OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Ready Biodegradability

INTRODUCTION

1. In this Guideline six methods are described that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium. They are:

301 A: DOC Die-Away
301 B: CO_2 Evolution (Modified Sturm Test)
301 C: MITI (I) (Ministry of International Trade and Industry, Japan)
301 D: Closed Bottle
301 E: Modified OECD Screening
301 F: Manometric Respirometry

Method 301 A is similar to the ISO Standard 7827-1984 and replaces the Modified AFNOR method; AFNOR has adopted the ISO standard. Methods 301 B, 301 D and 301 E are modified versions of the earlier OECD Guidelines adopted in 1981. Method 301 C is virtually identical with earlier Guideline 301 C (MITI I). Method 301 F is new; it is similar to 301 C differing mainly in the inocula employed.

2. Much experience has accumulated with the six methods over the years including an OECD inter-laboratory 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.

3. General considerations including those common to all six methods are given hereafter. Details of individual methods are given under separate headings (301 A to F). Throughout the text the reader is referred to the Annexes which contain definitions (Annex I), formulas and useful guidance material.

GENERAL PRINCIPLE OF THE TESTS

4. A solution, or suspension, of the test substance in a mineral 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 organic carbon 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 operation of the procedures.

5. 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 (301 C).

6. Normally, the test lasts for 28 days. Tests however 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 classed as readily biodegradable.

INFORMATION ON THE TEST SUBSTANCE

7. In order to select the most appropriate method, information on the chemical's solubility, vapour 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.

8. Information on the toxicity of the test substance to bacteria (Annex II) may be very useful for selecting appropriate test concentrations and may be essential for the correct interpretation of low biodegradation values.

APPLICABILITY AND SELECTION OF METHODS

9. Test substances which are soluble in water to at least 100 mg/l may be assessed by all methods, provided they are non-volatile and non-adsorbing. For those chemicals which 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 in Annex III, but in the MITI method neither solvents nor emulsifying agents are to be used. Moderately volatile chemicals may be tested by the DOC Die-Away method 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 which are:		
		poorly soluble	volatile	adsorbing
DOC Die-Away (301 A)	Dissolved organic carbon	-	-	+/-
CO_2 Evolution (301 B)	Respirometry: CO_2 evolution	+	-	+
MITI (I) (301 C)	Respirometry: oxygen consumption	+	+/-	+
Closed Bottle (301 D)	Respirometry: dissolved oxygen	+/-	+	+
Modified OECD Screening (301 E)	Dissolved organic carbon	-	-	+/-
Manometric Respirometry (301 F)	Oxygen consumption	+	+/-	+

PASS LEVELS

10. The pass levels for ready biodegradability are 70% removal of DOC and 60% of ThOD or $ThCO_2$ production for respirometric methods. They are lower in the respirometric methods since, as some of the carbon from the test chemical is incorporated into new cells, 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-d window within the 28-d period of the test, except where mentioned below. The 10-d window begins when the degree of biodegradation has reached 10% DOC, ThOD or $ThCO_2$ and must end before day 28 of the test. Chemicals which reach the pass levels after the 28-d period are not deemed to be readily biodegradable. The 10-d window concept does not apply to the MITI method. The value obtained in a 14-d window would be acceptable in the Closed Bottle method if it is considered that the number of bottles necessary to evaluate the 10-d window causes the test to become too unwieldy.

REFERENCE COMPOUNDS

11. In order to check the procedure, reference compounds which meet the criteria for ready biodegradability are tested by setting up an appropriate vessel in parallel as part of normal test runs. Suitable compounds are aniline (freshly distilled), sodium acetate and sodium benzoate. These reference compounds all degrade in these methods even when no inoculum is deliberately added. It was suggested that a reference compound should be sought which was readily biodegradable but required the addition of an inoculum. Potassium hydrogen phthalate has been proposed but more evidence needs to be obtained with this chemical before it can be accepted as a reference compound.

REPRODUCIBILITY OF TESTS

12. 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 micro-organisms 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.

GENERAL PROCEDURES AND PREPARATIONS

13. General conditions applying to the methods are summarised in Table 2. Apparatus and other experimental conditions pertaining specifically to an individual method are described later under the heading for that method.

Water

14. Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon 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 method, but the oxygen consumption of the water must be low (see 301 D, paragraph 25).

Mineral media

15. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate 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 (301 E), the medium for this test may need to be fortified with additional compounds. 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.

Methods of adding the test and reference substances

16. 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 medium to avoid diluting the buffer solution. Add substances which are even less soluble directly to the final mineral medium. Finally, refer to Annex III for the handling of poorly and insoluble substances, but note that in the MITI method (301 C) neither organic solvents nor emulsifying agents are to be used.

Inoculum

17. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents (unchlorinated); surface waters and soils; or from a mixture of these. For the DOC Die-Away (301 A), CO_2 Evolution (301 B) and Manometric Respirometry (301 F) methods if activated sludge is used, 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 Modified OECD Screening (301 E) and Closed Bottle (301 D)

methods, a more dilute inoculum without sludge flocs is needed and the preferred source is a secondary effluent from a domestic waste water treatment plant or laboratory-scale unit. For the MITI (I) method, 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.

Pre-conditioning of inoculum

18. Inoculum may be pre-conditioned to the experimental conditions, but not pre-adapted to the test substance. Pre-conditioning consists of aerating activated sludge (in mineral medium) or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test methods by reducing blank values. It is considered unnecessary to pre-condition MITI (I) inoculum.

Abiotic controls

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

Number of flasks and samples

20. 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 compounds plus inoculum and, when required, for toxicity, abiotic removal and adsorption controls. The Closed Bottle and MITI (I) methods have special requirements for the number of flasks. These are given under the specific headings. 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.

21. Although it is necessary to ensure that sufficient samples or readings are taken to allow the percentage removal in the 10-d window to be assessed, it is not possible to specify accurately the frequency of sampling because of the wide range of the lag phases and rates of degradation. In the MITI method (301 C) and, if an automatic respirometer is used in the Manometric Respirometry method (301 F), sampling for oxygen uptake presents no problems. In the latter method, daily readings are adequate when non-automatic respirometers are employed. Specific advice on sampling is given under the headings of the other four tests.

DATA AND REPORTING

Treatment of results

22. In the calculation of D_t, percentage 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 in the sections below on specific methods. The course of degradation is displayed graphically and the 10-d window is indicated where applicable. Calculate and report the percentage removal achieved and the value at the plateau, or at the end of the test, and/or at the end of the 10-d window, whichever is appropriate. In respirometric methods, N-containing chemicals may affect the oxygen uptake because of nitrification (see Annexes IV and V). Also, if the ThOD cannot be calculated because the test material is insufficiently defined, the COD value may be used to calculate the percentage 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 oxidised in the COD test, resulting in falsely high values for percentage biodegradation.

23. When specific chemical analytical data are available, calculate primary biodegradation from:

$$D_t = \frac{S_b - S_a}{S_b} \times 100$$

where:

D_t = % primary degradation at time t, normally 28 days;
S_a = residual amount of test chemical in inoculated medium at end of the test (mg);
S_b = residual amount of test chemical in the abiotic control at the end of the test (mg).

Validity of tests

24. A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage 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.

25. If in a toxicity test, containing both the test substance and a reference compound, less than 35% degradation (based on total DOC) or less than 25% (based on total ThOD or $ThCO_2$) occurred within 14 days, the test substance can be assumed to be inhibitory (see Annex II for other toxicity tests). 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 solids/l.

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

Test report

27. The test report must include the following:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Test conditions:

- inoculum: nature and sampling site(s), concentration and any pre-conditioning treatment;
- proportion and nature of industrial waste water in sewage, if known;
- test duration and temperature;
- in the case of poorly soluble test substances, methods of preparation of test solutions/suspensions;

- test method applied; scientific reasons and explanation for any change of procedure.

Results:

- data in tabular form;
- any observed inhibition phenomena;
- any observed abiotic degradation;
- specific chemical analytical data, if available;
- analytical data on intermediates, if available;
- the graph of percentage degradation against time for the test and reference substances, the lag phase, degradation phase, the 10-d window and slope (see Annex I for definitions);
- percentage removal at plateau, at end of test, and/or after 10-d window.

Discussion of results.

