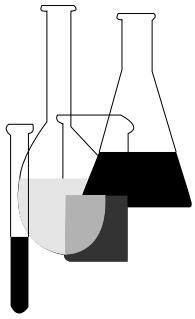
United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–076
January 1998

Fate, Transport, and Transformation Test Guidelines

OPPTS 835.3110
Ready Biodegradability



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202–512–1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202–512–0132 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from EPA's World Wide Web site (http://www.epa.gov/epahome/research.htm) under the heading ''Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines.''

OPPTS 835.3110 Ready biodegradability.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 796.3180 Ready Biodegradability: Modified AFNOR Test, 796.3200 Ready Biodegradability: Closed Bottle Test, 796.3220 Ready Biodegradability: Modified MITI Test (I), 796.3240 Ready Biodegradability: Modified OECD Test, and 796.3260 Ready Biodegradability: Modified Sturm Test, and OECD 301A DOC Die-Away Test, 301 B CO_2 Evolution Test, 301C Modified MITI Test (I), 301 D Closed Bottle Test, 301 E Modified OECD Screening Test, and 301 F Manometric Respirometry Test.

(b) **Preliminary information.** (1) In this guideline six methods are described that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium. They are:

(i) DOC Die-Away under paragraph (l) of this guideline.

(ii) CO_2 Evolution (Modified Sturm Test) under paragraph (m) of this guideline.

(iii) MITI (I) (Ministry of International Trade and Industry, Japan) under paragraph (n) of this guideline.

(iv) Closed Bottle under paragraph (o) of this guideline.

(v) Modified OECD Screening under paragraph (p) of this guideline.

(vi) Manometric Respirometry under paragraph (q) of this guideline.

(2) The DOC die-away method is similar to the ISO Standard 7827–1984 and replaces the Modified AFNOR method. The methods under paragraphs (m), (n), (o), and (p) of this guideline are modified versions of earlier OECD guidelines adopted in 1981. The manometric respirometry method under paragraph (q) of this guideline is similar to the MITI (I) method, differing mainly in the inocula employed.

(3) Much experience has accumulated with the six methods over the years including an OECD interlaboratory comparison exercise (ring test) in 1988. The accumulated experience, and the ring test, have confirmed that the methods may be used for the assessment of ready biodegradability. However, depending on the physical characteristics of the substance to be tested, a particular method may be preferred.

(4) General considerations including those common to all six methods are given under paragraphs (c) through (k) of this guideline. Details of

individual methods are given under paragraphs (l) through (q) of this guideline.

(c) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions and abbreviations also apply to this test guideline.

Biochemical oxygen demand (BOD) is the amount of oxygen in milligrams consumed by microorganisms when metabolizing a test compound; also expressed as milligrams of oxygen uptake per milligram of test compound.

Chemical oxygen demand (COD) is the amount of oxygen in milligrams consumed during oxidation of a test compound with hot, acidic dichromate; it provides a measure of the amount of oxidizable matter present; also expressed as milligrams of oxygen consumed per milligram of test compound.

Degradation phase is the time from the end of the lag period to the time when 90 percent of the maximum level of degradation has been reached.

Dissolved organic carbon (DOC) is the organic carbon present in solution or that which passes through a 0.45 μm filter or remains in the supernatant after centrifuging at approximately $4{,}000 \times g$ (about 40.000 m sec^{-2}) for 15 min.

Dissolved oxygen (DO) is the concentration of oxygen dissolved in an aqueous sample expressed as milligrams per liter.

EC50 is the concentration of the test substance at which the respiration rate is 50 percent of that shown by the control under the conditions of the guideline for the activated sludge respiration inhibition test (OPPTS 850.6800; ISO Standard 8192).

Inherently biodegradable is a classification of chemicals for which there is unequivocal evidence of biodegradation (primary or ultimate) in any test of biodegradability.

Inorganic carbon is abbreviated IC.

Lag phase is the period from inoculation in a die-away test until the degradation percentage has increased to about 10 percent. The lag time is often variable and poorly reproducible.

Organic carbon is abbreviated OC.

Primary biodegradation. is the alteration in the chemical structure of a substance, brought about by biological action, resulting in the loss of a specific property of that substance.

Readily biodegradable is an arbitrary classification of chemicals which have passed certain specified screening tests for ultimate biodegradability; these tests are so stringent that it is assumed that such compounds will rapidly and completely biodegrade in aquatic environments under aerobic conditions.

Suspended solids is abbreviated SS.

10–day window is the 10 days immediately following the attainment of 10 percent biodegradation.

Test substance is abbreviated TS.

Theoretical carbon dioxide ($ThCO_2$) is the quantity of carbon dioxide in milligrams calculated to be produced from the known or measured carbon content of the test compound when fully mineralized; also expressed as milligrams of carbon dioxide evolved per milligram of test compound.

Theoretical oxygen demand (ThOD) is the total amount of oxygen in milligrams required to oxidize a chemical completely; it is calculated from the molecular formula presented under paragraph (k)(2)(i) of this guideline and is also expressed as milligrams of oxygen required per milligram of test compound.

Total carbon (TC) is the sum of the organic carbon and the inorganic carbon present in a sample.

Total organic carbon (TOC) of a sample is the sum of the organic carbon in solution and in suspension.

Treatability is the amenability of compounds to removal during biological wastewater treatment without adversely affecting the normal operation of the treatment processes. Generally, readily biodegradable compounds are treatable, but this is not the case for all inherently biodegradable compounds. Abiotic processes may also operate.

Ultimate biodegradation (aerobic) is the level of degradation achieved when the test compound is totally utilized by microorganisms resulting in the production of carbon dioxide, water, mineral salts, and new microbial cellular constituents (biomass).

(d) **General principle of the tests.** (1) A solution, or suspension, of the test substance in a mineral nutrient medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC in the test solution due to the inoculum should be kept as low as possible compared with the amount of OC due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blanks with inoculum but without test substance, although the endogenous activity of cells in the presence of a chemical will not exactly match that in the endogenous control. A reference compound is run in

parallel to check the activity of the inoculum and to serve as a general check on the operation of the procedures.

(2) In general, degradation is followed by the determination of parameters such as DOC, CO_2 production and oxygen uptake, and measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation. With automatic respirometers the measurement is continuous. DOC is sometimes measured in addition to another parameter but this is usually done only at the beginning and end of the test. Specific chemical analysis can also be used to assess primary degradation of the test substance and to determine the concentration of any intermediate substances formed. It is obligatory in the MITI method under paragraph (n) of this guideline.

(3) Normally, the test lasts for 28 days. However, tests may be ended before 28 days, i.e. as soon as the biodegradation curve has reached a plateau for at least three determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but that the plateau has not been reached by day 28, but in such cases the chemical would not be classified as readily biodegradable.

(e) **Information on the test substance.** (1) In order to select the most appropriate method, information on the chemical's solubility, vapor pressure and adsorption characteristics is essential. The chemical structure or formula should be known in order to calculate theoretical values and/or check measured values of parameters, e.g. ThOD, $ThCO_2$, DOC, TOC, and COD. Information on the purity or the relative proportions of major components of the test material is required in order to interpret the results obtained, especially when the result lies close to the pass level.

(2) Information on the toxicity of the test substance to bacteria may be very useful for selecting appropriate test concentrations and may be essential for the correct interpretation of low biodegradation values. When a chemical is subjected to ready biodegradability testing and appears to be nonbiodegradable, the following procedure is recommended if a distinction between inhibition and inertness is desired (refer to paragraph (r)(1) of this guideline).

(i) Similar or identical inocula should be used for the toxicity and biodegradation tests.

(ii) To assess the toxicity of chemicals studied in ready biodegradability tests, the application of one or a combination of the inhibition of sludge respiration rate (OPPTS 850.6800; ISO Standard 8192), BOD and/or growth inhibition methods is appropriate.

(iii) If inhibition due to toxicity is to be avoided, it is suggested that the test substance concentrations used in ready biodegradability testing should be less than 0.1 of the EC50 values (or less than EC20 values)

obtained in toxicity testing. Compounds with an EC50 value greater than 300 mg/L are not likely to have toxic effects in ready biodegradability testing.

(iv) EC50 values of less than 20 mg/L are likely to pose serious problems for the subsequent testing. Low test concentrations should be employed, necessitating the use of the stringent and sensitive Closed Bottle test (under paragraph (o) of this guideline) or the use of ^{14}C-labeled material. Alternatively, an inoculum previously exposed to the test substance may permit higher test substance concentrations to be used. In the latter case, however, the stringency criterion of the ready biodegradability test is lost.

(f) **Applicability and selection of methods**—(1) **Evaluation of biodegradability of test substances.** Test substances that are soluble in water to at least 100 mg/L and are nonvolatile and nonadsorbing may be assessed by all methods described in this guideline. For those chemicals that are poorly soluble in water, volatile or adsorbing, suitable methods are indicated in Table 1. The manner in which poorly water-soluble chemicals and volatile chemicals can be dealt with is described under paragraph (f)(2) of this guideline, but in the MITI method under paragraph (n) of this guideline neither solvents nor emulsifying agents can be used. Moderately volatile chemicals may be tested by the DOC die-away method under paragraph (l) of this guideline if there is sufficient gas space in the test vessels (which should be suitably stoppered). In this case, an abiotic control must be set up to allow for any physical loss.

Table 1.—Applicability of Test Methods

Test	Analytical method	Suitability for compounds that are:		
		poorly soluble	volatile	adsorbing
DOC Die-Away	Dissolved organic carbon	–	–	+/–
CO_2 Evolution	Respirometry: CO_2 evolution	+	–	+
MITI (I)	Respirometry: Oxygen consumption	+	+/–	+
Closed Bottle	Respirometry: Dissolved oxygen	+/–	+	+
Modified OECD Screening	Dissolved organic carbon	–	–	+/–
Manometric Respirometry	Oxygen consumption	+	+/–	+

(2) **Evaluation of the biodegradability of poorly soluble compounds.** In biodegradability tests with poorly soluble compounds the following aspects should receive special attention. Additional information is available in paragraphs (r)(2), (r)(3), (r)(4), and (r)(5) of this guideline.

(i) While homogeneous liquids will seldom present sampling problems, it is recommended that solid materials be homogenized by appropriate means to avoid errors due to nonhomogeneity. Special care must

be taken when representative samples of a few milligrams are required from mixtures of chemicals or substances with large amounts of impurities.

(ii) Various forms of agitation may be used during the test. Care should be taken to use only sufficient agitation to keep the chemical dispersed, and to avoid overheating, excessive foaming, and excessive shear forces.

(iii) An emulsifier which gives a stable dispersion of the chemical may be used. It should not be toxic to bacteria and must not be biodegraded or cause foaming under test conditions.

(iv) The same criteria apply to solvents as to the emulsifiers.

(v) It is not recommended that solid carriers be used for solid test substances, but they may be suitable for oily substances.

(vi) When auxiliary substances such as emulsifiers, solvents, and carriers are used, a blank run containing the auxiliary substance(s) should be performed.

(vii) Any of the four respirometric tests (under paragraphs (m), (n), (o), or (q) of this guideline) can be used to study the biodegradability of poorly soluble compounds.

(g) **Pass levels.** The pass levels for ready biodegradability are 70 percent removal of DOC and 60 percent of ThOD or $ThCO_2$ production for respirometric methods. The pass levels are lower in the respirometric methods because some of the carbon from the test chemical is incorporated into new cells, and as a result, the percentage of CO_2 produced is lower than the percentage of carbon being used. These pass values have to be reached in a 10–day window within the 28–day period of the test, except where mentioned below. The 10–day window begins when the degree of biodegradation has reached 10 percent DOC disappearance, ThOD, or $ThCO_2$, and must end before day 28 of the test. Chemicals that reach a pass level after the 28–day period are not deemed to be readily biodegradable. The 10–day window concept does not apply to the MITI method under paragraph (n) of this guideline. A pass criterion based on a 14–day window may be acceptable in the Closed Bottle method if it is considered that the number of bottles necessary to evaluate the 10–day window causes the test to become too unwieldy.

(h) **Reference compounds.** In order to check the activity of the inoculum, one or more reference compounds that meet the criteria for ready biodegradability are tested by setting up appropriate vessels in parallel as part of normal test runs. Suitable compounds are aniline (freshly distilled), and phthalic or trimellitic acid, but sodium acetate, dextrose, and sodium benzoate are probably too biodegradable to be useful for this purpose.

