Designation: E 2170 – 01

Standard Test Method for Determining Anaerobic Biodegradation Potential of Organic Chemicals Under Methanogenic Conditions[1]

This standard is issued under the fixed designation E 2170; 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 covers screening procedures for the determining the biodegradation potential of organic chemicals under methanogenic conditions using an inoculum from anaerobic sewage treatment. The conditions described in this test method are not necessarily optimal for biodegradation, since it involves a dilute inoculum and a relatively high concentration of test chemical.

1.2 The test method is applicable to most organic chemicals, assuming that they can be accurately dosed into the test systems. The test is not applicable to chemicals that are inhibitory at the concentration used in the test or those whose volatility generates significant head pressure in the test vessels.

1.3 *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.* Specific hazard statements are given in Section 8.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1193 Specification for Reagent Water[2]

D 5904 Test Method for Total Carbon, Inorganic Carbon, and Organic Carbon in Water by Ultraviolet, Persulfate Oxidation, and Membrane Conductivity Detection[3]

D 5907 Test Method for Filterable and Non-filterable Matter in Water[2]

3. Terminology

3.1 *Definitions:*

3.1.1 *biodegradation potential*—the ability of an organic chemical to undergo partial or complete biodegradation under the conditions of the test. In the context of this test, biodegradation is evidenced by the production of methane and carbon dioxide, which is measured as an increased pressure in the headspace and increased dissolved inorganic carbon in the medium.

3.1.2 *digester sludge*—sludge which is obtained from an anaerobic reactor used to reduce the solids recovered and produced during the treatment of wastewater. Such reactors are generally operated at 35°C with a typical retention time of 25 to 30 days.

4. Summary of Test Method

4.1 The test chemical and an anaerobic digester sludge inoculum are suspended in a defined anaerobic medium and incubated in a sealed vessel at 35°C. The increase in headspace pressure resulting from the production of carbon dioxide and methane is measured at various time intervals using a pressure measuring device. At the termination of the test, the level of dissolved carbon dioxide is assayed by measuring dissolved inorganic carbon in the medium. The amount of total gas produced from the test chemical is determined by comparing total gas production in the experimental treatments and the blanks.

5. Significance and Use

5.1 Biodegradation is an important process for the removal of many chemical substances in anaerobic environments. In methanogenic environments, this process involves dissimilation to carbon dioxide and methane.

5.2 This test method has been developed to screen organic chemicals for anaerobic biodegradation potential under methanogenic conditions, where there is no ingress of oxygen. A high-biodegradability result in this test method is good evidence that the test substance will be biodegradable in waste treatment plant anaerobic digesters and in many natural environments. Conversely, a low-biodegradation result may have causes other than poor biodegradability of the test substance. Inhibition of the microbial inoculum by the test substance may have occurred at the concentrations tested, or conditions in the test may have been inappropriate for the development of an acclimated microbial population. Toxicity should be suspected when the gas production in the blanks exceeds that in the test vessels. In such cases, further work is needed to assess the anaerobic biodegradation potential of the chemical. An estimate of the expected environmental concentration will help to

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

[3] *Annual Book of ASTM Standards*, Vol 11.02.

put any observed toxic effects into perspective.

6. Apparatus

6.1 *Incubator*, water or sand bath, thermostatically controlled at 35 ± 2°C.

6.2 *Pressure-Resistant Glass Test Vessels*, each fitted with a gas-tight septum, capable of withstanding about 2 bar. From a practical point of view, the use of 160 mL serum bottles, which are often described in catalogs as 125 mL serum bottles, sealed with butyl rubber serum stoppers with crimped aluminum caps is recommended.

6.3 *Pressure Measuring Device*—An example of such a device would be a pressure transducer attached to a syringe needle, which may include a three-way gas-tight valve for releasing excess pressure. The device should be calibrated under conditions relevant to the test setup.

6.4 *Carbon Analyzer*, suitable for the direct determination of organic and inorganic carbon in the test vessels.

6.5 System for preparing and manipulating media, inocula and samples under anaerobic conditions. The system could be an anaerobic chamber or gas sparging system.

7. Reagents and Materials

7.1 *Purity of Reagents*—Reagent grade chemicals should be used in all tests. Unless otherwise indicated, it is intended that all reagents should conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 *Purity of the Water*—Unless otherwise indicated, reference to water shall be understood to mean reagent water as defined by Type IV of Specification D 1193.