TABLE 2: TEST CONDITIONS

<table>
<tr><th>TEST</th><th>DOC DIE-AWAY</th><th>CO_2 EVOLUTION</th><th>MANOMETRIC RESPIROMETRY</th><th>MODIFIED OECD SCREENING</th><th>CLOSED BOTTLE</th><th>MITI (I)</th></tr>
<tr><td colspan="7">Concentrations of test substance:</td></tr>
<tr><td>mg/l</td><td></td><td></td><td>100</td><td></td><td>2 - 10</td><td>100</td></tr>
<tr><td>mg DOC/l</td><td>10 - 40</td><td>10 - 20</td><td></td><td>10 - 40</td><td></td><td></td></tr>
<tr><td>mg ThOD/l</td><td></td><td></td><td>50 - 100</td><td></td><td>5 - 10</td><td></td></tr>
<tr><td colspan="7">Concentration of inoculum:</td></tr>
<tr><td>mg/l SS</td><td colspan="3">≤ 30</td><td></td><td></td><td>30</td></tr>
<tr><td>ml effluent/l</td><td colspan="3">≤ 100</td><td>0.5</td><td>≤ 5</td><td></td></tr>
<tr><td>approx. cells/l</td><td colspan="3">$10^7 - 10^8$</td><td>10^5</td><td>$10^4 - 10^6$</td><td>$10^7 - 10^8$</td></tr>
<tr><td colspan="7">Concentration of elements in mineral medium (in mg/l):</td></tr>
<tr><td>P
N
Na
K
Mg
Ca
Fe</td><td colspan="4">116
1.3
86
122
2.2
9.9
0.05 - 0.1</td><td>11.6
0.13
8.6
12.2
2.2
9.9
0.05 - 0.1</td><td>29
1.3
17.2
36.5
6.6
29.7
0.15</td></tr>
<tr><td>pH</td><td colspan="5">7.4 ± 0.2</td><td>preferably 7</td></tr>
<tr><td>Temperature ° C</td><td colspan="5">22 ± 2</td><td>$25 \pm 1°$</td></tr>
</table>

DOC = Dissolved Organic Carbon   ThOD = Theoretical Oxygen Demand   SS = Suspended Solids

301 A "DOC DIE-AWAY TEST"

INTRODUCTION

1. Matters of general interest concerning the assessment of biodegradability are discussed in "General Procedures and Preparations" 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 should be known. This test is virtually the same as the ISO Standard 7827-1984. It is similar to the Modified OECD Screening test (301 E) but allows the use of much higher microbial cell densities.

PRINCIPLE OF THE TEST

2. A measured volume of inoculated mineral medium, containing a known concentration of the test substance (10-40 mg DOC/l) as the nominal sole source of organic carbon, 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 blank 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.

DESCRIPTION OF THE METHOD

Apparatus

3. Normal laboratory apparatus and:

(a) Conical flasks, e.g. 250 ml to 2 litre, 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 membranes;

(d) DOC analyser;

(e) Apparatus for determining dissolved oxygen, to check that the flask contents are aerobic;

(f) Centrifuge.

Water

4. A description of the water to be used is given in the "General Procedures and Preparations" (p. 5).

Stock solutions for mineral medium

5. Prepare the following stock solutions using analytical grade reagents:

(a) Potassium dihydrogen orthophosphate, KH_2PO_4 . . . . 8.50 g
Dipotassium hydrogen orthophosphate, K_2HPO_4 . . . . 21.75 g
Disodium hydrogen orthophosphate dihydrate,
$Na_2HPO_4.2H_2O$ . . . . 33.40 g
Ammonium chloride, NH_4Cl . . . . 0.50 g

Dissolve in water and make up to 1 litre.
The pH of the solution should be 7.4.

(b) Calcium chloride, anhydrous, $CaCl_2$ . . . . 27.50 g
or
Calcium chloride dihydrate, $CaCl_2.2H_20$ . . . . 36.40 g

Dissolve in water and make up to 1 litre.

(c) Magnesium sulphate heptahydrate, $MgS0_4.7H_20$ . . . . 22.50 g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate, $FeCl_3.6H_20$ . . . . 0.25 g

Dissolve in water and make up to 1 litre.

Note: In order to avoid having to prepare this solution immediately before use, add one drop of concentrated HCl or 0.4 g ethylene-diaminetetra-acetic acid (EDTA disodium salt) per litre.

If a precipitate forms in a stock solution replace with a freshly made solution.

Preparation of mineral medium

6. 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 litre with water.

Stock solutions of test substances

7. 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 litre. Otherwise, prepare stock solutions in mineral medium or add the chemical directly to the mineral medium, making sure it dissolves.

Inoculum

8. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters; soils; or from a mixture of these.

Inoculum from activated sludge

9. 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.

10. Alternatively, after removal of any coarse particles, settle or centrifuge (e.g. at 1100 g for 10 minutes). Discard the supernatant. The sludge may be washed in the mineral medium. Suspend the concentrated sludge in mineral medium to yield a concentration of 3-5 g suspended solids/l. Thereafter aerate until required.

11. 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 re-suspended sludge after thorough mixing, discard the supernatant and again re-suspend the washed sludge in a further volume of mineral medium. Repeat this procedure until the sludge is considered to be free from excess substrate or inhibitor.

12. After complete re-suspension is achieved, or with untreated sludge, withdraw a sample just before use for the determination of the dry weight of the suspended solids.

13. A further alternative is to homogenise activated sludge
(3-5 g suspended solids/l). Treat the sludge in a Waring blender for 2 minutes at medium speed. Settle the blended sludge for 30 minutes or longer if required and decant liquid for use as inoculum at the rate of about 10 ml/l of mineral medium.

Other sources of inoculum

14. 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 hour 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 litre of medium.

15. A further 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.

Pre-conditioning of inoculum

16. Inoculum may be pre-conditioned to the experimental conditions, but not pre-adapted to the test substance. Pre-conditioning consists of aerating activated sludge (in mineral medium) or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test method by reducing blank values.

Preparation of flasks

17. As an example, introduce 800 ml portions of mineral medium into 2-litre 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, if necessary, to 7.4. Inoculate the flasks with activated sludge or other source of inoculum to give a final concentration not greater than 30 mg suspended solids/l. Also prepare inoculum controls in the mineral medium but without test or reference substance.

18. If needed, use one vessel to check the possible inhibitory effect of the test substance by inoculating a solution containing comparable concentrations of both the test and a reference substance in the mineral medium.

19. Also, if required, check whether the test substance is degraded abiotically by setting up a flask containing a sterilised uninoculated solution of the substance. Sterilise by filtering though a membrane (0.2-0.45 μm) or by the addition of a suitable toxic substance at an appropriate concentration.

20. Additionally, 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, p. 4). Set up a flask containing the test substance, inoculum and sterilising agent.

21. Make up the volumes in all flasks to 1 litre with mineral medium and, after mixing, take a sample from each flask to determine the initial concentration of DOC in duplicate (see Annex IV.4). Cover the openings of the flasks, e.g with aluminium foil, in such a way as to allow free exchange of air between the flask and the surrounding atmosphere. To start the test, insert the vessels into the shaking machine.

Number of flasks

22. In a typical run, the following flasks are used:

Flasks 1 & 2	-	containing test substance and inoculum (test suspension);
Flasks 3 & 4	-	containing only inoculum (inoculum blank);
Flask 5	-	containing reference compound and inoculum (procedure control); and, preferably and when necessary, also
Flask 6	-	containing test substance and sterilising agent (abiotic sterile control);
Flask 7	-	containing test substance, inoculum and sterilising agent (adsorption control);
Flask 8	-	containing test substance, reference compound and inoculum (toxicity control).

PROCEDURE

DOC determinations

23. 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. This may, however, not always be possible.

Sampling

24. Take only the minimal volume of test suspension necessary for each determination. Before sampling make good 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 re-dissolved or re-suspended before sampling. Membrane-filter or centrifuge the sample (see Annex IV.4) immediately after it has been taken. Analyse the filtered or centrifuged samples on the same day, otherwise store at 2-4°C for a maximum of 48 h or below -18°C for a longer period.

Frequency of sampling

25. Ensure that a sufficient number of samples are taken to allow the percentage removal in the 10-d 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, take samples daily or every two days. Analyse the last samples (28 d) 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. Of course, if the last samples (28 d) show no degradation, no further samples need be analysed.