(i) **Reproducibility of tests**. Because of the nature of biodegradation and of the mixed bacterial populations used as inocula, determinations should be carried out at least in duplicate. It is usually found that the larger the concentration of microorganisms initially added to the test medium, the smaller will be the variation between replicates. Ring tests have also shown that there can be large variations between results obtained by different laboratories, but good agreement is normally obtained with easily biodegradable compounds.

(j) **General procedures and preparations**—(1) **General conditions.** General conditions applying to the methods are summarized in the following Table 2. Apparatus and other experimental conditions pertaining specifically to an individual method are described later under the heading for that method.

Table 2.—Test Conditions

Test	DOC die-away	CO_2 evolution	MITI (I)	Closed bottle	Modified OECD screening	Manometric respirometry
Concentration of TS						
mg/L	–	–	100	2–10	–	100
mg DOC/L	10–40	10–20	–	–	10–40	–
mg ThOD/L	–	–	–	5–10	–	50–100
Concentration of inoculum						
mg/L SS	≤30	≤30	30	–	–	≤30
mL effluent/L	≤100	≤100	–	≤5	0.5	≤100
approx. cells/L	10^7–10^8	10^7–10^8	10^7–10^8	10^4–10^6	10^5	10^7–10^8
Concentration of elements in mineral nutrient medium in mg/mL						
P	116	116	29	11.6	116	116
N	1.3	1.3	1.3	0.13	1.3	1.3
Na	86	86	17.2	8.6	86	86
K	122	122	36.5	12.2	122	122
Mg	2.2	2.2	6.6	2.2	2.2	2.2
Ca	9.9	9.9	29.7	9.9	9.9	9.9
Fe	0.05–0.1	0.05–0.1	0.15	0.05–0.1	0.05–0.1	0.05–0.1
pH	7.4±0.2	7.4±0.2	preferably 7	7.4±0.2	7.4±0.2	7.4±0.2
Temperature, °C	22±2	22±2	25±1	22±2	22±2	22±2

(2) **Water.** Deionized or distilled water, free from inhibitory concentrations of toxic substances (e.g. $Cu^{2¶}$ ions), is used. It must contain no more than 10 percent of the OC content introduced by the test material. The high purity of the test water is necessary in order to eliminate high blank values. Contamination may result from inherent impurities and also from the ion-exchange resins and lysed material from bacteria and algae. For each series of tests, use only one batch of water, previously checked by DOC analysis. Such a check is not necessary for the closed bottle meth-

od, but the oxygen consumption of the water must be low (see paragraph (o)(5)(ii) of this guideline).

(3) **Mineral nutrient media.** Mineral nutrient media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulfate and iron(III) chloride. Since only a very small inoculum, containing low concentrations of trace elements and growth factors, is used in the Modified OECD Screening method under paragraph (p) of this guideline, the medium for this test may need to be fortified with additional nutrients. The details of the stock solutions of mineral salts, trace elements and growth factors and the proportions used are given under the headings for the separate tests.

(4) **Methods of adding the test and reference substances.** The method used for adding the test and reference substances to the reaction mixture depends upon the nature of the chemical, especially its water solubility. For substances of adequate solubility (greater than about 1 g/L), prepare stock solutions at appropriate concentrations and use aliquots to prepare the final test solution. Dissolve less soluble substances in the mineral nutrient medium to avoid diluting the medium. Add substances that are even less soluble directly to the final mineral nutrient medium. Finally, refer to paragraph (f)(2) of this guideline for the handling of poorly soluble substances, but note that in the MITI method under paragraph (n) of this guideline neither organic solvents nor emulsifying agents are to be used.

(5) **Inoculum.** The inoculum may be derived from a variety of sources: Activated sludge; sewage effluents (unchlorinated); surface waters and soils; or a mixture of these. If activated sludge is used for the DOC Die-Away, CO_2 Evolution, or Manometric Respirometry method, under paragraphs (l), (m), and (q) of this guideline, it should be taken from a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Inocula from other sources, usually yielding lower cell densities, have been found to give higher scattering of results. For the Closed Bottle and Modified OECD Screening methods under paragraphs (o) and (p) of this guideline, a more dilute inoculum without sludge flocs is needed and the preferred source is a secondary effluent from a domestic wastewater treatment plant or laboratory-scale unit. For the MITI (I) method under paragraph (n) of this guideline, the inoculum is derived from a mixture of sources. Details of the sources and preparation of inocula are described under the headings of the specific test methods.

(6) **Preconditioning of inoculum.** Inoculum may be preconditioned to the experimental conditions, but not preadapted to the test substance. Preconditioning consists of aerating activated sludge (in mineral nutrient medium) or secondary effluent for 5–7 days at the test temperature. Preconditioning sometimes improves the precision of the test methods by re-

ducing blank values. It is considered unnecessary to precondition MITI (I) inoculum under paragraph (n) of this guideline.

(7) **Abiotic controls.** When required, check for the possible abiotic degradation of the test substance by determining the removal of DOC, oxygen uptake or CO_2 evolution in sterile controls containing no inoculum. Sterilize by filtration through a membrane (0.2–0.45 μm pore size) or by the addition of a suitable toxic substance at an appropriate concentration. If membrane filtration is used, collect samples aseptically to maintain sterility. Unless adsorption of the test substance has been ruled out beforehand, tests that measure biodegradation as the removal of DOC, especially with activated sludge inocula, should include an abiotic control that is inoculated and poisoned.

(8) **Number of flasks and samples.** (i) At least two flasks or vessels containing the test substance plus inoculum, and at least two containing inoculum only should be used. Single vessels suffice for reference compound plus inoculum and, when required, for toxicity, abiotic removal and adsorption controls. The MITI (I) and Closed Bottle methods under paragraph (n) and (o) of this guideline have special requirements for the number of flasks. These are given under the specific headings for these methods. It is mandatory to follow DOC and/or the other parameters in the test suspension and inoculum blanks in parallel. It is advisable to follow DOC in the other flasks in parallel as well. This may, however, not always be possible.

(ii) Although it is necessary to ensure that sufficient samples or readings are taken to allow the percentage removal in the 10–day window to be assessed, it is not possible to specify accurately the frequency of sampling because of the wide range of lag phases and rates of degradation that may be observed. In the MITI method under paragraph (n) of this guideline and the Manometric Respirometry method under paragraph (q) of this guideline, if an automatic respirometer is used, sampling for oxygen uptake presents no problems. In the latter method, daily readings are adequate when nonautomatic respirometers are employed. Specific advice on sampling is given under the headings of the other four tests.

(k) **Data and reporting**—(1) **Treatment of results.** (i) In the calculation of D_t (percent degradation), the mean values of the duplicate measurement of the parameter in both test vessels and inoculum blank are used. The formulas are set out under paragraphs (l) through (q) of this guideline. The course of degradation is displayed graphically and the 10–day window is indicated where applicable. Calculate and report the percent removal achieved and the value at the plateau, or at the end of the test, and/or at the end of the 10–day window, whichever is appropriate. In respirometric methods, nitrogen-containing chemicals may affect oxygen uptake because of nitrification (see paragraphs (k)(2) and (3) of this guideline). If the ThOD cannot be calculated because the test material is insufficiently

defined, the COD value may be used to calculate percent degradation. However, it must be borne in mind that the COD is often not as high as the ThOD as some chemicals are very poorly oxidized in the COD test, resulting in falsely high values for percent biodegradation.

(ii) When specific chemical analytical data are available, calculate primary biodegradation from:

$$D_t = (S_b - S_a)/S_b \times 100$$

where:

D_t = percent primary degradation at time t, normally 28 days

S_a = residual amount of test chemical in inoculated medium at end of the test (in milligrams or mg/L)

S_b = residual amount of test chemical in the abiotic control at the end of the test (in milligrams or mg/L)

(2) **Calculation and determination of suitable measures of ultimate degradation.** Depending on the method chosen, certain parameters will be required. The following section describes the derivation of these values. Additional information is available in paragraphs (r)(6), (r)(7), and (r)(8) of this guideline. The use of these parameters is described in the individual methods in paragraphs (l) through (q) of this guideline.

(i) **Carbon content.** The carbon content is calculated from the known elemental composition or determined by elemental analysis of the test substance.

(ii) **ThOD.** The ThOD may be calculated if the elemental composition is determined or known. For the compound

$$C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$$

the ThOD, without nitrification, is

$$ThOD_{NH3} = \frac{(16[2c + (h - cl - 3n)/2 + 3s + 5p/2 + na/2 - o]\ mg/mg)}{\text{molecular weight}}$$

With nitrification, the formula is

$$ThOD_{NO3} = \frac{(16\ [2c + (h - cl)/2 + 5n/2 + 3s + 5p/2 + na/2 - o]mg/mg)}{\text{molecular weight}}$$

(iii) **COD.** The COD of water-soluble organic substances is determined by established procedures, e.g. according to ISO method 6060. COD is often, and especially in the case of poorly soluble substances, deter-

mined advantageously in a variant of the above analysis; i.e. in a closed system with a pressure equalizer (refer to paragraph (r)(9) of this guideline). In this modification, compounds which are determined by the conventional method (e.g. acetic acid) only with difficulty may often be successfully quantified. This method also fails, however, in the case of pyridine. If the potassium dichromate concentration is raised from 0.016 N (0.0026 M) as prescribed by under paragraph (r)(9) of this guideline to 0.25 N (0.0416 M), the direct weighing-in of 5–10 mg of substance, which is essential for the COD determination of poorly water-soluble substances, is facilitated (see paragraph (r)(5) of this guideline).

(iv) **DOC.** DOC is, by definition, the organic carbon of any chemical or mixture in water passing through a 0.45 μm pore size filter. Samples from the test vessels are withdrawn and filtered immediately in the filtration apparatus using an appropriate membrane filter. The first 20 mL (amount can be reduced when using small filters) of the filtrate are discarded. Volumes of 10–20 mL or lower, if injected (volume depending on the amount required for carbon analyzer) are retained for carbon analysis. The DOC concentration is determined by means of an organic carbon analyzer that is capable of accurately measuring a carbon concentration equivalent to or lower than 10 percent of the initial DOC concentration used in the test. Filtered samples that cannot be analyzed on the same working day can be preserved by storage in a refrigerator at 4 °C. Preserved samples can be retained for 48 h before analysis, or for longer at –18 °C.

(v) **Remarks.** (A) Membrane filters are often impregnated with surfactants for hydrophilization. Thus the filter may contain up to several milligrams of soluble organic carbon which may interfere in the biodegradability determinations. Surfactants and other soluble organic compounds may be removed by boiling the filters in deionized water for three periods each of 1 h unless this destroys the filters; washing is recommended in this case. The filters may then be stored in water for 1 week. If disposable filter cartridges are used, each lot must be checked to confirm that it does not release soluble organic carbon.

(B) Depending on the type of membrane filter, the test chemical may be retained by adsorption. It may therefore be advisable to ensure that the test chemical is not retained by the filter.

(C) Centrifugation at 4,000 × g (about 40.000 m sec^{-2}) for 15 min may be used for differentiation of TOC versus DOC instead of filtration. The method is not reliable at initial concentrations of <10 mg DOC/L since either not all bacteria are removed or carbon as part of the bacterial plasma is redissolved.

(3) **Correction for interference by nitrification in determination of biodegradation by oxygen uptake.** Respirometric methods with oxy-

gen uptake as the analytical procedure may be influenced significantly by the oxygen uptake resulting from ammonium oxidation.

(i) Errors due to not considering nitrification in the assessment by oxygen uptake of the biodegradability of test substances not containing nitrogen are marginal (not greater than 5 percent), even if oxidation of the ammonium ion nitrogen in the medium occurs erratically in test and blank vessels. However, for test substances containing nitrogen, serious errors can arise if the observed oxygen uptake is not corrected for the amount of oxygen used in oxidizing ammonium ion to nitrite ion and nitrate ion. In the case of complete nitrification, or transformation of ammonium ion to nitrate ion, the following equation applies:

$$NH_4\P + 2\ O_2 \longrightarrow NO_3^- + 2H\P + H_2O$$

(ii) The oxygen taken up by 14 g of nitrogen is 64 g and thus the oxygen consumed in nitrate formation is 4.57 × increase of nitrate ion nitrogen concentration. If incomplete nitrification takes place, the following equations apply:

$$NH_4\P + 3/2\ O_2 \longrightarrow NO_2^- + 2H\P + H_2O$$

$$NO_2^- + 1/2\ O_2 \longrightarrow NO_3^-$$

(iii) The oxygen taken up by 14 g of nitrogen in being oxidized to nitrite is 48 g, i.e. a factor of 3.43× increase of nitrite ion nitrogen concentration.