7.3 *Test Medium*—The test medium consists of the following constituents in water.

Anyhdrous potassium dihydrogen phosphate (KH_2PO_4)	0.27 g/L
Disodium hydrogen phosphate dodecahydrate ($NaHPO_4$- 12 H_2O)	1.12 g/L
Ammonium chloride (NH_4Cl)	0.53 g/L
Calcium chloride dihydrate ($CaCl_2$- 2 H_2O)	0.075 g/L
Magnesium chloride hexahydrate ($MgCl_2$- 6 H_2O)	0.10 g/L
Iron (II) chloride tetrahydrate ($FeCl_2$- 4 H_2O)	0.02 g/L
Resazurin (oxygen indicator)	0.001 g/L
Sodium sulfide nonahydrate (Na_2S - 9 H_2O)	0.1 g/L

7.4 With the exception of sodium sulfide, concentrated (100 ×) stock solutions can be prepared for each constituent.

8. Hazards

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

8.2 This test involves the use of digester sludge from a wastewater treatment plant. Avoid contact with sludge by using gloves and other appropriate protective equipment. Use good personal hygiene to minimize exposure to potentially harmful microbiological agents.

8.3 Since pressure can build-up in the test vessels, select inoculum levels, test substance concentrations and head space volumes that will avoid headspace pressures that could exceed safe pressure specifications established for the test vessels.

9. Preparation of the Test Medium

9.1 All constituents with the exception of the sodium sulfide are added to the water. The constituents can be added neat or as stock solutions. The use of stock solutions generally improves accuracy and convenience. The solution is autoclaved or alternatively heated to a boil while being mixed with a magnetic stirrer and being sparged with oxygen-free nitrogen. After being autoclave or reaching a boil, the medium is cooled in an anaerobic chamber or with continued nitrogen sparging. Once the medium has cooled to approximately 35°C, the sodium sulfide is added to the medium at which point, the resazurin in the medium should be colorless.

10. Inoculum

10.1 Collect anaerobic sludge from a well functioning anaerobic digester at a sewage treatment plant receiving predominantly domestic wastewater. Sludge must be sampled and transported in a manner that preserves its anaerobic integrity. One approach is to collect sludge in a wide necked polyethylene bottle filled to within 1 cm of the top and sealed tightly. Since pressure can build up in the bottle, the use of glass should be strictly avoided, and excess pressure must be periodically released during transport and storage.

10.2 If the sludge contains large particulates, it may be appropriate to sieve it through a 2-mm mesh screen or a single layer of cheese cloth prior to use. To reduce background gas production, it also may be appropriate to allow the sludge to digest in the laboratory without addition of nutrients or substrates for up to 10 days. Work by ECETOC **(1)**[4] suggests that 5 days gives an optimum decrease in background gas production without an unacceptable increase in lag or incubation period. Some laboratories monitor gas production and use the inoculum once gas production has reached a plateau. All procedures must be conducted under conditions that preserve the anaerobic integrity of the sludge (that is, in an anaerobic chamber).

10.3 Immediately prior to use, it may be appropriate to wash the sludge inoculum with anaerobic test medium to reduce levels of inorganic and organic carbon. This is accomplished by centrifuging sludge samples at a low speed (that is, 3000 g) and decanting the supernatant. The supernatant is discarded, and the pellet resuspended in an equal volume of the test medium. This process can be repeated as needed to reduce background DIC below 10 mg DIC/L **(2)**, which is usually accomplished with two washings. It is critical that all washing procedures be performed in a manner that preserves the anaerobic integrity of the sludge.

10.4 Prior to use, the total solids level of the sludge is determined so upon dilution in the test medium, the final TS concentration is 1-3 g/L in the inoculated medium.