DATA AND REPORTING

Treatment of results

26. Data from the test should be entered onto the attached data sheet.

27. The percentage degradation (Dt) 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) in order that the validity of the test can be assessed (see "Data and Reporting", p. 7). It is calculated using the following equation:

$$D_t = \left[1 - \frac{C_t - C_{bl(t)}}{C_o - C_{bl(o)}}\right] \times 100$$

where:

D_t	=	% degradation at time t,
C_o	=	mean starting concentration of DOC in the inoculated culture medium containing the 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),
$C_{bl(o)}$	=	mean starting concentration of DOC in blank inoculated mineral medium (mg DOC/l),
$C_{bl(t)}$	=	mean concentration of DOC blank inoculated mineral medium at time t (mg DOC/l).

All concentrations are measured experimentally.

28. 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-d 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-d window, whichever are appropriate.

29. When specific chemical analytical data are available, calculate primary biodegradation (see "Data and Reporting", p. 7).

30. When a abiotic sterile control is used calculate the percentage abiotic degradation using:

$$\%\ abiotic\ degradation = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

where,

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

Validity of tests

31. The validity criteria apply given in "Data and Reporting" (p. 7).

Test report

32. The test report should include the information described in "Data and Reporting" (p. 8).

DOC DIE-AWAY TEST
DATA SHEET

1. **LABORATORY:**

2. **DATE AT START OF TEST:**

3. **TEST SUBSTANCE:**

    Name:
    Stock solution concentration: mg/l as chemical
    Initial concentration in medium, t_o: mg/l as chemical

4. **INOCULUM:**

    Source:
    Treatment given:
    Pre-conditioning, if any:
    Suspended solids concentration in reaction mixture: mg/l

5. **CARBON DETERMINATIONS:**

    Carbon analyser:

	Flask no.		DOC after n days (mg/l)				
			0	n_1	n_2	n_3	n_x
Test substance plus inoculum	1	a_1					
		a_2					
		mean, $C_{a(t)}$					
	2	b_1					
		b_2					
		mean, $C_{b(t)}$					
Blank, inoculum without test substance	3	c_1					
		c_2					
		mean, $C_{c(t)}$					
	4	d_1					
		d_2					
		mean, $C_{d(t)}$					
	mean, $C_{bl(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$						

6. **EVALUATION OF RAW DATA:**

Flask no.	Calculation of results	% Degradation after n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left[1 - \frac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right] \times 100$	0				
2	$D_2 = \left[1 - \frac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right] \times 100$	0				
Mean*	$D_t = \frac{D_1 + D_2}{2}$	0				

* D_1 and D_2 should not be averaged if there is a considerable difference.

Note: Similar formats may be used for the reference compound and toxicity controls.

7. **ABIOTIC DEGRADATION (optional)**

	Time (days)	
	0	t
DOC conc. (mg/l) in sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\% \ abiotic\ degradation = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. **SPECIFIC CHEMICAL ANALYSIS (optional)**

	residual amount of test chemical at end of test	% primary degradation
Sterile control	S_b	
Inoculated test medium	S_a	$\frac{S_b - S_a}{S_b} \times 100$

301 B CO_2 EVOLUTION TEST

INTRODUCTION

1. Matters of general interest concerning the assessment of biodegradability are discussed in "General Procedures and Preparations" and it is advisable to read this before proceeding. For this method, the test substance should be non-volatile and its carbon content and, preferably, its purity or relative proportions of major components should be known.

PRINCIPLE OF THE TEST

2. A measured volume of inoculated mineral medium, containing a known concentration of the test substance (10-20 mg DOC or TOC/l) as the nominal sole source of organic carbon is aerated by the passage of carbon dioxide-free air at a controlled rate in the dark or in diffuse light. Degradation is followed over 28 days by determining the carbon dioxide produced. The CO_2 is trapped in barium or sodium hydroxide and is measured by titration of the residual hydroxide or as inorganic carbon. The amount of carbon dioxide produced from the test substance (corrected for that derived from the blank inoculum) is expressed as a percentage of $ThCO_2$. The degree of biodegradation may also be calculated from supplemental DOC analysis made at the beginning and end of incubation.

DESCRIPTION OF THE METHOD

Apparatus

3. Normal laboratory apparatus and:

(a) Flasks, 2-5 litres, each fitted with an aeration tube reaching nearly to the bottom of the vessel and an outlet (the tube must not interfere with the 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 carbon dioxide scrubbing, for preparation of air which is free from carbon dioxide; alternatively, a mixture of CO_2-free oxygen and $C0_2$-free nitrogen, from gas cylinders, in the correct proportions ($20\%0_2$:$80\%N_2$) may be used;

(f) Device for determination of carbon dioxide, either titrimetrically or by some form of inorganic carbon analyser;

(g) Membrane filtration device (optional);

(h) DOC analyser (optional).

Water

4. A description of the water to be used is given in the "General Procedures and Preparations" (p. 5).

Stock solutions for mineral medium

5. Prepare the same stock solutions as detailed in 301 A (paragraph 5).

Preparation of mineral medium

6. Refer to 301 A (paragraph 6).

Stock solutions of test substances

7. Prepare in the same way as described in 301 A (paragraph 7).
For the handling of poorly soluble substances see Annex III.

Inoculum

8. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters; soils; or from a mixture of these.

Inoculum from activated sludge

9. Refer to 301 A (paragraphs 9 to 13) for details on the collection and preparation of inoculum from activated sludge.

Other sources of inoculum

10. 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. Refer to 301 A (paragraphs 14 and 15) for details.

Pre-conditioning of inoculum

11. Refer to 301 A (paragraph 16).

Preparation of flasks

12. As an example, the following volumes and weights indicate the values for 5-litre flasks containing 3 litres of suspension. If smaller volumes are used modify the values accordingly, but ensure that the carbon dioxide formed can be measured accurately. To each 5-litre flask add 2,400 ml mineral medium. Add an appropriate volume of the prepared activated sludge to give a concentration of suspended solids of not more than 30 mg/l in the final 3 litres of inoculated mixture. Alternatively, first dilute the prepared sludge to give a suspension of 500-1000 mg/l in the mineral medium before adding an aliquot to the contents of the 5-litre flask to attain a concentration of 30 mg/l; this ensures greater precision (other sources of inoculum may be used, see paragraphs 14 and 15 of 301 A). Aerate these inoculated mixtures with $C0_2$-free air overnight to purge the system of carbon dioxide.

13. Add the test material and reference compound, separately, as known volumes of stock solutions, to replicate flasks to yield concentrations, contributed by the added chemicals, of 10 to 20 mg DOC or TOC/l; 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 in Annex III. Make up the volumes of suspensions in all flasks to 3 litres by the addition of mineral medium previously aerated with CO_2-free air.

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

15. Also, if required, check whether the test substance is degraded abiotically by using a sterilised uninoculated solution of the chemical. Sterilise by the addition of a toxic substance at an appropriate concentration.

16. If barium hydroxide is used, connect three absorption bottles, each containing 100 ml of 0.0125 M barium hydroxide solution, in series to each 5-litre flask. The solution must be free of precipitated sulphate and carbonate and its strength must be determined immediately before use. If sodium hydroxide is used, connect two traps, the second acting as a control to demonstrate that all the carbon dioxide was absorbed in the first. Absorption bottles fitted with serum bottle closures are suitable. Add 200 ml 0.05 M sodium hydroxide to each bottle. This is sufficient to absorb the total quantity of carbon dioxide evolved when the test substance is completely degraded. The sodium hydroxide solution, even when freshly prepared, will contain traces of carbonates; this is corrected by deduction of the carbonate in the blank.

17. Optionally, samples may be withdrawn for analysis of DOC (see Annex IV.4) and/or specific chemical analysis.

Number of flasks

18. In a typical run, the following flasks are used:

Flasks 1 & 2	-	containing test substance and inoculum (test suspension);
Flasks 3 & 4	-	containing only inoculum (inoculum blank);
Flask 5	-	containing reference compound and inoculum (procedure control); and, preferably and when necessary, also
Flask 6	-	containing test substance and sterilising agent (abiotic sterile control);
Flask 7	-	containing test substance, reference compound and inoculum (toxicity control).

PROCEDURE

19. Start the test by bubbling $C0_2$-free air through the suspensions at a rate of 30-100 ml/min.

CO_2 determinations

20. It is mandatory to follow the CO_2 evolution from the test suspensions and inoculum blanks in parallel and it is advisable to do the same for the other test vessels.