(iv) Since the reactions are sequential, being carried out by distinct and different bacterial species, it is possible for the concentration of nitrite ion to increase or decrease during the test. In the latter case an equivalent concentration of nitrate ion would be formed. Thus, the oxygen consumed in the formation of nitrate ion is 4.57× the increase in concentration of nitrate ion nitrogen, whereas the oxygen associated with the formation of nitrite ion is 3.43× the increase in the concentration of nitrite ion nitrogen; or, with the decrease in its concentration, the oxygen ‘‘loss’’ is 3.43 multiplied by the decrease in nitrite concentration.

(v) Alternatively, if only ‘‘total oxidized N’’ is determined, the oxygen uptake due to nitrification may be taken to be, as a first approximation, 4.57 × increase in oxidized nitrogen.

(vi) The corrected value for oxygen consumption due to carbon oxidation is then compared with $ThOD_{NH3}$, as calculated under paragraph (k)(2)(ii) of this guideline.

(4) **Validity of tests.** (i) A test is considered valid if the difference in extremes of replicate values of biodegradation of the test chemical at the plateau, at the end of the test or at the end of the 10–day window, as appropriate, is less than 20 percent, and if the percent degradation of

the reference compound has reached the pass levels by day 14. If either of these conditions is not met, the test should be repeated. Because of the stringency of the methods, low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

(ii) If, in a toxicity test containing both the test substance and a reference compound in the same test vessel, less than 35 percent degradation (based on total DOC) or less than 25 percent (based on total ThOD or $ThCO_2$) occurred within 14 days, the test substance can be assumed to be inhibitory (see paragraph (e)(2) of this guideline for other toxicity tests). In this case the test series should be repeated, using a lower concentration of test substance (if this can be done without seriously impairing the accuracy of the DOC determination) and/or a higher concentration of inoculum, but not greater than 30 mg of solids per liter.

(iii) Other conditions for the validity of test results specific to individual methods are set out under the headings for those tests.

(5) **Test report.** The test report must include the following:

(i) *Test substance*. (A) Physical nature and, where relevant, physical/chemical properties.

(B) Identification data.

(ii) *Test conditions*. (A) Inoculum: Nature and sampling site(s), concentration, and any preconditioning treatment.

(B) Proportion and nature of industrial waste water in sewage, if known.

(C) Test duration and temperature.

(D) In the case of poorly soluble test substances, methods of preparation of test solutions/suspensions.

(E) Test method applied; scientific reasons and explanation for any change of procedure.

(iii) *Results.* (A) Data in tabular form.

(B) Any observed inhibition phenomena.

(C) Any observed abiotic degradation.

(D) Specific chemical analytical data, if available.

(E) Analytical data on intermediates, if available.

(F) The graph of percent degradation vs. time for the test and reference substances, the lag phase, the degradation phase, the 10–day window, and the maximum slope of the degradation curve (see paragraph (c) of this guideline for definitions).

(G) Percent removal at the plateau, at the end of the test, and/or after the 10–day window.

(iv) *Discussion of results.*

(l) **DOC die-away test**—(1) **General.** Matters concerning the assessment of biodegradability are discussed under paragraph (j) of this guideline and it is advisable to read this material before proceeding. For this method, the test substance should be nonvolatile and have a solubility in water of at least 100 mg/L. Also, the carbon content and preferably the purity or relative proportions of major components must be known. This test is virtually the same as the ISO Standard 7827–1984. It is similar to the Modified OECD Screening test under paragraph (p) of this guideline but allows the use of much higher microbial cell densities.

(2) **Principle of the test.** A measured volume of inoculated mineral nutrient medium, containing a known concentration of the test substance (10–40 mg DOC/L) as the nominal sole source of OC, is aerated in the dark or diffuse light at 22 ± 2 °C. Degradation is followed by DOC analysis at frequent intervals over a 28–day period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the inoculum control) as a percentage of the concentration initially present. Primary biodegradation may also be calculated from supplemental chemical analysis for parent compound made at the beginning and end of incubation.

(3) **Description of the method**—(i) **Apparatus.** In addition to normal laboratory equipment, the following apparatus should be available:

(A) Conical flasks, e.g. 250–mL to 2–L, depending on the volume needed for DOC analysis. The flasks must be carefully cleaned with, for example, alcoholic hydrochloric acid, rinsed, and dried before each test.

(B) Shaking machine—to accommodate the conical flasks, either with automatic temperature control or used in a constant temperature room—and of sufficient power to maintain aerobic conditions in all flasks.

(C) Filtration apparatus, with suitable membrane filters.

(D) DOC analyzer.

(E) Apparatus for determining DO, to confirm that the flask contents are aerobic.

(F) Centrifuge.

(ii) **Water.** A description of the water to be used is given under paragraph (j)(2) of this guideline.

(iii) **Stock solutions for mineral nutrient medium.** Prepare the following stock solutions using analytical grade reagents:

(A) Dissolve 8.50 g potassium dihydrogen orthophosphate (KH_2PO_4), 21.75 g dipotassium hydrogen orthophosphate (K_2HPO_4), 33.40 g disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), and 0.5 g ammonium chloride (NH_4Cl) in water and make up to 1 L. The pH of the solution should be 7.4.

(B) Dissolve 27.50 g calcium chloride, anhydrous ($CaCl_2$), or 36.40 g calcium chloride dihydrate, $CaCl_2 \cdot 2H_2O$), in water and make up to 1 L.

(C) Dissolve 22.50 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in water and make up to 1 L.

(D) Dissolve 0.25 g iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in water and make up to 1 L.

(E) In order to avoid having to prepare this solution immediately before use, add one drop of concentrated HCl or 0.4 g ethylenediaminetetraacetic acid (EDTA disodium salt) per liter. If a precipitate forms in a stock solution replace with a freshly made solution.

(iv) **Preparation of mineral nutrient medium.** Mix 10 mL of solution (A) with 800 mL water, then add 1 mL of solutions (B), (C) and (D) and make up to 1 L with water.

(v) **Stock solution of test substance.** When the solubility of the substance exceeds 1 g/L, dissolve 1–10 g, as appropriate, of test or reference substance in water and make up to 1 L. Otherwise, prepare stock solutions in mineral nutrient medium or add the chemical directly to the mineral nutrient medium, making sure it dissolves.

(vi) **Inoculum.** The inoculum may be derived from a variety of sources: Activated sludge; sewage effluents; surface waters; soils; or a mixture of these.

(A) **Inoculum from activated sludge.** (*1*) Collect a fresh sample of activated sludge from the aeration tank of a sewage treatment plant or laboratory-scale unit treating predominantly domestic sewage. Remove coarse particles if necessary by filtration through a fine sieve and keep the sludge aerobic thereafter.

(*2*) Alternatively, after removal of any coarse particles, settle or centrifuge (e.g. at 1,100 × g for 10 min). Discard the supernatant. The sludge may be washed in the mineral nutrient medium. Suspend the concentrated

sludge in mineral nutrient medium to yield a concentration of 3–5 g SS/L. Thereafter aerate until required.

(*3*) Sludge should be taken from a properly working conventional treatment plant. If sludge has to be taken from a high-rate treatment plant, or is thought to contain inhibitors, it should be washed. Settle or centrifuge the resuspended sludge after thorough mixing, discard the supernatant and again resuspend the washed sludge in a further volume of mineral nutrient medium. Repeat this procedure until the sludge is considered to be free from excess substrate or inhibitor.

(*4*) After complete resuspension is achieved, or with untreated sludge, withdraw a sample just before use for the determination of the dry weight of the SS.

(*5*) A further alternative is to homogenize activated sludge (3–5 g SS/L). Treat the sludge in a blender for 2 min at medium speed. Settle the blended sludge for 30 min or longer if required and decant liquid for use as inoculum at the rate of about 10 mL/L of mineral nutrient medium.

(B) **Other sources of inoculum.** (*1*) Alternatively, the inoculum can be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Collect a fresh sample and keep it aerobic during transport. Allow to settle for 1 h or filter through a coarse filter paper and keep the decanted effluent or filtrate aerobic until required. Up to 100 mL of this type of inoculum may be used per liter of medium.

(*2*) Another source for the inoculum is surface water. In this case, collect a sample of an appropriate surface water (e.g. river, lake) and keep aerobic until required. If necessary, concentrate the inoculum by filtration or centrifugation.

(vii) **Preconditioning of inoculum.** Preconditioning is discussed in paragraph (j)(6) of this guideline.

(viii) **Preparation of flasks.** (A) Introduce 800–mL–portions of mineral nutrient medium into 2–L conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10–40 mg DOC/L. Check the pH values and adjust to 7.4, if necessary. Inoculate the flasks with activated sludge or other source of inoculum to give a final concentration not greater than 30 mg SS/L. Also prepare inoculum controls in the mineral nutrient medium but without test or reference substance.

(B) If required, use one vessel to check the possible inhibitory effect of the test substance by inoculating a solution containing comparable con-

centrations of both the test and a reference substance in the same test vessel.

(C) If required, check whether the test substance is degraded abiotically by setting up a flask containing a sterilized, uninoculated solution of the substance. Abiotic controls are discussed in paragraph (j)(7) of this guideline.

(D) If the test substance is suspected of being significantly adsorbed onto glass, sludge, etc., make a preliminary assessment to determine the likely extent of adsorption and thus the suitability of the test for the chemical (see Table 1, under paragraph (f)(1) of this guideline). Set up a flask containing the test substance, inoculum and sterilizing agent.

(E) Make up the volumes in all flasks to 1 L with mineral nutrient medium and, after mixing, take a sample from each flask to determine the initial concentration of DOC in duplicate (see paragraph (k)(2)(iv) of this guideline). Cover the openings of the flasks in such a way as to allow free exchange of air between the flask and the surrounding atmosphere, for example using aluminum foil. To start the test, place the vessels in the shaking machine.

(ix) **Number of flasks.** In a typical run, the following flasks are used:

(A) Flasks 1 and 2—containing test substance and inoculum (test suspension).

(B) Flasks 3 and 4—containing only inoculum (inoculum control).

(C) Flask 5—containing reference compound and inoculum (procedure control).

(D) Flask 6—containing test substance and sterilizing agent (abiotic sterile control: Optional).

(E) Flask 7—containing test substance, inoculum and sterilizing agent (adsorption control: Optional).

(F) Flask 8—containing test substance, reference compound and inoculum (toxicity control: Optional).

(4) **Procedure**—(i) **DOC determinations.** Throughout the test, determine the concentrations of DOC in samples from each flask in duplicate at known time intervals. It is mandatory to follow DOC in the test suspension and inoculum blanks in parallel. It is advisable to follow DOC in the other flasks in parallel as well, although this may not always be possible.

(ii) **Sampling.** Take only the minimum volume of test suspension necessary for each determination. Before sampling make up for any evaporation losses from the flasks by adding water in the required amount. Mix

the culture medium thoroughly before withdrawing a sample and ensure that material adhering to the walls of the vessels is redissolved or resuspended before sampling. Membrane-filter or centrifuge the sample immediately after it has been taken. For other information on sampling, consult paragraph (k)(2)(iv) of this guideline.

(iii) **Frequency of sampling.** Ensure that a sufficient number of samples are collected to allow the percent removal in the 10–day window to be assessed. No precise sampling pattern can be described. If analyses are performed on the day of sampling, assess the next sampling time by considering the result of the analysis. If the samples are preserved, collect samples daily or every 2 days. Analyze the last samples (28–day) first and, by a stepwise ‘‘backwards’’ selection of appropriate samples for analysis, it is possible to obtain a good description of the biodegradation curve with a relatively small number of determinations. If the last samples (28–d) show no degradation, no further samples need be analyzed.