11. Preparation and Addition of Test Substances

11.1 In the case of water soluble or dispersible test chemicals, prepare an aqueous stock solution, suspension or emulsion, and dose aliquots to each test vessel. Ideally, the stock

[4] The boldface numbers in parentheses refer to a list of refernces at the end of this test method.

should be prepared in anaerobic medium. Since addition of a test solution can alter the ratio of liquid to headspace and the total solids content of the test vessels, the amount of added liquid should be controlled or minimized. The volume of liquid and the level of sludge solids must be identical in the blank vessels and those receiving test material. Test substances that are insoluble can be directly added to the test vessels. Test substance may be weighed directly into tared empty test vessels prior to purging oxygen from the vessels. Since weighing such and transferring such low levels can be inaccurate, the test substance can be ground into a powder and mixed with a known excess of fine sand, which will increase the mass being added. Alternatively, the test material can be dissolved in a volatile solvent, and aliquots transferred to the empty test vessels with subsequent evaporation of the solvent. Dosing of deoxygenated test substance stock solutions to the test vessels should be done in an anaerobic chamber or in a manner that maintains the anaerobic integrity of all solutions.

12. Procedure

12.1 *Preparation of Test Vessels*—At least three test vessels should be prepared for each test chemical. In addition, at least three blanks should be prepared for each test. Multiple test chemicals can be included in a single test. At least one vessel should be prepared for each reference compound. An optional inhibition control can be included that is dosed with equal concentrations of both the test and reference chemical. Following the additions of test substances and solvent blanks as needed, inoculated anaerobic test medium is added to each vessel. Constant mixing of inoculated medium using a magnetic bar is recommended during this process to ensure uniform solids addition to each vessel. The recommended final added concentration of the sludge inoculum is between 1 and 3 g/L. The typical concentration of test material is 100 mg C/L. Lower concentrations can be tested if an adequate signal can be achieved above the background in the blanks. All manipulations should be conducted in an anaerobic chamber or in a manner that preserves the anaerobic integrity of the test systems.

12.2 Every test vessel should contain the same final volume of liquid and have the same headspace volume. The ratio of liquid volume to headspace should be based upon the predicted pressure increase resulting from the complete mineralization of the chemical and the measurement range of the transducer. At no time should ratios be utilized that could result in the development of pressures that exceed the pressure tolerance of the test vessels. Furthermore, high headspace pressures could contribute to leakage. A typical configuration is 100 mL of test media in a 160 mL serum bottle.

12.3 Once inoculated and dosed, each vessel should be stoppered and crimp sealed. The vessels are allowed to equilibrate for 1 h at 35 ± 2. At this point, the starting pressure is recorded or equilibrated to atmospheric pressure by inserting a needle through the stopper to release any excess pressure within the bottles.

12.4 *Incubation*—Incubate the sealed bottles at 35 ± 2 in the dark. After 24 to 48 h, examine the bottles and eliminate any vessels that exhibit pink coloration. Vessels can be incubated statically or with intermittent or continual gentle mixing. A common practice is to gently swirl or mix the bottles 2 or 3 times per week. Mixing resuspends the inoculum and ensures gaseous equilibrium. It is recommended that vessels be thoroughly mixed prior to each measurement.

12.5 *Pressure Measurements*—Each vessel can be outfitted with an individual pressure measurement device that is periodically monitored. Alternatively, a single measurement device can be used by inserting the syringe needle through the septum and taking a reading. It is critical that the test vessel be maintained at the incubation temperature during the measurement process. After recording the pressure, the pressure can be re-equilibrated to atmospheric pressure by releasing any headspace pressure through the 3-way valve before removing the syringe needle from the septum. It is recommended that measurements be taken weekly to allow the kinetics to be followed and to provide guidance as to when the test can be terminated. The typical duration of a test is 60 days.

12.6 *Measurement of Inorganic Carbon*—After the last pressure measurement, allow the sludge to settle and open the test vessels. Immediately determine the concentration of inorganic carbon (IC) in the clear supernatants. Centrifugation or filtration should be avoided since it could result in changes in the IC.

13. Calculation and Expression of Results

13.1 For practical reasons, the pressure of the gas is measured in millibars or psi (1 mbar = 100 Pascals (Pa) and 1 psi = 6 895 Pa). Psi should be converted to mbar (1 psi = 69 mbar).

13.2 *Carbon in Headspace*—One mol of carbon dioxide and 1 mol of methane each contain 12 g of carbon. The carbon content (C) of any volume of a given volume of evolved gas is calculated by:

$$C = 12 \times 10^3 \times n \tag{1}$$

where:

C = mass of carbon in milligrams in a given volume of evolved gas,
12 = atomic mass of carbon, and
n = moles of gas.

n is calculated from the gas law in the following manner:

$$n = \frac{pV}{RT} \tag{2}$$

where:

p = headspace pressure in Pascals (Pa),
V = headspace volume in cubic meters,
R = gas constant (8.314 J/(mol K), and
T = temperature in Kelvin.