21. During the first ten days it is recommended that analyses of CO_2 should be made every second or third day and then at least every fifth day until the 28th day so that the 10-d window period can be identified. On the days of $C0_2$ measurement, disconnect the barium hydroxide 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 barium hydroxide at the far end of the series. Make titrations as needed, for example, when substantial precipitation is seen in the first trap and before any is evident in the second, or at least weekly. Alternatively, with Na0H as absorbent, withdraw a sample of the

sodium hydroxide solution from the absorber nearest to the test vessel using a syringe. The sample volume needed will depend on the carbon analyser used, but sampling should not significantly change the absorbent volume over the test period. Inject the sample into the IC part of the carbon analyser for analysis of evolved carbon dioxide directly. Analyse the contents of the second trap only at the end of the test in order to correct for any carry over of carbon dioxide.

22. On the 28th day withdraw samples, optionally, for DOC and/or specific chemical analysis. Add 1 ml of concentrated hydrochloric acid to each test vessel and aerate them overnight to drive off the carbon dioxide present in the test suspensions. On day 29 make the last analysis of evolved carbon dioxide.

DATA AND REPORTING

Treatment of results

23. Data from the test should be entered onto the attached data sheet.

24. 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 would be needed to titrate 100 ml $Ba(OH)_2$).

25. Since 1 mmol of CO_2 is produced for every mmol of $Ba(OH)_2$ reacted to $BaCl_2$ and 2 mmol of HCl are needed for the titration of the remaining $Ba(OH)_2$, and given that the molecular weight of CO_2 is 44 g, the weight of CO_2 produced (mg) is calculated by:

$$\frac{0.05 \times (50 - ml\ HCl\ titrated) \times 44}{2} = 1.1 \times (50 - ml\ HCl\ titrated)$$

Thus, in this case, the factor to convert volume of HCl titrated to mg 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,

CO_2 from inoculum = 1.1 x (50-48) = 2.2 mg
CO_2 from inoculum plus test substance = 1.1 x (50-45) = 5.5 mg

and thus the weight of CO_2 produced from the test substance is 3.3 mg.

26. The percentage biodegradation is calculated from:

$$\%\ degradation = \frac{mg\ CO_2\ produced}{ThCO_2 \times mg\ test\ substance\ added} \times 100$$

or,

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

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

27. When NaOH is used as the absorbent, calculate the amount of CO_2 produced after any time interval from the concentration of inorganic carbon and the volume of absorbent used.
Calculate the percentage degradation from:

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

28. Display the course of degradation graphically and indicate the 10-d 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-d window, whichever is appropriate.

29. When appropriate, calculate DOC removals using the equation given in 301 A (paragraph 27).

30. When an abiotic control is used, calculate the percentage abiotic degradation by:

$$\% \; abiotic\ degradation = \frac{CO_2\ produced\ by\ sterile\ flask\ after\ 28d\ (mg)}{ThCO_2\ (mg)} \times 100$$

Validity of tests

31. The IC content of the test substance suspension in the mineral medium at the beginning of the test must be less than 5% of the TC, and the total $C0_2$ evolution in the inoculum blank 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.

32. The other validity criteria given in "Data and Reporting (p. 7) also apply.

Test report

33. The test report should include the information described in "Data and Reporting" (p. 8).

CO_2 EVOLUTION TEST
DATA SHEET

1. **LABORATORY:**

2. **DATE AT START OF TEST:**

3. **TEST SUBSTANCE:**

Name:
Stock solution concentration: mg/l as chemical
Initial conc. in medium: mg/l as chemical
Total C added to flask: mg C
$ThCO_2$: mg CO_2

4. **INOCULUM:**

Source:
Treatment given:
Pre-conditioning, if any:
Suspended solids concentration in reaction mixture: mg/l

5. **CO_2 PRODUCTION AND DEGRADABILITY:**

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

Time (day)	CO_2 produced (mg)					Cumulative CO_2 produced (mg) test - blank mean		% $ThCO_2$ cumulative CO_2 x 100 / $ThCO_2$		
	Test chemical		Blank							
	Flask 1	Flask 2	Flask 3	Flask 4	Mean	Flask 1	Flask 2	Flask 1	Flask 2	Mean*
0										
n_1										
n_2										
n_3										
n_4										
28										

Note: Similar formats may be used for the reference and toxicity controls.

* Do not take mean if there is a considerable difference between replicates.

6. **CARBON ANALYSIS (optional):**

Carbon analyser:

Time (day)	Test chemical (mg/l)	Blank (mg/l)
0	(C_o)	($C_{bl(0)}$)
28*	(C_t)	($C_{bl(t)}$)

* or at end of incubation

$$\% \text{ DOC removed} = \left[1 - \frac{C_t - C_{bl(t)}}{C_o - C_{bl(o)}}\right] \times 100$$

7. **ABIOTIC DEGRADATION (optional)**

$$\% \text{ abiotic degradation} = \frac{CO\ 2 \text{ formation in sterile flask after 28 d (mg)}}{ThCO_2 \text{ (mg)}} \times 100$$

301 C MODIFIED MITI TEST (I)

INTRODUCTION

1. Matters of general interest concerning the assessment of biodegradability are discussed in "General Procedures and Preparations" 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, should 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.

PRINCIPLE OF THE TEST

2. The oxygen uptake by a stirred solution, or suspension, of the test substance in a mineral medium, inoculated with specially grown, unadapted micro-organisms, is measured automatically over a period of 28 days in a darkened, enclosed respirometer at 25 ± 1°C. Evolved carbon dioxide is absorbed by soda lime. Biodegradation is expressed as the percentage oxygen uptake (corrected for blank uptake) of 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 by DOC analysis.

DESCRIPTION OF THE METHOD

Apparatus

3. Normal laboratory apparatus and:

(a) Automatic electrolytic BOD meter or respirometer normally equipped with 6 bottles, 300 ml each and equipped with cups to contain CO_2 absorbent;

(b) Constant temperature room and/or water-bath at 25°C ± 1°C or better;

(c) Membrane-filtration assembly (optional);

(d) Carbon analyser (optional).

Water

4. A description of the water to be used is given in the "General Procedures and Preparations" (p. 5).

Stock solutions for mineral medium

5. Prepare the following stock solutions, using analytical grade reagents:

(a) Potassium dihydrogen orthophosphate, KH_2PO_4 . . . . 8.50 g
Dipotassium hydrogen orthophosphate, K_2HPO_4 . . . . 21.75 g
Disodium hydrogen orthophosphate
dodecahydrate, $Na_2HPO_4.12H_2O$ . . . . 44.60 g

Ammonium chloride, NH_4Cl . . . . 1.70 g

Dissolve in water and make up to 1 litre.
The pH value of the solution should be 7.2.

(b) Magnesium sulphate heptahydrate, $MgS0_4.7H_20$ . . . . 22.50 g

Dissolve in water and make up to 1 litre.

(c) Calcium chloride anhydrous, $CaCl_2$ . . . . 27.50 g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate, $FeCl_3.6H_20$ . . . . 0.25 g

Dissolve in water and make up to 1 litre. The pH of the solution should be 7.2.

Preparation of mineral medium

6. Take 3 ml of each solution (a), (b), (c) and (d) and make up to 1 litre.

Stock solutions of test substances

7. When the solubility exceeds 1 g/l dissolve 1-10 g, as appropriate, of test or reference substance in water and make up to 1 litre. Otherwise prepare stock solutions in the mineral medium or add the chemical directly to the mineral medium. For the handling of poorly soluble substances, see paragraph 11.

Preparation of inoculum

8. Collect fresh samples from no fewer than ten sites, mainly in areas where a variety of chemicals are used and discharged. From sites such as sewage treatment works, industrial waste-water treatment works, rivers, lakes, seas, collect 1 litre samples of sludge, surface soil, water, etc. and mix thoroughly together.

9. After removing floating matter and allowing to stand, adjust the supernatant to pH 7 ± 1 with sodium hydroxide or phosphoric acid. Use an appropriate volume of the filtered supernatant to fill a fill-and-draw activated sludge vessel and aerate the liquid for about 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 % each of glucose, peptone and potassium orthophosphate, to the settled material and re-commence aeration. Repeat this procedure once per day.