(5) **Data and reporting**—(i) **Treatment of results**. (A) Data from the test should be entered on a data sheet, such as the DOC Die-Away Test Data Sheet following paragraph (l) of this guideline.

(B) The percent degradation (D_t) at each time a sample was taken should be calculated separately for both flasks containing test substance (i.e. flasks 1 and 2) using mean values of duplicate DOC measurements (see data sheet) so that the validity of the test can be assessed (see paragraph (k)(4) of this guideline). It is calculated using the following equation:

$$D_t = [1 - (C_t - C_{bl(t)})/(C_0 - C_{bl(0)})] \times 100$$

where:

D_t = percent degradation at time t (days)

C_0 = mean starting concentration of DOC in the inoculated culture medium containing test substance (mg DOC/L)

C_t = mean concentration of DOC in the inoculated culture medium containing test substance at time t (mg DOC/L)

$C_{bl(0)}$ = mean starting concentration of DOC in blank inoculated mineral nutrient medium (mg DOC/L)

$C_{bl(t)}$ = mean concentration of DOC blank inoculated mineral nutrient medium at time t (mg DOC/L)

All concentrations are measured experimentally.

(C) If the test has complied with the validity criteria, display the course of degradation graphically using the mean of both flasks containing test substance. Indicate the 10–day window. Calculate and report the per-

centage removal achieved at the plateau, at the end of the test and/or at the end of the 10–day window, as appropriate.

(D) When specific chemical analytical data are available, calculate percent primary biodegradation (see paragraph (k)(1)(ii) of this guideline).

(E) When an abiotic sterile control is used calculate the percent abiotic degradation as follows:

$$\% \text{ abiotic degradation} = (C_{s(0)} - C_{s(t)})/C_{s(0)} \times 100$$

where:

$C_{s(0)}$ = DOC concentration in sterile control at day 0

$C_{s(t)}$ = DOC concentration in sterile control at day t.

(ii) **Validity of tests.** The validity criteria apply as given under paragraph (k)(4) of this guideline.

(iii) **Test report.** The test report should include the information described in under paragraph (k)(5) of this guideline. Data from the test should be entered on the data sheet. A similar format may be used for the procedure and toxicity controls.

DOC DIE-AWAY TEST

DATA SHEET

LABORATORY: _____

DATE AT START OF TEST:_____

TEST SUBSTANCE:_____

Name:_____

Stock solution concentration (mg/L as chemical):_____

Initial concentration in medium (mg/L as chemical):_____

INOCULUM:

Source:_____

Treatment given:_____

Preconditioning, if any:_____

SS concentration in reaction mixture (mg/L):_____

1. CARBON DETERMINATIONS

Carbon analyzer used:_____

	Flask no.		DOC after *t* days (mg/L)				
			0	t_1	t_2	t_3	t_4
Test substance plus inoculum							
	1	a_1	.....	.....	.....	.....	.....
	1	a_2	.....	.....	.....	.....	.....
	1	mean, $C_{a(t)}$	.....	.....	.....	.....	.....
	2	b_1	.....	.....	.....	.....	.....
	2	b_2	.....	.....	.....	.....	.....
	2	mean, $C_{b(t)}$	.....	.....	.....	.....	.....
Control: Inoculum but no test substance							
	3	c_1	.....	.....	.....	.....	.....
	3	c_2	.....	.....	.....	.....	.....
	3	mean, $C_{c(t)}$	.....	.....	.....	.....	.....
	4	d_1	.....	.....	.....	.....	.....
	4	d_2	.....	.....	.....	.....	.....
	4	mean, $C_{d(t)}$	.....	.....	.....	.....	.....
		mean, $C_{bl(t)} = (C_{c(t)} + C_{d(t)})/2$	.....	.....	.....	.....	.....

2. EVALUATION OF RAW DATA

Flask no.	Calculation of results	% degradation after *t* days				
		0	t_1	t_2	t_3	t_4
1	$D_1 = [1 - (C_{a(t)} - C_{bl(t)})/(C_{a(0)} - C_{bl(0)})] \times 100$	0	.....	.....	.....	.....
2	$D_2 = [1 - (C_{b(t)} - C_{bl(t)})/(C_{b(0)} - C_{bl(0)})] \times 100$	0	.....	.....	.....	.....
Mean[1]	$D_t = (D_1 + D_2)/2$	0	.....	.....	.....	.....

[1] D_1 and D_2 should not be averaged if there is a considerable difference between replicates

3. ABIOTIC DEGRADATION (optional)

	Time (days)	
	0	t
DOC concentration in sterile control (mg/L)	$C_{s(0)}$	$C_{s(t)}$
.....	.....	.....
.....	.....	.....

$$\% \text{ abiotic degradation} = (C_{s(0)} - C_{s(t)})/C_{s(0)} \times 100$$

4. SPECIFIC CHEMICAL ANALYSIS (optional)

	residual amount of test chemical at end of test	% primary degradation
Sterile control	S_b	
.....	.....	.....
.....	.....	.....
.....	.....	.....
.....	.....	.....
Inoculated test medium	S_a	$(S_b - S_a)/S_b \times 100$
.....	.....	.....
.....	.....	.....
.....	.....	.....
.....	.....	.....

(m) **Carbon dioxide evolution test**—(1) **General.** Matters concerning the assessment of biodegradability are discussed under paragraph (j) of this guideline and it is advisable to read this before proceeding. For this method, the test substance should be nonvolatile and its carbon content and degree of purity or relative proportions of major components must be known.

(2) **Principle of the test.** A measured volume of inoculated mineral nutrient medium, containing a known concentration of the test substance (10–20 mg DOC or TOC/L) as the nominal sole source of OC is aerated by the passage of carbon dioxide-free air at a controlled rate in the dark or in diffuse light, at 22 ± 2 °C. Degradation is followed over 28 days by determining the CO_2 produced. The CO_2 is trapped in $Ba(OH)_2$ or NaOH and is measured by titration of the residual hydroxide, or as IC. The amount of CO_2 produced from the test substance (corrected for that derived from the inoculum control) is expressed as a percentage of the $ThCO_2$. The degree of biodegradation may also be calculated from supplemental DOC analysis made at the beginning and end of incubation.

(3) **Description of the method**—(i) **Apparatus.** In addition to normal laboratory equipment, the following apparatus should be available:

(A) Flasks, 2– to 5–L, each fitted with an aeration tube reaching nearly to the bottom of the vessel and an outlet (the tube must not interfere with a magnetic stirrer when used).

(B) Magnetic stirrers, when assessing poorly soluble chemicals.

(C) Gas absorption bottles.

(D) Device for controlling and measuring air flow.

(E) Apparatus for CO_2 scrubbing, for preparation of air which is free from CO_2; alternatively, a mixture of CO_2-free oxygen and CO_2-free nitrogen supplied from a gas cylinder in the correct proportions (20% O_2:80% N_2) may be used.

(F) Device for determination of CO_2, either titrimetrically or using some form of IC analyzer.

(G) Membrane filtration device (optional).

(H) DOC analyzer (optional).

(ii) **Water.** A description of the water to be used is given under paragraph (j)(2) of this guideline.

(iii) **Stock solutions for mineral nutrient medium.** Prepare the same stock solutions as described under paragraph (l)(3)(iii) of this guideline.

(iv) **Preparation of mineral nutrient medium.** See paragraph (l)(3)(iv) of this guideline.

(v) **Stock solution of test substance.** Prepare stock solutions as described in paragraph (l)(3)(v) of this guideline. For the handling of poorly soluble substances see paragraph (f)(2) of this guideline.

(vi) **Inoculum.** The inoculum may be derived from a variety of sources: Activated sludge; sewage effluents; surface waters; soils; or from a mixture of these.

(vii) **Inoculum from activated sludge.** See paragraphs (l)(3)(vi)(A)(*1*) through (*5*) of this guideline for details on the collection and preparation of inoculum from activated sludge.

(viii) **Other sources of inoculum.** Alternatively, the inoculum can be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage or from surface water. See paragraph (l)(3)(vi)(B) of this guideline for details.

(ix) **Preconditioning of inoculum.** Preconditioning is discussed in paragraph (j)(6) of this guideline.

(x) **Preparation of flasks.** (A) As an example, the following volumes and weights indicate the values for 5–L flasks containing 3 L of inoculated mineral nutrient medium. If smaller volumes are used modify the values accordingly, but ensure that the CO_2 formed can be measured accurately. To each 5–L flask add 2,400 mL of mineral nutrient medium. Add an appropriate volume of the prepared activated sludge to give a concentration of SS of not more than 30 mg/L in the final 3 L of inoculated mixture. Alternatively, first dilute the prepared sludge to give a suspension of 500–1,000 mg/L in the mineral nutrient medium before adding an aliquot to the contents of the 5–L flask to attain a concentration of 30 mg/L; this ensures greater precision (other sources of inoculum may be used; see paragraph (l)(3)(vi)(B) of this guideline). Aerate these inoculated mixtures with CO_2-free air overnight to purge the system of CO_2.

(B) Add the test material and reference compound to separate replicate flasks as known volumes of stock solutions, to yield concentrations of 10 to 20 mg DOC or TOC per liter; leave some flasks without addition of chemicals as inoculum controls. Add poorly soluble test substances directly to the flasks on a weight or volume basis or handle as described under paragraph (f)(2) of this guideline. Make up the volumes of inoculated medium in all flasks to 3 L by the addition of mineral nutrient medium previously aerated with CO_2-free air.

(C) If required, use one flask to check the possible inhibitory effect of the test substance by adding both the test and reference substances to the same flask at the same concentrations as present in the other flasks.

(D) If required, check whether the test substance is degraded abiotically by setting up a flask containing a sterilized, uninoculated solution of the chemical. Abiotic controls are discussed in paragraph (j)(7) of this guideline.

(E) If $Ba(OH)_2$ is used, connect three absorption bottles, each containing 100 mL of 0.0125 M $Ba(OH)_2$ solution, in series, to each 5–L flask. The solution must be free of precipitated sulfate and carbonate and its concentration must be determined immediately before use. If NaOH is used, connect two bottles, the second acting as a control to demonstrate that all of the CO_2 was absorbed in the first. Absorption bottles fitted with serum bottle closures are suitable. Add 200 mL of 0.05 M NaOH to each bottle. This is sufficient to absorb the total quantity of CO_2 evolved when the test substance is completely degraded. The NaOH solution, even when freshly prepared, will contain traces of carbonates; this is corrected by deduction of the carbonate in the blank control.

(F) Optionally, samples may be withdrawn for analysis of DOC (see paragraph (k)(2)(iv) of this guideline) and/or specific chemical analysis.

(xi) **Number of flasks.** In a typical run, the following flasks are used:

(A) Flasks 1 and 2—containing test substance and inoculum (test suspension).

(B) Flasks 3 and 4—containing only inoculum (inoculum control).

(C) Flask 5—containing reference compound and inoculum (procedure control).

(D) Flask 6—containing test substance and sterilizing agent (abiotic sterile control: Optional).

(E) Flask 7—containing test substance, reference compound and inoculum (toxicity control: Optional).

(4) **Procedure.** (i) Start the test by bubbling CO_2-free air through the suspensions at a rate of 30–100 mL/min.

(ii) **CO_2 determinations.** (A) It is mandatory to follow the CO_2 evolution from the test suspensions and inoculum controls in parallel and it is advisable to do the same for the other test vessels.

(B) It is recommended that during the first 10 days analyses of CO_2 be made every 2nd or 3rd day, and after this at least every 5th day until the 28th day so that the 10–day window period can be identified.

(C) On the days of CO_2 measurement, disconnect the $Ba(OH)_2$ absorber closest to the test vessel and titrate the hydroxide solution with 0.05 M HCl using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the test vessel and place a new absorber

containing 100 mL fresh 0.0125 M $Ba(OH)_2$ at the far end of the series. Perform titrations as needed; for example, when substantial precipitation is seen in the first bottle and before any is evident in the second. Alternatively, with NaOH as absorbent, withdraw a sample of the NaOH solution from the absorber nearest to the test vessel using a syringe. The sample volume needed will depend on the carbon analyzer used, but sampling should not significantly change the absorbent volume over the test period. Inject the sample into the IC part of the carbon analyzer for analysis of evolved CO_2 directly. Analyze the contents of the second bottle only at the end of the test in order to correct for any carryover of CO_2.