Thus, the carbon evolved (Ce) as mg of gas in each vessel equals:

$$Ce = \frac{12000 \times 0.1 \times Pcum \times Vh}{R \times T} \tag{3}$$

where:

12 000 = converts moles to mg,
$Pcum$ = cumulative pressure produced through the study in mbar,

Vh = headspace volume in liters, and
0.1 converts cubic meters to liters.

For a normal incubation temperature of 35°C (308°K), *Ce* can be calculated as:

$$Ce = 0.468(Pcum \times Vh) \quad (4)$$

13.3 *Dissolved Inorganic Carbon*—The level of dissolved inorganic carbon (*Cd*) in each vessel is calculated as:

$$Cd = IC * VI \quad (5)$$

where:
IC = dissolved inorganic carbon in mg/L, and
VI = volume of liquid in the test vessel in liters.

13.4 *Total Carbon Mineralized*—The total carbon (*Ct*) converted to gas in each vessel is calculated as:

$$Ct = Ce + Cd \quad (6)$$

13.5 *Total Carbon Derived from Test Substance*—The total gaseous carbon (*Cn*) derived from the test substance is calculated as follows:

$$Cn = Ct(test) - Ct(blank) \quad (7)$$

where:
Ct(test) = total carbon (mg) converted to gas in each vessel with test substance, and
Ct(blank) = total carbon (mg) converted to gas in the blanks. This value generally is a mean of the blanks.

13.6 *Carbon of Test Substance*—The carbon (*Ca*) in the form of test substance added to each test vessel in mg is calculated as follows:

$$Ca = Ctm \times VI \quad (8)$$

where:
Ctm = concentration of test substance carbon in mg/L, and
VI = volume of liquid in the test vessel in liters.

13.7 *Extent of Biodegradation*—The percent of the test material converted to gaseous products (*D*) is calculated as follows:

$$D = \frac{(100 \times Cn)}{Ca} \quad (9)$$

14. Interpretation of Results

14.1 Information on the toxicity of the chemical may be useful in the interpretation of low results.

14.2 In some cases, reference substances may be useful. Commonly used reference compounds include sodium benzoate, phenol and polyethylene glycol 400 (PEG 400) as positive controls. The biodegradation of these materials must exceed 60 % within 60 days to verify healthy anaerobic digester sludge was used as an inoculum.

14.3 The sensitivity of the test is a function of the difference between gas production in the test vessels and blanks. The lower the background gas production in the blanks, the lower the test substance concentration, which can be assessed. Twenty mg of added organic carbon can theoretically produce 4.4 mL of gas at 35°C, while 50 and 100 mg can produce 11.1 and 22.2 mL, respectively. Actual gas production will be lower due to incorporation of carbon into biomass and other products. The larger the ratio of potential gas production from the added test material to the background gas production in the blank, the greater potential accuracy of the method.

14.4 This method is only appropriate for screening anaerobic biodegradation potential under methanogenic conditions. More definitive results can be obtained using more realistic test methods **(3)** with radiolabeled test materials and actual environmental samples.

15. Report

15.1 Report the following data and information:

15.1.1 Information on the inoculum includes information on the source, operating conditions of the digester, percent solids, date of collection, and potential previous exposure to the test materials. Much of this information is generally available from the operator.

15.1.2 Average cumulative gas production in blank, reference, and test substance treatments at each sampling as well as the standard deviation.

15.1.3 Average percent of theoretical biodegradation for each test substance and reference compound at each sampling time including standard deviation.

16. Precision and Bias

16.1 A specific precision and bias statement cannot be made for this test method, since interlaboratory testing has not been conducted. However, the test method is based on published procedures involving the use of multiple compounds **2(4-6)**.

16.2 Interlaboratory testing was conducted on two compounds (palmitic acid and PEG 400) using a closely related procedure **(6)**. Standard deviations in replicate tests with the same compound within the same test were mainly <20 %. Deviations were larger when small test vessels (<150 mL) or headspace volumes exceeded 20 % of the vessel volume. In the interlaboratory comparison, both compounds were well degraded in most laboratories. Eliminating extremely low and high values, which were attributed to sub-optimal experimental conditions, the standard deviations among laboratories was less than 20 % for both compounds.

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