use Test Method D2593 to measure and record the molecular weight of the test material.

11. Procedure

11.1 *Inoculum Buildup and Preparation:*

11.1.1 All media shall be prepared in accordance with the directions described on the label. Forty mL portions of the marine broth shall be placed in up to thirteen 125-mL Erlenmeyer flasks (one for every organism) and autoclaved for 20 min at 121°C at 15 lb of pressure.

11.1.2 Each flask shall be inoculated with 250-µL stock cultures of the microorganisms that were grown overnight or to an optical density of approximately 2.0 at a wavelength of 660 and placed in a shaker/incubator at 30°C.

11.1.3 When the cultures have reached the state of growth where the cells are in late logarithmic growth or early stationary phase (24 h), each separate culture must be centrifuged at a relative centrifugal force (RCF) of approximately 26890 (g) for 8 min to obtain pellets.

11.1.4 After centrifuging, decant the media away from the pellets and resuspend the pellets in 20 mL of minimal marine solution. Repeat the centrifuging and decanting to remove any carbon source from the microorganisms. After decanting for the second time, resuspend the pellets in 10 mL of minimal marine solution. Repeat the centrifuging and decanting of the solution and then resuspend the pellets in 4 mL of minimal marine solution.

11.1.5 Place the 4-mL resuspensions of all the microorganisms into a single sterile flask with cap. Mix the various resuspensions together by the use of a vortex on the flask.

11.2 *Preparation of Respirometry Flasks:*

11.2.1 A stock solution containing enough minimal marine solution for the number of flasks to be used shall be autoclaved at 121°C for 20 min prior to the day of the experiment. A tube with attached air sparger, as well as all of the empty respirometer flasks and lids shall also be autoclaved.

11.2.2 Seventy–five mL of the minimal marine stock solution or natural sea water with inorganic nutrients shall be placed aseptically into each respirometry 125-mL bottle in a sterile environment.

11.3 *Test and Reference (Control) Specimen:*

11.3.1 The test and control specimens must be sterilized before placement into the respirometry bottles. Specimen size is usually 20 mg depending on the carbon content of the sample (pre-weighed, ±0.1 mg). Sterilization of polymers usually cannot be performed through the use of an autoclave due the limited thermal stability of the polymers and the effect of high temperatures on a polymer's structure. For this reason, ethylene oxide sterilization of samples is recommended.

11.3.2 Within a sterile environment constituted by a laminar flow hood, the samples shall be added to the respirometry flask and the flask lid screwed on. The sampled reference materials and blanks shall be prepared in triplicate.

11.4 *Inoculating the Respirometry Flasks:*

11.4.1 Inoculate each of the respirometry flasks containing the minimal marine stock solution with 100 ± µL of the inoculum established in 11.1.5. If natural seawater with inorganic nutrients is used, then no inoculation of pre-grown microorganisms is done.

11.4.2 Reserve one of the bottles for the sodium bicarbonate control, and do not inoculate it.

11.5 *System Start-Up and Maintenance:*

11.5.1 After attaching all of the respirometry bottles to the respirometer, perform standard diagnostic tests to find any system leaks. The respirometry bottles shall be placed in either a water bath or shaker apparatus set to 30 ± 1°C and a rotation of 175 ± 5 rpm.

11.5.2 Before starting the experiment, place the sample of sodium bicarbonate and sodium sulfite into its assigned flask and close the lid immediately to avoid letting any of the evolved CO_2 escape.

11.5.3 Once the system has started, continuously monitor CO_2 production and the condition of the system as a whole. Follow the manufacturer's instructions for maintenance, monitoring, and parts replacement.

11.5.4 Typical respirometer runs last anywhere from 10-90 days in duration; although, if the specimens are extremely biodegradable, then it is acceptable for the experiment to be terminated when cumulative CO_2 production plateaus or extended if no biodegradation has occurred in a specific time.

12. Calculation

12.1 *Theoretical Maximum CO_2 Production:*

12.1.1 Determine the total organic carbon content of the test material (by elemental analysis or, if the chemical composition is well-established, by calculation).

12.1.2 Calculate the maximum amount CO_2 that theoretically can be evolved during the aerobic biodegradation of the test materials. The relevant chemical reactions are:

$$C_{polymer} + O_2 \rightarrow CO_2$$

Each mmole (12 mg) of polymer-C can be converted to 1 mmole of gaseous CO_2. The theoretical maximum amount of CO_2 that can be produced is calculated as follows:

$$\text{Maxbiogas} - \text{C} = \frac{W(\%C_T)}{100} \times \frac{1 \text{ mmol gaseous C}}{12 \text{ mg C}}$$

where:

W = total weight (mg) of the test material, and
$\%C_T$ = percent organic carbon in the test material.

12.1.3 Calculate the percentage of mineralization (that is, aerobic biodegradation) of the test material as follows:

$$\% \text{ mineralization} = \left(\frac{(C_{\text{Test}}) - (C_{\text{Blank}})}{C_i} \right) \times 100$$

where:

C_{Test} = mean amount of biogas-C produced in the test reactors,
C_{Blank} = mean amount of biogas-C produced in the blank (inoculum only) reactors, and
C_i = total amount of polymer-C added to the test reactors.

13. Interpretation of Results

13.1 A reference material that is known to biodegrade (for example, chromatography-grade cellulose or chitin) must be included in each test run to check the activity of the inoculum. When the test yields less than 70 % of theoretical biogas for the reference material or there is excessive biogas production, the test is invalid, and rerun with fresh inoculum and appropriate corrections.

13.2 The plateau level of the carbon dioxide evolved compared to the positive control will indicate the degree of biodegradability of the test material. The evolved carbon dioxide will be converted to % mineralization to compare to other materials and results obtained from different methods.

13.3 It is possible that toxicity data for the test materials will be useful in interpreting low results.

14. Report

14.1 Report the following data and information:

14.1.1 Information on the inoculum including source, percent volatile solids, date of collection, storage, handling and potential acclimation to the test material or information on the natural sea water including the source and date of the sample collection.

14.1.2 Carbon content of the plastic material.

14.1.3 Cumulative average carbon dioxide evolution over time until plateau shall be reported and displayed graphically as lag-phase and slope (rate).

14.1.4 Percent of theoretical aerobic biodegradation for each plastic material tested and the standard control polymer.

14.1.5 Temperature range of test.

14.1.6 Microorganisms per mL in original inoculum (optionally).

14.1.7 If a more mathematical treatment of the data is required, the cumulative carbon dioxide evolution versus time data can be fitted to a nonlinear regression model to generate rate constants for mineralization and a final extent of biodegradation at infinite time (asymptote, if no plateau is reached).

14.1.8 Molecular weight of the plastic material if measured and form in which it is used.

15. Precision and Bias

15.1 The precision of this test method is being determined and will be available on or before August 2010. It is not feasible to specify the precision at this time because further experiments are being performed.

16. Keywords

16.1 aerobic; biodegradation; marine environment; plastics