(D) Optionally, on the 28th day withdraw samples for DOC and/or specific chemical analysis. Add 1 mL of concentrated HCl to each test vessel and aerate overnight to drive off the CO_2 present in the test suspensions. On day 29 make the last analysis of evolved CO_2.

(5) **Data and reporting**—(i) **Treatment of results.** (A) Data from the test should be entered on a data sheet, such as the CO_2 Evolution Test Data Sheet following paragraph (m) of this guideline.

(B) The amount of CO_2 produced is calculated from the amount of base remaining in the absorption bottle. When 0.0125 M $Ba(OH)_2$ is used as the absorbent, the amount remaining is assessed by titrating with 0.05 M HCl. Thus 50 mL HCl is needed to titrate 100 mL of $Ba(OH)_2$.

(C) Since 1 mmol of CO_2 is produced for every millimole of $Ba(OH)_2$ reacted to $BaCl_2$ and 2 mmol of HCl is needed for the titration of the remaining $Ba(OH)_2$, and given that the molecular weight of CO_2 is 44 g/mol, the weight of CO_2 produced (given in milligrams) is calculated as follows:

$$\text{mg } CO_2 = [0.05 \times (50 - \text{ml HCl titrated}) \times 44)/2] =$$

$$1.1 \times (50 - \text{ml HCl titrated})$$

In this case, the factor to convert volume of HCl titrated to milligrams of CO_2 produced is 1.1. Calculate the weights of CO_2 produced from the inoculum alone and from the inoculum plus test substance using the respective titration values; the difference is the weight of CO_2 produced from the test substance alone. For example, if the inoculum alone gives a titration of 48 mL and inoculum plus test substance gives 45 mL,

(a) = CO_2 from inoculum = $1.1 \times (50 - 48) = 2.2$ mg

(b) = CO_2 from inoculum + test substance =

$$1.1 \times (50 - 45) = 5.5 \text{ mg}$$

and the weight of CO_2 produced from the test substance is given by (b) – (a) = 3.3 mg.

(D) Calculate the weight of $ThCO_2$ as follows:

$$\text{mg } ThCO_2 = \frac{\text{no. of carbons in TS} \times \text{molecular weight of } CO_2 \times \text{mg of test substance added}}{\text{molecular weight of test substance}}$$

(E) The percent biodegradation is calculated from:

$$\text{\% degradation} = \frac{\text{mg } CO_2 \text{ produced}}{\text{mg } ThCO_2} \times 100$$

or

$$\text{\% degradation} = \frac{\text{mg } CO_2 \text{ produced}}{\text{mg TOC added in test} \times 3.67} \times 100$$

where 3.67 is the conversion factor (44/12) for carbon to CO_2. Obtain the percent degradation after any time interval by adding the percent $ThCO_2$ values calculated for each of the days, up to that time on which it was measured.

(F) When NaOH is used as the absorbent, calculate the amount of CO_2 produced after any time interval from the concentration of IC and the volume of absorbent used. Calculate the percent degradation from:

$$\text{\% of } ThCO_2 = \frac{\text{mg IC from test flask} - \text{mg of IC from inoculum control}}{\text{mg TOC added as test substance}} \times 100$$

(G) Display the course of degradation graphically and indicate the 10–day window. Calculate and report the percentage removal achieved at the plateau, at the end of the test and/or at the end of the 10–day window, whichever is appropriate.

(H) When appropriate, calculate DOC removals using the equation given in paragraph (l)(5)(i)(B) of this guideline.

(I) When an abiotic control is used, calculate the percent abiotic degradation as follows:

$$\text{\% abiotic degradation} = \frac{\text{mg } CO_2 \text{ produced by sterile flask after 28 days}}{\text{mg } ThCO_2} \times 100$$

(ii) **Validity of tests.** (A) The IC content of the test suspension at the beginning of the test must be less than 5 percent of the TC, and the total CO_2 evolution in the inoculum control at the end of the test should not normally exceed 40 mg/L medium. If values greater than

70 mg CO_2/L are obtained, the data and experimental technique should be examined critically.

(B) The other validity criteria given under paragraph (k)(4) of this guideline also apply.

(iii) **Test report.** The test report should include the information described under paragraph (k)(5) of this guideline. Data should be entered on the data sheet.

CO_2 Evolution Test

DATA SHEET

LABORATORY: _____

DATE AT START OF TEST:_____

TEST SUBSTANCE:_____

Name:_____

Stock solution concentration (mg/L as chemical):_____

Initial concentration in medium (mg/L as chemical):_____

Total C added to flask (mg C):_____

$ThCO_2$:_____

INOCULUM:

Source:_____

Treatment given:_____

Preconditioning, if any:_____

SS concentration in reaction mixture (mg/L):_____

1. CO_2 PRODUCTION AND DEGRADABILITY

Method: $Ba(OH)_2$/NaOH/other

Time (days)	CO_2 produced (mg)					Cumulative CO_2 produced (mg)		% $ThCO_2$ = mg cumulative CO_2/mg $ThCO_2$		
	test chemical		inoculum control			test-control mean				
	flask 1	flask 2	flask 3	flask 4	mean	flask 1	flask 2	flask 1	flask 2	mean[1]
0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
t_1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
t_2	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
t_3	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
t_4	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
28	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

[1] Do not take mean if there is a considerable difference between replicates.

NOTE: Similar formats may be used for the procedure control and toxicity controls (if any).

2. CARBON ANALYSIS (optional; see paragraph (l)(5)(i)(B) of this guideline)

Carbon analyzer used:_____

Time (days)	Test chemical (mg/L)	Blank (mg/L)
0	C_0	$C_{bl(0)}$
	.....	.....
28[1]	C_t	$C_{bl(t)}$
	.....	.....

[1] or at end of incubation

$$\% \text{ DOC removed} = [1 - (C_t - C_{bl(t)})/C_0 - C_{bl(0)}] \times 100$$

3. ABIOTIC DEGRADATION (optional)

% abiotic degradation =

mg CO_2 formed in sterile flask after 28 days/mg $ThCO_2 \times 100$

(n) **Modified MITI test (I)**—(1) **General.** Matters concerning the assessment of biodegradability are discussed under paragraph (j) of this guideline and it is advisable to read that material before proceeding. For this method, the formula of the test substance and its purity, or relative proportions of major components, must be known so that the ThOD may be calculated. Insoluble and volatile substances may be assessed provided precautions are taken. Insoluble substances should be dispersed, for example, by using very finely ground material or ultrasonics, but not solvents or emulsifying agents. For volatile substances the volume of ''dead'' gas space in the automatic respirometer should be kept to a minimum.

(2) **Principle of the test.** The oxygen uptake by a stirred solution, or suspension, of the test substance in a mineral nutrient medium, inoculated with specially grown, unadapted microorganisms, is measured automatically over a period of 28 days in a darkened, enclosed respirometer at 25 ± 1 °C. Evolved CO_2 is absorbed by soda lime. Biodegradation is expressed as the oxygen uptake relative to the theoretical uptake (ThOD). The percentage primary biodegradation is also calculated from supplemental specific chemical analysis made at the beginning and end of incubation, and optionally, ultimate biodegradation is determined by DOC analysis.

(3) **Description of the method—** (i) **Apparatus.** In addition to normal laboratory equipment, the following apparatus should be available:

(A) Automatic electrolytic BOD meter or respirometer normally equipped with six bottles of 300–mL capacity each, and equipped with cups to contain CO_2 absorbent.

(B) Constant temperature room and/or water bath maintained at 25 ± 1 °C.

(C) Membrane filtration assembly (optional).

(D) Carbon analyzer (optional).

(ii) **Water.** A description of the water to be used is given under paragraph (j)(2) of this guideline.

(iii) **Stock solutions for mineral nutrient medium.** Prepare the following stock solutions using analytical grade reagents:

(A) Dissolve 8.50 g potassium dihydrogen orthophosphate (KH_2PO_4), 21.75 g dipotassium hydrogen orthophosphate (K_2HPO_4), 44.60 g disodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), and 1.70 g ammonium chloride (NH_4Cl) in water and make up to 1 L. The pH value of the solution should be 7.2

(B) Prepare calcium chloride, magnesium sulfate, and iron(III) chloride solutions as described in paragraphs (l)(3)(iii)(B) through (D) of this guideline.

(iv) **Preparation of mineral nutrient medium.** Take 3 mL of each solution and make up to 1 L.

(v) **Stock solution of test substance.** Prepare stock solutions in the mineral nutrient medium or add the chemical directly to the medium. When the solubility of the test or reference substance exceeds 1 g/L, dissolve 1–10 g, as appropriate, of the substance in water and make up to 1 L. For the handling of poorly soluble substances, see paragraph (f)(2) of this guideline.

(vi) **Preparation of inoculum.** (A) Collect fresh samples from no fewer than 10 sites, mainly in areas where a variety of chemicals are used and discharged. Collect 1–L samples of sludge, surface soil, water, etc. from sites such as sewage treatment works, industrial wastewater treatment works, rivers, lakes and seas, and mix thoroughly together.

(B) After removing floating matter and allowing the samples to stand, adjust the supernatant to pH 7 ± 1 with NaOH or H_3PO_4. Use an appropriate volume of the filtered supernatant to fill a fill-and-draw activated sludge vessel and aerate the liquid for 23.5 h. Thirty minutes after stopping aeration, discard about one-third of the whole volume of supernatant and add an equal volume of a solution (pH 7) containing 0.1 percent each of glucose, peptone, and potassium orthophosphate to the settled material, and recommence aeration. Repeat this procedure once per day.

(C) The sludge unit must be operated according to normal sound laboratory practice. Effluents should be clear; temperature should be kept at 25 ± 2 °C and pH at 7 ± 1; sludge should settle well; there should be sufficient aeration to keep the mixture aerobic at all times; protozoa should be present; and the activity of the sludge should be tested against a reference substance at least every 3 months. Do not use sludge as inoculum until after at least 1 month of operation, but not after more than 4 months. Thereafter, sample from at least 10 sites at regular intervals, once every 3 months. In order to maintain fresh and old sludge at the same activity, mix the filtered supernatant of an activated sludge in use with an equal volume of the filtered supernatant of a freshly collected 10–source mixture and culture the combined liquor as above. Take sludge for use as inoculum 18–24 h after the unit has been fed.

(vii) **Preparation of bottles.** Prepare the following six bottles:

(A) Bottle 1—test substance in water at 100 mg/L (abiotic control).

(B) Bottles 2, 3, and 4—test substance in mineral nutrient medium at 100 mg/L.

(C) Bottle 5—reference compound (e.g. aniline) in mineral nutrient medium at 100 mg/L (procedure control).

(D) Bottle 6—mineral nutrient medium only (inoculum control).

(E) Add poorly soluble test substances directly on a weight or volume basis, or handle as described under paragraph (f)(2) of this guideline except that neither solvent nor emulsifying agent should be used. Add the CO_2 absorbent to all test vessels in the special cups provided. Adjust the pH in bottles 2, 3, and 4 to 7.0 before inoculation, if necessary.

(4) **Procedure.** (i) Inoculate vessels 2, 3, and 4 (test suspensions), 5 (procedure control), and 6 (inoculum control) with a small volume of the inoculum to give a concentration of 30 mg/L SS. No inoculum is added to Bottle 1, which serves as an abiotic control. Assemble the equipment, check that it is air-tight, start the stirrers, and start the measurement of oxygen uptake under conditions of darkness. Check the temperature, stirrer, and coulometric oxygen uptake recorder, and note any changes in color of the contents of the vessels on a daily basis. Read the oxygen uptake for the six bottles directly by an appropriate method, for example, from the 6–point chart recorder, which produces a BOD curve.

(ii) At the end of incubation, normally 28 days, measure the pH of the contents of the bottles and determine the concentration of the residual test substance and any intermediates and, in the case of water-soluble substances, the concentration of DOC (see paragraph (k)(2) of this guideline). Take special care in the case of volatile substances. If nitrification is anticipated, determine nitrate and nitrite ion concentrations, if possible.

(5) **Data and reporting—**(i) **Treatment of results—** (A) Data from the test should be entered on a data sheet, such as the Modified MITI Test Data Sheet following paragraph (n) of this guideline.