10. The sludge unit must be operated according to good practice: effluents should be clear; temperature should be kept at 25 ± 2°C and at pH 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 three months. Do not use sludge as inoculum until after at least one month's operation, but not after more than four months. Thereafter, sample from at least 10 sites at regular intervals, once every three 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 ten-source mixture and culture the combined liquor as above. Take sludge for use as inoculum 18-24 h after the unit has been fed.

Preparation of bottles

11. Prepare the following six bottles:

Bottle 1	-	test substance in water at 100 mg/l;
Bottles 2, 3 and 4	-	test substance in mineral medium at 100 mg/l;
Bottle 5	-	reference compound (e.g. aniline) in mineral medium at 100 mg/l;
Bottle 6	-	mineral medium only.

Add poorly soluble test substances directly on a weight or volume basis or handle as described in Annex III except that neither solvent nor emulsifying agent should be used. Add the $C0_2$ 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.

PROCEDURE

12. Inoculate vessels 2, 3 and 4 (test suspensions), 5 (activity control) and 6 (inoculum blank) with a small volume of the inoculum to give a concentration of 30 mg/l suspended solids. 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 colour of the contents of the vessels on a daily basis. Read the oxygen uptakes for the six bottles directly by an appropriate method, for example, from the six-point chart recorder, which produces a BOD curve.

13. 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 (Annex IV.4). Take special care in the case of volatile substances. If nitrification is anticipated, determine nitrate and nitrite concentrations, if possible.

DATA AND REPORTING

Treatment of results

14. Data from the test should be entered onto the attached data sheet.

15. Divide the oxygen uptake (mg) by the test substance (mg) after a given time, corrected for that taken up by the blank inoculum control after the same time, by the weight of the test substance used. This yields the BOD expressed as mg oxygen/mg test substance, that is,

$$BOD = \frac{mg\ O_2\ uptake\ by\ test\ substance\ -\ mg\ O_2\ uptake\ by\ blank}{mg\ test\ substance\ in\ vessel} = mg\ O_2/mg\ test\ substance$$

The percentage biodegradation is then obtained from:

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

16. For mixtures, calculate the ThOD from the elemental analysis, as for a single compound. Use the appropriate ThOD ($ThOD_{NH4}$ or $ThOD_{NO3}$) according to whether nitrification is absent or complete (Annex IV.2). 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 (Annex V).

17. Calculate the percentage primary biodegradation from loss of specific (parent) chemical using the equation given in "Data and Reporting" (p. 7). If there has been a loss of test substance in Bottle 1, measuring abiotic removal, report this and use the concentration of test substance (S_b) after 28 days in this bottle to calculate percentage biodegradation.

18. When determinations of DOC are made (optional), calculate the percentage ultimate biodegradation at time t using the equation given in 301 A (paragraph 27). 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 percentage biodegradation.

Validity of results

19. The oxygen uptake of the inoculum blank is normally 20-30 mg 0_2/l and should not be greater than 60 mg 0_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%, the test could be repeated with a lower concentration of test substance.

20. A test is considered valid if the difference of extremes of 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% and if the percentage degradation of aniline calculated from the oxygen consumption exceeds 40% after 7 days and 65% 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.

Test report

21. The test report should include the information outlined in "Data and Reporting" (p. 8).

MODIFIED MITI TEST (I)
DATA SHEET

1. **LABORATORY:**

2. **DATE AT START OF TEST:**

3. **TEST SUBSTANCE:**

Name:	
Stock solution concentration:	mg/l as chemical
Initial concentration in medium, C_o:	mg/l as chemical
Volume of reaction mixture, V:	ml
ThOD:	mg O_2/l

4. **INOCULUM:**

Sludge sampling sites:

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

Concentration of suspended solids in activated sludge after acclimation with synthetic sewage = mg/l

Volume of sludge added/litre of final medium = ml

Concentration of sludge in final medium = mg/l

5. **OXYGEN UPTAKE: BIODEGRADABILITY**

Type of respirometer used:

	Time (days)			
	n_1	n_2	n_3	n_x
O_2 uptake by test substance (mg): a1 a2 a3				
O_2 uptake by blank (mg): b				
Corrected O_2 uptake (mg): a1-b a2-b a3-b				
BOD (mgO_2/mg test substance): $(a1-b)/C_oV$ $(a2-b)/C_oV$ $(a3-b)/C_oV$				
% degradation BOD/ThOD x 100: 1 2 3 mean*				

Note: Similar formats may be used for the reference compound

* Do not take a mean if there are considerable differences between replicates

6. **CARBON ANALYSIS (OPTIONAL)**

Carbon analyser:

Flask	**DOC**				**%DOC removed**	**Mea n**
	Measured		**Corrected**			
Water + test substance	a				-	-
Sludge + test substance	b1		b1-c			
Sludge + test substance	b2		b2-c			
Sludge + test substance	b3		b3-c			
Control blank	c		-		-	-

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

7. SPECIFIC CHEMICAL ANALYSIS

	residual amount of test substance at end of test	% primary degradation
Blank test with water	Sb	
Inoculated medium	Sa1 Sa2 Sa3	

$$\% \ degradation = \frac{Sb - Sa}{Sb} \times 100$$

Calculate % primary degradation for bottles a1, a2, and a3 respectively.

8. REMARKS

BOD curve against time, if available, should be attached.

301 D CLOSED BOTTLE TEST

INTRODUCTION

1. Matters of general interest concerning the assessment of biodegradability are discussed in "General Procedures and Preparations" 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, should 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 oxidised 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.

PRINCIPLE OF THE TEST

2. The solution of the test substance in mineral medium, usually at 2-5 mg/l, is inoculated with a relatively small number of micro-organisms from a mixed population and kept in completely full, closed bottles in the dark at constant temperature. Degradation is followed by analysis of dissolved oxygen over a 28-d period. The amount of oxygen taken up by the microbial population during biodegradation of the test substance, corrected for uptake by the blank inoculum run in parallel, is expressed as a percentage of ThOD or, less satisfactorily COD.

DESCRIPTION OF THE METHOD

Apparatus

3. Normal laboratory apparatus and:

(a) BOD bottles, with glass stoppers e.g. 250-300 ml or 100-125 ml;

It is important that the bottles are thoroughly clean before use. If the Winkler method for determining dissolved oxygen is used, it is sufficient to rinse the bottle several times with tap water then deionised 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 potassium iodide per litre of 1% w/v sulfuric acid) shaking well to coat the bottle walls. Leave to stand for 15 min., pour off the solution and rinse thoroughly with tap water and finally deionised water.

(b) Water bath or incubator, for keeping bottles at constant temperature ($\pm$ 1°C/or better) with the exclusion of light;

(c) Large glass bottles (2-5 litres) for the preparation of media and for filling the BOD bottles;

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

Water

4. A description of the water to be used is given in "General Procedures and Preparations" (p. 5).

Stock solutions for mineral medium

5. Prepare the following stock solutions, using analytical grade reagents:

(a) Potassium dihydrogen orthophosphate, KH_2PO_4 . . . . 8.50 g
Dipotassium hydrogen orthophosphate, K_2HPO_4 . . . . 21.75 g
Disodium hydrogen orthophosphate dihydrate,
$Na_2HPO_4.2H_2O$ . . . . 33.40 g
Ammonium chloride, NH_4Cl . . . . 0.50 g

Dissolve in water and make up to 1 litre.
The pH of the solution should be 7.4

(b) Calcium chloride, anhydrous, $CaCl_2$ . . . . 27.50 g
or
Calcium chloride dihydrate, $CaCl_2.2H_20$ . . . . 36.40 g

Dissolve in water and make up to 1 litre.

(c) Magnesium sulphate heptahydrate, $MgS0_4.7H_20$ . . . . 22.50 g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate, $FeCl_3.6H_20$ . . . . 0.25 g

Dissolve in water and make up to 1 litre.

Note: In order to avoid having to prepare this solution immediately before use, add one drop of concentrated HCl or 0.4 g ethylene-diaminetetra-acetic acid (EDTA) (disodium salt) per litre.

If a precipitate forms in a stock solution, replace it with a freshly made solution.

Preparation of mineral medium

6. Mix 1 ml of solutions (a), (b), (c) and (d) with 800 ml water and then make up to 1 litre.

Stock solutions of test substances

7. If the solubility exceeds 1 g/l, dissolve 1-10 g, as appropriate, of test or reference substance in water and make up to 1 litre. Otherwise, prepare stock solutions in mineral medium or add the chemical directly to the mineral medium making sure that the chemical dissolves.