(B) Divide the oxygen uptake (in milligrams) in the presence of test substance after a given time, corrected for uptake by the inoculum control after the same time, by the weight of the test substance used (in milligrams). This yields the BOD expressed as milligrams of oxygen per milligram of mg test substance (TS), that is,

$$\text{BOD} = \frac{\text{mg } O_2 \text{ uptake with TS} - \text{mg } O_2 \text{ uptake by inoculum control}}{\text{mg test substance in bottle}} = \text{mg } O_2/\text{mg TS}$$

The percent biodegradation is then obtained as follows:

$$\%\ \text{biodegradation} = \%\ \text{ThOD} = \frac{\text{BOD (mg } O_2/\text{mg substance)}}{\text{ThOD (mg } O_2/\text{mg substance)}} \times 100$$

(C) For mixtures, calculate the ThOD from the elemental analysis, as for a single compound. Use the appropriate ThOD ($ThOD_{NH3}$ or $ThOD_{NO3}$) according to whether nitrification is absent or complete (see paragraph (k)(2)(ii) of this guideline). If, however, nitrification occurs but is incomplete, make a correction for the oxygen consumed by nitrification calculated from the changes in concentrations of nitrite and nitrate ions (see paragraph (k)(3) of this guideline).

(D) Calculate the percentage primary biodegradation from loss of specific (parent) chemical using the equation given under paragraph (k)(1)(ii) of this guideline. If there has been a loss of test substance in Bottle 1, (abiotic control), report this and use the concentration of test substance (S_b) after 28 days in this bottle to calculate percent biodegradation.

(E) When determinations of DOC are made (optional), calculate the percentage ultimate biodegradation at time t using the equation given under paragraph (l)(5)(i)(B) of this guideline. If there has been a loss of DOC in Bottle 1, measuring abiotic removal, use the DOC concentration in this vessel at day 28 to calculate the percent biodegradation.

(ii) **Validity of results.** (A)The oxygen uptake of the inoculum control is normally 20–30 mg O_2/L and should not be greater than 60 mg O_2/L in 28 days. Values higher than 60 mg/L require critical examination of the data and the experimental technique. If the pH value is outside the range 6–8.5 and the oxygen consumption by the test substance is less than 60 percent, the test could be repeated with a lower concentration of test substance.

(B) A test is considered valid if the greatest difference between replicate values of the removal of the test substance at the plateau or at the end of the test, as appropriate, is less than 20 percent, and if the percent degradation of aniline calculated from the oxygen consumption exceeds 40 percent after 7 days and 65 percent after 14 days. If either of these conditions is not met, the test should be repeated. Low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

(iii) **Test report.** The test report should include the information outlined under paragraph (k)(5) of this guideline. Data from the test should be entered on the data sheet. If a BOD curve (BOD vs. time) is available, it should be attached to the data sheet.

MODIFIED MITI TEST

DATA SHEET

LABORATORY: _____

DATE AT START OF TEST:_____

TEST SUBSTANCE:_____

Name:_____

Stock solution concentration (mg/L as chemical):_____

Initial concentration in medium (mg/L as chemical):_____

Volume of reaction mixture, V (mL):_____

ThOD (mg O_2/L):_____

INOCULUM:

Sludge sampling sites:

1. _____
2. _____
3. _____
4. _____
5. _____
6. _____
7. _____
8. _____
9. _____
10. _____

Concentration of SS in activated sludge after acclimation with synthetic sewage (mg/L): _____

Volume of sludge added per liter of final medium (mL):_____

Concentration of sludge in final medium (mg/L):_____

1. OXYGEN UPTAKE: BIODEGRADABILITY

Type of respirometer:_____

	Time (days)			
	t_1	t_2	t_3	t_x
O_2 uptake with test substance (mg)				
a_1	.....	.....	.....	.....
a_2	.....	.....	.....	.....
a_3	.....	.....	.....	.....
O_2 uptake by inoculum control (mg)				
b	.....	.....	.....	.....
Corrected O_2 uptake (mg)				
$a_1 - b$	.....	.....	.....	.....
$a_2 - b$	.....	.....	.....	.....
$a_3 - b$	.....	.....	.....	.....
BOD (mg O_2/mg test substance)				
$a_1 - b/C_0V$	.....	.....	.....	.....
$a_2 - b/C_0V$	.....	.....	.....	.....
$a_3 - b/C_0V$	.....	.....	.....	.....
% degradation (BOD/ThOD × 100)				
1	.....	.....	.....	.....
2	.....	.....	.....	.....
3	.....	.....	.....	.....
mean[1]	.....	.....	.....	.....

[1] Do not take a mean if there are considerable differences between replicates.

Note: Similar formats may be used for the other controls.

2. CARBON ANALYSIS: (optional)

Carbon analyzer used:_____

Flask	DOC				% DOC re-moved	Mean *n*
	Measured		Corrected			
Sludge + test substance	a_1	.....	$a_1 - b$	.....	.....	.....
Sludge + test substance	a_2	.....	$a_2 - b$	.....	.....	.....
Sludge + test substance	a_3	.....	$a_3 - b$	.....	.....	.....
Inoculum control	b	.....	–	.....	–	–
Water + test substance (abiotic control)	c	.....	.....	.....	–	–

$$\% \text{ DOC removed} = c - (a - b)/c \times 100$$

3. SPECIFIC CHEMICAL ANALYSIS

	residual amount of test substance at end of test	% primary degradation
blank test with water (abiotic control)	S_b	
	.....	.....
inoculated medium	S_{a1}	
	.....	.....
	S_{a2}	
	.....	.....
	S_{a3}	
	.....	.....

$$\% \text{ degradation} = S_b - S_a/S_b \times 100$$

Calculate percent primary degradation for bottles a_1, a_2, and a_3, respectively.

(o) **Closed bottle test**—(1) **General.** Matters concerning the assessment of biodegradability are discussed under paragraph (j) of this guideline and it is advisable to read this before proceeding. For this method, the formula of the substance and its purity, or relative proportions of major components, must be known so that the ThOD may be calculated. If the ThOD cannot be calculated the COD should be determined, but falsely high values of percent biodegradation may be obtained if the test substance is incompletely oxidized in the COD test. Insoluble and volatile substances may be assessed provided that precautions are taken. Degradation values for insoluble substances may be falsely low unless the bottles are agitated periodically during the incubation.

(2) **Principle of the test.** The solution of the test substance in mineral nutrient medium, usually at 2–5 mg/L, is inoculated with a relatively small number of microorganisms from a mixed population and kept in completely full, closed bottles in the dark at constant temperature (22 ± 2 °C). Degradation is followed by analysis of DO over a 28–day period. The amount of oxygen taken up by the microbial population during biodegradation of the test substance, corrected for uptake in the inoculum control run in parallel, is expressed as a percentage of ThOD or, less satisfactorily, COD.

(3) **Description of the method**—(i) **Apparatus.** In addition to normal laboratory equipment, the following apparatus should be available:

(A) BOD bottles with glass stoppers; e.g. 250– to 300–mL or 100– to 125–mL. It is important that the bottles are thoroughly cleaned before use. If the Winkler method for determining DO is used, it is sufficient to rinse the bottle several times with tap water followed by deionized water. However, if the electrode method is used, a more stringent cleaning procedure is required. Add to the empty bottle 5–10 mL of a wash solution (e.g. 2.5 g iodine plus 12.5 g KI per liter of 1 percent w/v H_2SO_4), shaking well to coat the bottle walls. Allow to stand for 15 min, pour off the solution, and rinse thoroughly with tap water and finally deionized water.

(B) Water bath or incubator for keeping bottles at constant temperature with the exclusion of light.

(C) Large glass bottles (2– to 5–L) for the preparation of media and filling the BOD bottles.

(D) Oxygen electrode and meter, or equipment and reagents for Winkler titration.

(ii) **Water.** A description of the water to be used is given under paragraph (j)(2) of this guideline.

(iii) **Stock solutions for mineral nutrient medium.** Prepare the stock solutions given under paragraph (l)(3)(iii) of this guideline, using analytical grade reagents.

(iv) **Preparation of mineral nutrient medium.** Add 1 mL of solutions (A), (B), (C) and (D) as prepared according to paragraph (l)(3)(iii) of this guideline to 800 mL water, mix, and then make up to 1 L.

(v) **Stock solution of test substance.** If the solubility of test or reference substance exceeds 1 g/L, dissolve 1–10 g, as appropriate, in water and make up to 1 L. Otherwise, prepare stock solutions in mineral nutrient medium or add the chemical directly to the nutrient medium making sure that the chemical dissolves.

(vi) **Inoculum.** (A) The inoculum is normally derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Collect and handle as described under paragraph (l)(3)(vi)(B)(*1*) of this guideline. Normally use from one drop (0.05 mL) to 5 mL of filtrate per liter of medium; trials may be needed to discover the optimum volume for a given effluent.

(B) An alternative source for the inoculum is surface water. In this case, collect a sample of an appropriate surface water, e.g. river or lake water, and keep aerobic until required. As with effluents, the optimum volume to be used as inoculum may have to be determined by trial tests.

(vii) **Preconditioning of inoculum.** If required, the inoculum may be preconditioned by aerating the secondary effluent, without other treatment or addition, for 5–7 days at the test temperature.

(viii) **Preparation of bottles.** (A) Strongly aerate mineral nutrient medium for at least 20 min and allow to stand. Generally, the medium is ready for use after standing for 20 h at the test temperature. Carry out each test series with medium derived from the same batch. Determine the concentration of DO for control purposes; the value should be about 9 mg/L at 20 °C. Conduct all transfer and filling operations of the air-saturated medium bubble-free, for example, by the use of siphons.

(B) Prepare parallel groups of BOD bottles for the determination of the test and reference substances in simultaneous experimental series. Assemble a sufficient number of BOD bottles, including inoculum controls, to allow at least duplicate measurements of oxygen consumption to be made at the desired test intervals, for example, after 0, 7, 14, 21 and 28 days. To ensure that the 10–day window can be identified, more bottles than this will probably be required.

(C) Add fully-aerated mineral nutrient medium to large bottles so that they are about one-third full. Then add sufficient amounts of the stock solutions of the test and reference substances or add by other means (see

paragraph (f)(2) of this guideline) to separate large bottles so that the final concentration of the chemicals is not greater than 10 mg/L (see paragraph (o)(3)(viii)(D) of this guideline). Do not add test or reference chemical to the inoculum control medium set up in a separate large bottle.

(D) In order to ensure that the inoculum activity is not limited, the concentration of DO must not fall below 0.5 mg/L in the BOD bottles. Generally, this limits the concentration of test substance to about 2 mg/L. An idea of the highest concentration to be used can be obtained from the ThOD (mg O_2/mg chemical) of the test substance. For poorly degradable compounds and those with a low ThOD, 5–10 mg/L can be used. In some cases, it would be advisable to run parallel series of test substance at two different concentrations, for example, 2 and 5 mg/L. Normally, calculate the ThOD on the basis of formation of ammonium salts, but if nitrification is expected or known to occur, calculate on the basis of the formation of nitrate (i.e. $ThOD_{NO3}$); (see paragraph (k)(2)(ii) of this guideline). However, if nitrification is not complete but does occur, correct for the changes in concentration of nitrite and nitrate as determined by analysis (see paragraph (k)(3) of this guideline)

(E) If the toxicity of the test substance is to be investigated (in the case, for example, of a low biodegradability value having been found previously), another series of bottles is necessary. Prepare another large bottle to contain aerated mineral nutrient medium (to about one-third of its volume) plus test and reference substances at final concentrations the same as those in the other large bottles.

(F) Inoculate the solutions in the large bottles with secondary effluent (one drop, or about 0.05 mL, to 5 mL/L (see paragraph (o)(3)(vi)(A) of this guideline) or with another source such as river water (see paragraph (o)(3)(vi)(B) of this guideline). Finally, make up the solutions to volume with aerated mineral nutrient medium using a hose that reaches down to the bottom of the bottle to achieve adequate mixing.

(ix) **Number of bottles.** (A) In a typical run the following bottles are used:

(*1*) At least 10 containing test substance and inoculum (test suspension).

(*2*) At least 10 containing only inoculum (inoculum control).

(*3*) At least 10 containing reference compound and inoculum (procedure control).

(*4*) When necessary, six bottles containing test and reference substances, and inoculum (toxicity control).

(B) About twice as many bottles are required to ensure the ability to identify the 10–day window as are needed otherwise.

(4) **Procedure.** (i) Dispense each prepared solution or suspension immediately into the respective group of BOD bottles by hose from the lower quarter (not the bottom) of the appropriate large bottle, so that all the BOD bottles are completely filled. When testing poorly soluble substances, added by methods described in paragraph (f)(2) of this guideline, ensure that the contents of the large bottles are well mixed by stirring. Tap gently to remove any air bubbles.

(ii) Analyze the 0–time bottles immediately for DO by the Winkler or electrode method. The contents of the bottles can be preserved for later analysis by the Winkler method by adding $MnSO_4$ and NaOH (the first Winkler reagent). Store the carefully stoppered bottles, containing the oxygen fixed as brown hydrated manganese(III) oxide, in the dark at 10–20 °C for no longer than 24 h before proceeding with the remaining steps of the Winkler method. Stopper the remaining replicate bottles ensuring that no air bubbles are enclosed, and incubate at 20 °C in the dark.

(iii) Each series must be accompanied by a complete parallel series for the determination of the inoculum control. Withdraw at least duplicate bottles of all series for DO analysis at time intervals (at least weekly) over the 28 days incubation. Weekly samples should allow the assessment of percent removal in a 14-day window, whereas sampling every 3–4 days should allow the 10–day window to be identified, and will require about twice as many bottles.

(iv) For nitrogen-containing test substances, corrections for uptake of oxygen by any nitrification occurring should be made. To do this, use the O_2-electrode method for determining the concentration of DO and then withdraw a sample from the BOD bottle for analysis for nitrite and nitrate. From the increase in concentration of nitrite and nitrate, calculate the oxygen used (see paragraph (k)(3) of this guideline)

(5) **Data and reporting**—(i) **Treatment of results.** (A) Data from the test should be entered on a data sheet, such as the Closed Bottle Test Data Sheet followimg paragraph (o) of this guideline.

(B) (*1*) First calculate the BOD exerted after each time period by subtracting the oxygen depletion (mg O_2/L) of the inoculum control from that exhibited by the test substance. Divide this corrected depletion by the concentration (mg/L) of the test substance, to obtain the specific BOD as milligrams of oxygen per milligrams of test substance:

$$\text{BOD} = \frac{(\text{mg } O_2\text{/L uptake by TS} - \text{mg } O_2\text{/L uptake by inoculum control})}{\text{mg TS/L in bottle}} = \frac{\text{mg } O_2}{\text{mg TS}}$$

(*2*) Calculate the percent biodegradation by dividing the specific BOD by the specific ThOD (calculated according to paragraph (k)(2)(ii) of this

guideline) or COD (determined by analysis; see paragraph (k)(2)(iii)), as follows:

$$\% \text{ degradation} = \frac{\text{BOD (mg } O_2\text{/mg TS)}}{\text{ThOD or COD (mg } O_2\text{/mg TS)}} \times 100$$

(C) It should be noted that the ThOD and COD methods do not necessarily give the same value; it is preferable to use the former method.

(D) For test substances containing nitrogen, use the appropriate ThOD (NH_4 or NO_3) according to what is known or expected about the occurrence of nitrification (see paragraph (k)(2)(ii) of this guideline). If nitrification occurs but is not complete, calculate a correction for the oxygen consumed by nitrification from the changes in concentration of nitrite and nitrate during the 28 days of the test (see paragraph (k)(3) of this guideline).

(ii) **Validity of results.** (A) Oxygen depletion in the inoculum control should not exceed 1.5 mg DO/L after 28 days. Values higher than this require investigation of the experimental techniques. The residual concentration of oxygen in the test bottles should not fall below 0.5 mg/L at any time. Such low oxygen levels are acceptable only if the method used for determining DO is sufficiently accurate.

(B) The other validity criteria given under paragraph (k)(4) of this guideline also apply.

(iii) **Test report.** The test report should include the information described under paragraph (k)(5) of this guideline. Data from the test should be entered on the data sheet.

CLOSED BOTTLE TEST

DATA SHEET

LABORATORY: _____

DATE AT START OF TEST:_____

TEST SUBSTANCE:_____

Name:_____

Stock solution concentration (mg/L as chemical):_____

Initial concentration in bottle (mg/L as chemical):_____

ThOD or COD (mg O_2/mg TS):_____

INOCULUM:

Source:_____

Treatment given:_____

Preconditioning, if any:_____

Concentration in reaction mixture (mg/L):_____

1. DO DETERMINATION

Method: Winkler/electrode

	flask no.		mg O_2/L after *t* days			
			0	t_1	t_2	t_x
Control—with inoculum but without TS	1	c_1	.....	.....	.....	.....
	2	c_2	.....	.....	.....	.....
	mean	$m_b = c_1 + c_2/2$	.....	.....	.....	.....
TS plus inoculum	1	a_1	.....	.....	.....	.....
	2	a_2	.....	.....	.....	.....

Note: Similar formats may be used for the procedure control and the toxicity controls, if any.

2. CORRECTION FOR NITRIFICATION (see paragraph (k)(3) of this guideline)

	Time of incubation (days)				
	0	t_1	t_2	t_3	t_x
(i) Concentration of nitrate (mg N/L)	–	.....	.....	.....	.....
(ii) Change in nitrate concentration (mg N/L)	–	.....	.....	.....	.....
(iii) Oxygen equivalent (mg/L)	.....	.....	.....	.....	.....
(iv) Concentration of nitrite (mg N/L)	.....	.....	.....	.....	.....
(v) Change in nitrite concentration (mg N/L)	–	.....	.....	.....	.....
(vi) Oxygen equivalent (mg/L)	–	.....	.....	–	.....
(iii+vi) Total oxygen equivalent (mg/L)	–	.....	.....	.....	.....

3. DO DEPLETION: % DEGRADATION (% D)

	DO depletion after *t* days (mg/L)			
	t_1	t_2	t_3	t_x
$(m_b - a_1)$[1]	.....	.....	.....	.....
$(m_b - a_2)$[1]	.....	.....	.....	.....
% $Da_1 = (m_b - a_1)$[1]/TS (mg/L) × ThOD × 100	.....	.....	.....	.....
% $Da_2 = (m_b - a_2)$[1]/TS (mg/L) × ThOD × 100	.....	.....	.....	.....
% D_{mean}[2] = $Da_1 + Da_2/2$	.....	.....	.....	.....

[1] This assumes that $m_{b(0)} = a_{1(0)} = a_{2(0)}$, where $m_{b(0)}$ = blank value at day 0; $a_{1(0)}$ = test substance value at day 0 in Bottle 1; $a_{2(0)}$ = test substance value at day 0 in Bottle 2. If $m_{b(0)}$ does not equal $a_{1(0)}$ or $a_{2(0)}$, use $[(a_{1(0)} - a_{1(x)}) - (m_{b(0)} - m_{b(x)})]$ and $[(a_{2(0)} - a_{2(x)}) - (m_{b(0)} - m_{b(x)})]$, where $m_{b(x)}$ = mean blank value at day x, $a_{1(x)}$ = TS value at day *x* in Bottle 1, and $a_{2(x)}$ = TS value at day *x* in Bottle 2.

[2] Do not take a mean if there are considerable differences between replicates.

4. INOCULUM CONTROL DO DEPLETIONS:

Oxygen consumption by control = $(m_{b(0)} - m_{b(28)})$ mg/L.

This consumption is important for the validity of the test and should be less than 1.5 mg/L. Apply any correction for nitrification.

(p) **Modified OECD screening test**—(1) **Introduction.** Matters of general interest concerning the assessment of biodegradability are discussed under paragraph (j) of this guideline, and it is advisable to read this before proceeding. For this method, the test substance should be non-volatile and have a solubility in water of at least 100 mg/L. Also, the carbon content and, preferably, the purity or relative proportions of major components must be known. This method is similar to the DOC die-away test under paragraph (l) of this guideline but employs a relatively low concentration of microorganisms.

(2) **Principle of the test.** A measured volume of mineral nutrient medium containing a known concentration of the test substance (10–40 mg DOC/L) as the nominal sole source of OC is inoculated with 0.5 mL effluent per liter of medium. The mixture is aerated in the dark or diffuse light at 22 ± 2 °C. Degradation is followed by DOC analysis at frequent intervals over a 28–day period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the inoculum control) as a percentage of the concentration initially present. Primary biodegradation may also be calculated from supplemental chemical analysis for the parent compound made at the beginning and end of incubation.

(3) **Description of the method**—(i) **Apparatus.** In addition to normal laboratory equipment, the following apparatus should be available:

(A) Conical flasks, e.g. 250–mL to 2–L, depending on the volume needed for DOC analysis. The flasks must be carefully cleaned with e.g. alcoholic hydrochloric acid, rinsed, and dried before each test.

(B) Shaking machine to accommodate the conical flasks, either with automatic temperature control or used in a constant temperature room, and of sufficient power to maintain aerobic conditions in all flasks.

(C) Filtration apparatus, with suitable membranes.

(D) DOC analyzer.

(E) Apparatus for determining DO, to confirm that the flask contents are aerobic.

(F) Centrifuge.

(ii) **Water.** A description of the water to be used is given under paragraph (j)(2) of this guideline.

(iii) **Stock solutions for mineral nutrient medium.** Prepare the same stock solutions as described in paragraph (l)(3)(iii) of this guideline, using analytical grade reagents.

(iv) **Preparation of mineral nutrient medium.** (A) Prepare medium as described in paragraph (l)(3)(iv) of this guideline.

(B) The OECD screening test uses only 0.5 mL effluent/L as inoculum and therefore the medium may need to be fortified with trace elements and growth factors. This is done by adding 1 mL each of the following solutions per liter of final medium:

(*1*) **Trace element solution.** Dissolve the following salts in water in a 1-L flask: 39.9 mg manganese sulfate tetrahydrate, $MnSO_4 \cdot 4H_2O$; 57.2 mg boric acid, H_3BO_3; 42.8 mg zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$; 34.7 mg ammonium heptamolybdate $(NH_4)_6Mo_7O_{24}$; and 100.0 mg iron(III)-chelate, (Fe[ethylenediaminetetraacetic acid]Cl_3) Make up to 1 L with water.

(*2*) **Vitamin solution.** Dissolve 15.0 mg of yeast extract in 100 mL of water. Sterilize by passage through a 0.2 μm pore-size membrane, or prepare immediately before use.

(v) **Stock solution of test substance.** Prepare stock solution as described in paragraph (l)(3)(v) of this guideline.

(vi) **Inoculum.** The inoculum is derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage and should be prepared as described in paragraph (l)(3)(vi)(B) of this guideline. Use 0.5 mL of the filtrate per liter of medium.

(vii) **Preconditioning of inoculum.** If required, the inoculum may be preconditioned as described in paragraph (j)(6) of this guideline.

(viii) **Preparation of flasks.** (A) Introduce 800–mL–portions of mineral nutrient medium into 2–L conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10–40 mg DOC/L. Check the pH value and adjust to pH 7.4 if necessary. Inoculate the flasks with sewage effluent at 0.5 mL/L medium. Prepare inoculum controls in the mineral nutrient medium but without test or reference substance.

(B) Toxicity, abiotic, and adsorption controls can be set up, if required, by following the same procedures as described under paragraphs (l)(3)(viii)(B) through (D) of this guideline.

(C) Make up the volumes in all flasks to 1 L with mineral nutrient medium and, after mixing, take a sample from each flask to determine the initial concentration of DOC in duplicate (see paragraph (k)(2)(iv) of this guideline). Cover with aluminium foil or other material in such a way as to allow free exchange of air between the flask and the surrounding atmosphere, then place the vessels in the shaking machine to start the test.

(ix) **Number of flasks.** In a typical run, the following flasks are used:

(A) Flasks 1 and 2—containing test substance and inoculum (test suspension).

(B) Flasks 3 and 4—containing only inoculum (inoculum control).

(C) Flask 5 containing reference compound and inoculum (procedure control).

(D) Flask 6—containing test substance and sterilizing agent (abiotic sterile control: Optional).

(E) Flask 7—containing test substance, inoculum, and sterilizing agent (adsorption control: Optional).

(F) Flask 8—containing test substance, reference compound and inoculum (toxicity control: Optional).

(4) **Procedure.** See paragraph (l)(4) of this guideline.