Inoculum

8. 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 in 301 A (paragraph 14). Normally use from one drop (0.05 ml) to 5 ml of filtrate per litre of medium; trials may be needed to discover the optimum volume for a given effluent.

9. An alternative 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. As with effluents, the optimum volume to be used as inoculum may have to be determined by trial tests.

Pre-conditioning of inoculum

10. If required, the inoculum may be pre-conditioned by aerating the secondary effluent, without other treatment or addition, for 5-7 days at the test temperature.

Preparation of bottles

11. Strongly aerate mineral medium for at least 20 minutes 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 mineral medium derived from the same batch. Determine the concentration of dissolved oxygen 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.

12. 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 blanks, 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 d. To ensure that the 10-day window could be identified, more bottles would be required.

13. Add fully-aerated mineral medium to large bottles so that they are about one-third full. Then add sufficient of the stock solutions of the test and reference substances (or add by other means, see Annex III) to separate large bottles so that the final concentration of the chemicals is normally not greater than 10 mg/l (see paragraph 14 below). Add no chemicals to the blank control medium contained in a further large bottle.

14. In order to ensure that the inoculum activity is not limited, the concentration of dissolved oxygen must not fall below 0.5 mg/l in the BOD bottles. This limits the concentration of test substance in general to about 2 mg/l. An idea of the highest concentration to be used can be obtained from the ThOD (mg 0_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 $ThOD_{NO3}$ (see Annex IV.2). However, if nitrification is not complete but does occur, correct for the changes in concentration of nitrite and nitrate, determined by analysis (see Annex V).

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

16. Inoculate the solutions in the large bottles with secondary effluent (one drop, or about 0.05 ml, to 5 ml/l, see paragraph 8) or with another source such as river water (see paragraph 9). Finally, make up the solutions to volume with aerated mineral medium using a hose which reaches down to the bottom of the bottle to achieve adequate mixing.

Number of bottles

17. In a typical run the following bottles are used:

- at least 10 containing test substance and inoculum (test suspension);
- at least 10 containing only inoculum (inoculum blank);
- at least 10 containing reference compound and inoculum (procedure control), and, when necessary,

- 6 bottles containing test substance, reference compound and inoculum (toxicity control).

However, to ensure being able to identify the 10-d window about twice as many bottles would be necessary.

PROCEDURE

18. 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 Annex III, ensure that the contents of the large bottles are well mixed by stirring. Tap gently to remove any air bubbles.

19. Analyse the zero-time bottles immediately for dissolved oxygen by the Winkler or electrode methods. The contents of the bottles can be preserved for later analysis by the Winkler method by adding manganese (II) sulphate and sodium hydroxide (the first Winkler reagent). Store the carefully stoppered bottles, containing the oxygen fixed as a brown manganese (III) hydrated 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.

20. Each series must be accompanied by a complete parallel series for the determination of the inoculated blank medium. Withdraw at least duplicate bottles of all series for dissolved oxygen analysis at time intervals (at least weekly) over the 28 days incubation. Weekly samples should allow the assessment of percentage removal in a 14-d window, whereas sampling every 3-4 days should allow the 10-d window to be identified, and would require about twice as many bottles.

21. For N-containing test substances, corrections for uptake of oxygen by any nitrification occurring should be made. To do this, use the 0_2-electrode method for determining the concentration of dissolved oxygen 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 Annex V).

DATA AND REPORTING

Treatment of results

22. Data from the test should be entered onto the attached data sheet.

23. First calculate the BOD exerted after each time period by subtracting the oxygen depletion (mg 0_2/l) of the inoculum blank 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 mg oxygen per mg test substance. Calculate the percentage biodegradation by dividing the specific BOD by the specific ThOD (calculated according to Annex IV.2) or COD (determined by analysis, see Annex IV.3). Thus:

$$BOD = \frac{mg\ O_2/l\ uptake\ by\ test\ substance\ -\ O_2/l\ uptake\ by\ blank}{mg\ test\ substance/l\ in\ vessel} = mg\ O_2/mg\ test\ substance$$

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

or

$$\% \text{ degradation} = \frac{BOD \ (mg \ O_2/mg \ test \ substance)}{COD \ (mg \ O_2/mg \ test \ substance)} \times 100$$

It should be noted that these two methods do not necessarily give the same value; it is preferable to use the former method.

24. For test substances containing nitrogen, use the appropriate ThOD (NH_4 or $N0_3$) according to what is known or expected about the occurrence of nitrification (Annex IV.2). 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 d of the test (Annex V).

Validity of results

25. Oxygen depletion in the inoculum blank should not exceed 1.5 mg dissolved oxygen/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 of determining dissolved oxygen used is capable of measuring such levels accurately.

26. The other validity criteria given in the "Data and Reporting" (p. 7) also apply.

Test report

27. The test report should include the information described in the "Data and Reporting" (p. 8).

CLOSED BOTTLE TEST
DATA SHEET

1. **LABORATORY:**

2. **DATE AT START OF TEST:**

3. **TEST SUBSTANCE:**

   Name:
   Stock solution concentration: mg/l
   Initial concentration in bottle: mg/l
   ThOD or COD: mg O_2/mg test substance

4. **INOCULUM:**

   Source:
   Treatment given:
   Pre-conditioning, if any:
   Concentration in reaction mixture: ml/l

5. **DO DETERMINATION:**

   Method: Winkler/electrode

	Flask no.		mg O_2/l after n days			
			0	n_1	n_2	n_x
Blank - with inoculum but without test substance	1	c_1				
	2	c_2				
	Mean blank	$m_b = \frac{C_1 + C_2}{2}$				
Test substance plus inoculum	1	a_1				
	2	a_2				

Note: Similar format may be used for reference compound and toxicity controls.

6. CORRECTION FOR NITRIFICATION (see Annex V)

		Time of incubation (d)				
		0	n_1	n_2	n_3	n_x
(i)	Concentration of nitrate (mg N/1)					
(ii)	Change in nitrate conc. (mg N/1)	-				
(iii)	Oxygen equivalent (mg/l)	-				
(iv)	Concentration of nitrite (mg N/1)					
(v)	Change in nitrite conc. (mg/l)	-				
(vi)	Oxygen equivalent (mg/l)	-				
(iii+vi)	Total oxygen equivalent (mg/l)	-				

7. **DO DEPLETION: % DEGRADATION (%D):**

	DO depletion after n days (mg/l)			
	n_1	n_2	n_3	n_x
$(m_b - a_1)^{(1)}$				
$(m_b - a_2)^{(1)}$				
$\% Da_1 = \frac{(m_b - a_1)^{(1)}}{test\ substance\ (mg/1) \times ThOD} \times 100$				
$\% Da_2 = \frac{(mb - a_2)^{(1)}}{test\ substance\ (mg/l) \times ThOD} \times 100$				
$\% D_{mean}* = \frac{Da_1 + Da_2}{2}$				

* Do not take mean if there are considerable differences between replicates.

---

(1) This assumes that $m_{b(0)} = a_{1(0)} = a_{2(0)}$, where

$m_{b(o)}$ = blank value at day 0,
$a_{1(o)}$ = test substance value at day 0 in Flask 1
$a_{2(o)}$ = test substance value at day 0 in Flask 2

If $m_{b(o)}$ does not equal $a_{1(o)}$ or $a_{2(o)}$, use

$(a_{1(o)} - a_{1(x)}) - (m_{b(o)} - m_{b(x)})$ and $(a_{2(o)} - a_{2(x)}) - (m_{b(o)} - m_{b(x)})$, where

$m_{b(x)}$ = mean blank value at day x,
$a_{1(x)}$ = test substance value at day x in Flask 1
$a_{2(x)}$ = test substance value at day x in Flask 2

8. **BLANK DO DEPLETIONS:**

Oxygen consumption by blank: ($m_{b(o)}$ - $m_{b(28)}$) 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 from (iii + iv) in section 5.

301 E MODIFIED OECD SCREENING TEST

INTRODUCTION

1. Matters of general interest concerning the assessment of biodegradability are discussed in "General Procedures and Preparations", 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 should be known. This method is similar to the DOC Die-Away test (301 A) but employs a relatively low concentration of micro-organisms.