(5) **Data and reporting.** See paragraph (l)(5) of this guideline.

(q) **Manometric respirometry test**—(1) **General.** Matters concerning the assessment of biodegradability are discussed under paragraph (j) of this guideline and it is advisable to read this before proceeding. For this method, the formula of the test substance and its purity, or relative proportions of major components, must be known so that the ThOD may be calculated. If the ThOD cannot be calculated, the COD should be determined, but falsely high values of percentage biodegradation may be obtained if the test substance is incompletely oxidized in the COD test. Insoluble and volatile substances may be assessed provided precautions are taken.

(2) **Principle of the test.** A measured volume of inoculated mineral nutrient medium, containing a known concentration of test substance (100 mg TS/L giving at least 50–100 mg ThOD/L) as the nominal sole source of OC, is stirred in a closed flask at a constant temperature (±1 °C or better) for up to 28 days. The consumption of oxygen is determined either by measuring the quantity of oxygen (produced electrolytically) required to maintain constant gas volume in the respirometer flask, or from the change in volume or pressure (or a combination of the two) in the apparatus. Evolved CO_2 is absorbed in a solution of potassium hydroxide or other suitable absorbent. The amount of oxygen taken up by the microbial population during biodegradation of the test substance (corrected for uptake by the inoculum control, run in parallel) is expressed as a percentage of ThOD or, less satisfactorily, COD. Optionally, primary biodegradation may also be calculated from supplemental specific chemical analysis made at the beginning and end of incubation, and ultimate biodegradation may be determined by DOC analysis.

(3) **Description of the method**—(i) **Apparatus.** In addition to normal laboratory equipment, the following apparatus should be available:

(A) Suitable respirometer.

(B) Apparatus to maintain temperature at 22 ± 2 °C or better.

(C) Membrane filtration assembly (optional).

(D) Carbon analyzer (optional).

(ii) **Water.** A description of the water to be used is given under paragraph (j)(2) of this guideline.

(iii) **Stock solutions for mineral nutrient medium.** Prepare the same stock solutions as described in paragraph (l)(3)(iii) of this guideline, using analytical grade reagents.

(iv) **Preparation of mineral nutrient medium.** Prepare medium as described in paragraph (l)(3)(iv) of this guideline.

(v) **Stock solutions of test substances.** Prepare stock solutions as described in paragraph (l)(3)(v) of this guideline.

(vi) **Inoculum.** The inoculum may be derived from any of a variety of sources. See paragraph (l)(3)(vi) for details. If the inculum is derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage, (see paragraph (l)(3)(vi)(B) of this guideline), use only 0.5 mL of the filtrate per liter of medium.

(vii) **Preconditioning of inoculum.** If required, the inoculum may be preconditioned as described in paragraph (j)(6) of this guideline.

(viii) **Preparation of flasks.** (A) Prepare solutions of the test and reference substances in separate batches in mineral nutrient medium at 100 mg chemical/L (giving 50–100 mg ThOD/L), using the stock solutions. Calculate the ThOD on the basis of formation of ammonium salts unless nitrification is anticipated; in this case, the calculation should be based on nitrate formation (see paragraph (k)(2)(ii) of this guideline). Determine the pH values and if necessary adjust to 7.4 ± 0.2. Poorly soluble substances should be added at a later stage.

(B) If the toxicity of the test substance is to be determined, prepare a solution in mineral nutrient medium containing both test and reference substances at the same concentrations as in the individual solutions.

(C) If measurement of abiotic degradation is required, prepare a solution of the test substance in mineral nutrient medium at 100 mg ThOD/L, and sterilize by adding a toxic substance at an appropriate concentration. Abiotic controls are discussed in paragraph (j)(7) of this guideline.

(D) Introduce the requisite volume of solutions of test and reference substances into at least duplicate respirometer flasks. Add to additional, separate flasks mineral nutrient medium only (for inoculum controls) and, if required, the mixed test/reference substance solution and sterile solution of test substance. If the test substance is poorly soluble, add it directly at this stage on a weight or volume basis or handle it as described in paragraph (f)(2) of this guideline. Add potassium hydroxide, soda-lime pellets, or other absorbent to the CO_2-absorber compartments.

(ix) **Number of flasks.** In a typical run the following flasks are used:

(A) Flasks 1 and 2—containing test substance and inoculum (test suspension).

(B) Flasks 3 and 4—containing only inoculum (inoculum control).

(C) Flask 5—containing reference compound and inoculum (procedure control).

(D) Flask 6—containing test substance and sterilizing agent (abiotic sterile control).

(E) Flask 7—containing test and reference substances, and inoculum (toxicity control).

(4) **Procedure.** (i) Allow the vessels to reach the desired temperature and inoculate appropriate vessels with activated sludge or other inoculum to give a concentration of SS not greater than 30 mg/L. Assemble the equipment, start the stirrer, check that the equipment is air-tight, and start the measurement of oxygen uptake. Usually no further attention is required other than taking the necessary readings and making daily checks to see that the correct temperature and adequate stirring are maintained.

(ii) When an automatic respirometer is used, a continuous record of oxygen uptake is obtained so that the 10–day window is easily recognized. For nonautomatic respirometers daily readings will be adequate.

(iii) Calculate the oxygen uptake from the readings taken at regular and frequent intervals, using the methods given by the manufacturer of the equipment. At the end of incubation, normally 28 days, measure the pH of the contents of the flasks, especially if oxygen uptake is low or greater than $ThOD_{NH4}$, for nitrogen-containing chemicals.

(iv) If required, withdraw samples from the respirometer flasks initially and at the end of the experiment, for analysis of DOC (see paragraph (k)(2)(iv) of this guideline) and/or specific chemical. At the initial withdrawal, ensure that the volume of test suspension remaining in the flask is known. When oxygen is taken up by a nitrogen-containing test substance, determine the increase in concentration of nitrite and nitrate over

28 days and calculate the correction for the oxygen consumed by nitrification (see paragraph (k)(3) of this guideline).

(5) **Data and reporting**—(i) **Treatment of results.** (A) Data should be entered on a data sheet, such as the Manometric Respirometry Test Data Sheet following paragraph (q) of this guideline.

(B) (*1*) Calculate the BOD (mg O_2/mg test chemical) exerted after each time period by dividing the oxygen uptake (in milligrams) in the presence of the test chemical, corrected for uptake by the inoculum control, by the weight of the test chemical used in milligrams. That is,

$$\text{BOD} = \frac{\text{mg } O_2 \text{ uptake with TS} - \text{mg } O_2 \text{ uptake by inoculum control}}{\text{mg TS in vessel}}$$

(*2*) Calculate the percent biodegradation as follows:

$$\%\ \text{degradation} = \frac{\text{BOD (mg } O_2\text{/mg TS)}}{\text{ThOD or COD (mg } O_2\text{/mg TS)}} \times 100$$

(C) When optional determinations of specific chemical and/or DOC are made, calculate the percent degradation as described in paragraphs (k)(1)(ii) and (l)(5)(i)(B) of this guideline, respectively.

(ii) **Validity of results.** (A) The oxygen uptake of the inoculum control is normally 20–30 mg O_2/L and should not be greater than 60 mg/L in 28 days. Values higher than 60 mg/L require critical examination of the data and experimental technique. If the pH value is outside the range 6–8.5 and the oxygen consumption by the test substance is less than 60 percent, the test should be repeated with a lower concentration of test substance.

(B) The validity criteria given in paragraph (k)(4) of this guideline also apply.

(iii) **Test report.** The test report should include the information described in paragraph (k)(5) of this guideline. Data should be entered on the data sheet.

MANOMETRIC RESPIROMETRY TEST

DATA SHEET

LABORATORY: _____

DATE AT START OF TEST:_____

TEST SUBSTANCE:_____

Name:_____

Stock solution concentration (mg/L as chemical):_____

Initial concentration in medium (mg/L as chemical):_____

Volume in test flask, V (mL):_____

ThOD or COD (mg O_2/mg TS (NH_3, NO_3):_____

INOCULUM:

Source:_____

Treatment given:_____

Preconditioning, if any:_____

SS concentration in reaction mixture (mg/L):_____

1. OXYGEN UPTAKE, BIODEGRADABILITY

Type of respirometer:_____

		Time (days)				
		t_1	t_2	t_3	t_4	t_x
O_2 uptake with test substance (mg)	a_1	.....	.....	.....	.....	.....
.....	a_2	.....	.....	.....	.....	.....
O_2 uptake by inoculum control (mg)	b_1	.....	.....	.....	.....	.....
.....	b_2	.....	.....	.....	.....	.....
.....	b_{mean}	.....	.....	.....	.....	.....
Corrected O_2 uptake (mg)	$(a_1 - b_{mean})$	.....	.....	.....	.....	.....
.....	$(a_2 - b_{mean})$	.....	.....	.....	.....	.....
BOD (mg O_2/mg TS)	$(a_1 - b_{mean})$/mg TS	.....	.....	.....	.....	
.....	$(a_2 - b_{mean})$/mg TS	.....	.....	.....	.....	.....
% degradation, D (BOD/ThOD × 100) ...	$D_{1(a1)}$	.....	.....	.....	.....	.....
.....	$D_{2(a2)}$	.....	.....	.....	.....	.....
.....	mean[1]	.....	.....	.....	.....	.....

[1] D_1 and D_2 should not be averaged if there is a considerable difference between the values.

Note: Similar formats may be used for the other controls.

2. CORRECTION FOR NITRIFICATION (see paragraph (k)(3) of this guideline)

		Time of incubation (days)		
		0	28	difference
(i)	concentration of nitrate (mg N/L)	.....	.....	(N)
(ii)	oxygen equivalent (4.57 × N × V) (mg)	–	–	.....
(iii)	concentration of nitrite (mg N/L)	.....	.....	(N)
(iv)	oxygen equivalent (3.43 × N × V) (mg)	–	–	.....
(ii + iv)	total oxygen equivalent	–	–	.....

3. CARBON ANALYSIS (optional; see paragraph (l)(5)(i)(B))

Time (days)	Test chemical (mg/L)	Inoculum control (mg/L)
0	C_0	$C_{bl(0)}$
.....	.....	
28[1]	C_t	$C_{bl(t)}$
.....	.....	

[1] or at end of incubation

$$\% \text{ DOC removed} = [1 - (C_t - C_{bl(t)}/C_0 - C_{bl(0)})] \times 100$$

4. SPECIFIC CHEMICAL ANALYSIS (optional)

	residual amount of TS at end of test	% primary degradation
Sterile control	S_b	
.....	.....	
Inoculated test medium	S_a	$(S_b - S_a)/S_b \times 100$
.....	.....	

5. ABIOTIC DEGRADATION (optional)

a = O_2 consumption in sterile flasks at end of test (mg)

a/mg TS = O_2 consumption per milligram test chemical

$$\% \text{ abiotic degradation} = \frac{a}{\text{mg TS} \times \text{ThOD}} \times 100$$

(r) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Reynolds, L. et al. Evaluation of the toxicity of substances to be assessed for biodegradability. *Chemosphere* 16:2259 (1987).

(2) Boethling, R. Biodegradation testing of insoluble chemicals. *Environmental Toxicology and Chemistry* 3:5 (1984).

(3) de Morsier A. et al. Biodegradation tests for poorly soluble compounds. *Chemosphere* 16:833 (1987).

(4) Draft ISO Standard 10634. Water Quality, Evaluation in an aqueous medium of the ‘‘ultimate’’ biodegradability of low-soluble organic compounds (1990).

(5) Gerike P. The Biodegradability testing of poorly water soluble compounds. *Chemosphere* 13:169 (1984).

(6) American Public Health Association. Standard Methods for the Examination of Water and Wastewater, 12th ed, American Water Pollution Control Federation, Oxygen Demand, p 65 (1965).

(7) Deutsche Einheitsverfahren zur Wasser, Abwasser und Schlammuntersuchung, Summarische Wirkungs- und Stoffkenngrossen (Gruppe H). DIN 38 409 Teil 41. Bestimmung des Chemischen Sauerstofbedarfs (CSB) (H 41), Normenausschuss Wasserwesen (NAW) in DIN Deutsches Institut fur Normung e.V.

(8) Wagner R. *Vom Wasser* 46:139 (1976).

(9) Kelkenberg, H. *Zur Wasser und Abwasserforschung* 8:146 (1975).