PRINCIPLE OF THE TEST

2. A measured volume of mineral medium containing a known concentration of the test substance (10-40 mg DOC/l) as the nominal sole source of organic carbon is inoculated with 0.5 ml effluent per litre of medium. The mixture is aerated in the dark or diffused light at 22 $\pm$ 2°C. Degradation is followed by DOC analysis at frequent intervals over a 28 d period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the blank 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.

DESCRIPTION OF THE METHOD

Apparatus

3. Normal laboratory apparatus and:

(a) Conical flasks, e.g. 250 ml to 2 litre, 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 analyser;

(e) Apparatus for determining dissolved oxygen, to check that the flask contents are aerobic;

(f) Centrifuge.

Water

4. A description of the water to be used is given in the "Procedures and Preparations" (p. 5).

Stock solutions for mineral medium

5. Prepare the same stock solutions as detailed in 301 A (paragraph 5).

Preparation of mineral medium

6. Prepare mineral medium in the same way as described in 301 A (paragraph 6). 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 litre of final medium:

(i) Trace element solution:

Manganese sulphate tetrahydrate, $MnSO_4.4H_20$ .... 39.9 mg
Boric acid, H_3B0_3 .... 57.2 mg
Zinc sulphate heptahydrate, $ZnSO_4.7H_20$ .... 42.8 mg
Ammonium heptamolybdate $(NH_4)_6Mo_70_{24}$ .... 34.7 mg
Fe-chelate ($FeCl_3$ ethylenediamine-tetra-
acetic acid) .... 100.0 mg

Dissolve in, and make up to 1 litre with water.

(ii) Vitamin solution:

Yeast extract .... 15.0 mg

Dissolve the yeast extract in 100 ml water. Sterilise by passage through a 0.2 μm membrane, or make up freshly.

Stock solutions of test substances

7. Prepare in the same way as described in 301 A (paragraph 7).

Inoculum

8. The inoculum is derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage and should be prepared in the same way as in 301 A (paragraph 14). Use 0.5 ml of the filtrate per litre of mineral medium.

Pre-conditioning of inoculum

9. If required, the inoculum may be pre-conditioned as described in 301 A (paragraph 16).

Preparation of flasks

10. As an example, introduce 800 ml portions of mineral medium into 2-litre 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. Also prepare inoculum controls in the mineral medium but without test or reference substance (see paragraph 13).

11. Toxicity, abiotic and adsorption controls can also be set up, if required, by following the same procedure as described in 301 A (paragraphs 18 to 20).

12. Make up the volumes in all flasks to 1 litre with mineral medium and, after mixing, take a sample from each flask to determine the initial concentration of DOC in duplicate (see Annex IV.4). Cover the openings of the flasks, e.g with aluminium foil, in such a way as to allow free exchange of air between the flask and the surrounding atmosphere. Then insert the vessels into the shaking machine for starting the test.

Number of flasks

13. In a typical run, the same number of flasks as used in 301 A are used, i.e.:

Flasks 1 & 2	-	containing test substance and inoculum (test suspension);
Flasks 3 & 4	-	containing only inoculum (inoculum blank);
Flask 5	-	containing reference compound and inoculum (procedure control); and, preferably and when necessary, also
Flask 6	-	containing test substance and sterilising agent (abiotic sterile control);
Flask 7	-	containing test substance, inoculum and sterilising agent (adsorption control);
Flask 8	-	containing test substance, reference compound and inoculum (toxicity control).

PROCEDURE

DOC determinations

14. Refer to 301 A (paragraph 23).

Sampling

15. Refer to 301 A (paragraph 24).

Frequency of sampling

16. Refer to 301 A (paragraph 25).

DATA AND REPORTING

Treatment of results

17. Data from the test should be entered onto the attached data sheet.

18. Calculate the percentage degradation (D_t) at each time a sample was analysed using the equation given in 301 A (paragraph 27).

19. Display the course of degradation graphically and indicate the 10-d 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-d window, whichever are appropriate.

20. When specific chemical analytical data are available, calculate primary biodegradation using the equation given in the "Data and Reporting" (p. 7).

Validity of results

21. A test is considered valid if the criteria given in "Data and Reporting" (p. 7) are met.

Test Report

22. The test report should include the information described in "Data and Reporting" (p. 8).

MODIFIED OECD SCREENING TEST
DATA SHEET

1. **LABORATORY:**

2. **DATE AT START OF TEST:**

3. **TEST SUBSTANCE:**

   Name:
   Stock solution concentration: mg/l as chemical
   Initial concentration in medium: mg/l as chemical

4. **INOCULUM:**

   Source of sewage effluent:
   Treatment given:
   Pre-conditioning, if any:
   Concentration of effluent in reaction mixture: ml/l

5. **CARBON DETERMINATIONS:**

   Carbon analyser:

	Flask no.		DOC after n days (mg/l)				
			0	n_1	n_2	n_3	n_x
Test substance plus inoculum	1	a_1					
		a_2					
		mean, $C_{a(t)}$					
	2	b_1					
		b_2					
		mean, $C_{b(t)}$					
Blank, inoculum without test substance	3	c_1					
		c_2					
		mean, $C_{c(t)}$					
	4	d_1					
		d_2					
		mean, $C_{d(t)}$					
	mean, $C_{bl(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$						

6. **EVALUATION OF RAW DATA:**

Flask no.	Calculation of results	% Degradation after n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left[1 - \frac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right] \times 100$	0				
2	$D_2 = \left[1 - \frac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right] \times 100$	0				
Mean (*)	$D_t = \frac{D_1 + D_2}{2}$	0				

* D_1 and D_2 should not be averaged if there is a considerable difference.

Note: Similar formats may be used for the reference compound and toxicity controls.

7. **ABIOTIC DEGRADATION (optional)**

	Time (days)	
	0	t
DOC conc. (mg/l) in sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\%\ abiotic\ degradation = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. **SPECIFIC CHEMICAL ANALYSIS (optional)**

	residual amount of test chemical at end of test	% primary degradation
Sterile control	S_b	
Inoculated test medium	S_a	$\frac{S_b - S_a}{S_b} \times 100$

301 F MANOMETRIC RESPIROMETRY TEST

INTRODUCTION

1. Matters of general interest concerning the assessment of biodegradability are discussed in "General Procedures and Preparations" 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, should 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 oxidised in the COD test. Insoluble and volatile substances may be assessed provided precautions are taken.

PRINCIPLE OF THE TEST

2. A measured volume of inoculated mineral medium, containing a known concentration of test substance (100 mg test substance/l giving at least 50-100 mg ThOD/l) as the nominal sole source of organic carbon, is stirred in a closed flask at a constant temperature ($\pm$ 1°C or closer) 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 carbon dioxide is absorbed in a solution of potassium hydroxide or another suitable absorbent. The amount of oxygen taken up by the microbial population during biodegradation of the test substance (corrected for uptake by blank inoculum, 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 by DOC analysis.

DESCRIPTION OF THE METHOD

Apparatus

3. Normal laboratory apparatus and:

(a) Suitable respirometer;
(b) Temperature control, maintaining $\pm$ 1ºC or better;
(c) Membrane-filtration assembly (optional);
(d) Carbon analyser (optional).

Water

4. A description of the water to be used is given in the "General Procedures and Preparations" (p. 5).

Stock solutions for mineral medium

5. Prepare the same stock solutions as detailed in 301 A (paragraph 5).

Preparation of mineral medium

6. Refer to 301 A (paragraph 6).

Stock solutions of test substances

7. Prepare and handle in the same way as described in 301 A (paragraph 7). For the handling of poorly soluble substances see Annex III.

Inoculum

8. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters and soils; or from a mixture of these as described in 301 A (paragraphs 9 to 15).

Pre-conditioning of inocula

9. Inocula may be pre-conditioned to the experimental conditions as described in 301 A (paragraph 16).

Preparation of flasks

10. Prepare solutions of the test and reference substances, in separate batches, in mineral medium equivalent to a concentration, normally, of 100 mg chemical/l (giving 50-100 mg ThOD/l), using stock solutions. Calculate the ThOD on the basis of formation of ammonium salts unless nitrification is anticipated, when the calculation should be based on nitrate formation (see annex IV.2). Determine the pH values and if necessary adjust to 7.4 ± 0.2. Poorly soluble substances should be added at a later stage (paragraph 13).

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

12. If measurement of any abiotic degradation is required, prepare a solution of the test substance at, normally, 100 mg ThOD/l which has been sterilised by the addition of a toxic substance at an appropriate concentration.

13. Introduce the requisite volume of solutions of test and reference substances, respectively, into at least duplicate respirometer flasks. Add to further flasks mineral medium only (for inoculum controls) and, if required, the mixed test/reference substance solution and the sterile solution. 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 Annex III. Add potassium hydroxide, soda-lime pellets or other absorbent to the CO_2-absorber compartments.

Number of flasks

14. In a typical run, the same number of flasks as used in 301 A are used, i.e.;

Flasks 1 & 2 - containing test substance and inoculum (test suspension);
Flasks 3 & 4 - containing only inoculum (inoculum blank);
Flask 5 - containing reference compound and inoculum (procedure control); and, preferably and when necessary, also
Flask 6 - containing test substance and sterilising agent (abiotic sterile control);
Flask 7 - containing test substance, reference compound and inoculum (toxicity control).

PROCEDURE

15. Allow the vessels to reach the desired temperature and inoculate appropriate vessels with prepared activated sludge or other source of inoculum to give a concentration of suspended solids 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.

16. When an automatic respirometer is used, a continuous record of oxygen uptake is obtained so that the 10-d window is easily recognised. For non-automatic respirometers daily readings will be adequate.

17. 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 uptakes are low or greater than ThOD NH_4, for N-containing chemicals.

18. If required, withdraw samples from the respirometer flasks, initially and at the end of the experiment, for analysis of DOC and/or specific chemical (see annex IV.4). At the initial withdrawal, ensure that the volume of test suspension remaining in the flask is known. When oxygen is taken up by a N-containing test substance, determine the increase in concentration of nitrite and nitrate over 28 d and calculate the correction for the oxygen consumed by nitrification (Annex V).

DATA AND REPORTING

Treatment of results

19. Data should be entered onto the attached data sheet.

20. First calculate the BOD (mg 0_2/mg test chemical) exerted after each time period by dividing the oxygen uptke (mg) of the test chemical, corrected for that by the blank inoculum control, by the weight of the test chemical used, i.e.:

$$BOD = \frac{mg\ O_2\ uptake\ by\ test\ substance\ -\ mg\ O_2\ uptake\ by\ blank}{mg\ test\ substance\ in\ vessel}$$

Calculate the % biodegradation as described in 301 D (paragraphs 23 and 24)

21. When optional determinations of specific chemical and/or DOC are made, calculate the percentage degradation, as described in the "Data and Reporting" (p. 7) and in 301 A (paragraph 27) respectively.

Validity of results

22. The oxygen uptake of the inoculum blank is normally 20-30 mg 0_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%, the test should be repeated with a lower concentration of test substance.

23. The validity criteria given in "Data and Reporting" (p. 7) apply.

Test report

24. The test report should include the information described in "Data and Reporting" (p. 8).

MANOMETRIC RESPIROMETRY TEST
DATA SHEET

1. **LABORATORY:**

2. **DATE AT START OF TEST:**

3. **TEST SUBSTANCE:**

   Name:
   Stock solution concentration: mg/l
   Initial concentration in medium, C_o: mg/l
   Volume in test flask (V): ml
   ThOD or COD: mg O_2/mg test substance (NH_4, NO_3)

4. **INOCULUM:**

   Source:
   Treatment given:
   Pre-conditioning treatment, if any:
   Suspended solids concentration in reaction mixture: mg/l

5. **O_2 UPTAKE, BIODEGRADABILITY:**

Type of respirometer:

		Time (days)				
		n_1	n_2	n_3	n_4	n_x
O_2 uptake by test chemical (mg)	a_1 a_2					
O_2 uptake by blank (mg)	b_1 b_2 b_m mean					
Corrected O_2 uptake (mg)	$(a_1 - b_m)$ $(a_2 - b_m)$					
BOD (mgO_2/mg test substance)	$\frac{a_1 - b_m}{C_o V}$ $\frac{a_2 - b_m}{C_o V}$					
% degradation D $\frac{BOD}{ThOD} x\ 100$	$D_{1(a1)}$ $D_{2(a2)}$ mean*					

* D_1 and D_2 should not be averaged if there is a considerable difference.

Note: Similar formats may be used for the reference compound and toxicity control.

6. **CORRECTION FOR NITRIFICATION (see Annex V)**

		Time of incubation (d)		
		0	28	difference
(i)	Concentration of nitrate (mgN/1)			(N)
(ii)	oxygen equivalent (4.57 x N x V) (mg)	_	_	
(iii)	concentration of nitrite (mgN/1)			(N)
(iv)	oxygen equivalent (3.43 x N x V) (mg)	_	_	
(ii+iv)	total oxygen equivalent	_	_	

7. **CARBON ANALYSIS (optional)**

Carbon analyser:

Time (day)	Test chemical (mg/l)	Blank (mg/l)
0	(C_o)	$(C_{bl(0)})$
28*	(C_t)	$(C_{bl(t)})$

* or at end of incubation

$$\% \ DOC \ removed = \left[1 - \frac{C_t - C_{bl(t)}}{C_o - C_{bl(o)}} \right] \times 100$$

8. **SPECIFIC CHEMICAL ANALYSIS (optional)**

	residual amount of test substance at end of test	% primary degradation
Sterile control	S_b	
Inoculated test medium	S_a	$\frac{S_b - S_a}{S_b} \times 100$

9. **ABIOTIC DEGRADATION**

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

$$O_2 \text{ consumption per mg test chemical} = \frac{a}{C_o V}$$

$$\% \text{ abiotic degradation} = \frac{a}{C_o V \times ThOD} \times 100$$

ANNEX I

ABBREVIATIONS AND DEFINITIONS

DO: Dissolved oxygen (mg/l) is the concentration of oxygen dissolved in an aqueous sample.

BOD: Biochemical oxygen demand (mg) is the amount of oxygen consumed by micro-organisms when metabolising a test compound; also expressed as mg oxygen uptake per mg test compound.

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

DOC: Dissolved organic carbon is the organic carbon present in solution or that which passes through a 0.45 micrometre filter or remains in the supernatant after centrifuging at approx. 4000 g (about 40.000 m sec^{-2}) for 15 min.

ThOD: Theoretical oxygen demand (mg) is the total amount of oxygen required to oxidise a chemical completely; it is calculated from the molecular formula (see Annex IV.2) and is also expressed as mg oxygen required per mg test compound.

$ThC0_2$: Theoretical carbon dioxide (mg) is the quantity of carbon dioxide calculated to be produced from the known or measured carbon content of the test compound when fully mineralized; also expressed as mg carbon dioxide evolved per mg test compound.

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

IC: Inorganic carbon

TC: Total carbon, is the sum of the organic and inorganic carbon present in a sample.

Primary Biodegradation: 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.

Ultimate Biodegradation (aerobic): The level of degradation achieved when the test compound is totally utilised by micro-organisms resulting in the production of carbon dioxide, water, mineral salts and new microbial cellular constituents (biomass).

Readily Biodegradable: 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.

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

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.

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

Degradation phase: The time from the end of the lag period to the time when 90% of the maximum level of degradation has been reached.

10-d window: The 10 days immediately following the attainment of 10% biodegradation.

ANNEX II

EVALUATION OF THE BIODEGRADABILITY OF CHEMICALS SUSPECTED TO BE TOXIC TO THE INOCULUM

When a chemical is subjected to ready biodegradability testing and appears to be non-biodegradable, the following procedure is recommended if a distinction between inhibition and inertness is desired (Reynolds et al., 1987).

1. Similar or identical inocula should be used for the toxicity and biodegradation tests.

2. 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 (OECD Guideline 209; ISO Standard 8192), BOD and/or growth inhibition methods would seem appropriate.

3. 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 1/10 of the EC_{50} values (or less than EC_{20} values) obtained in toxicity testing. Compounds with an EC_{50} value greater than 300 mg/l are not likely to have toxic effects in ready biodegradability testing.

4. EC_{50} 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 or the use of C^{14}-labelled 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 specific criterion of the ready biodegradability test is lost.

LITERATURE

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

ANNEX III

EVALUATION OF THE BIODEGRADABILITY OF POORLY SOLUBLE COMPOUNDS

In biodegradability tests with poorly soluble compounds the following aspects should receive special attention.

1. While homogeneous liquids will seldom present sampling problems, it is recommended that solid materials be homogenised by appropriate means to avoid errors due to non-homogeneity. 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.

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

3. 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.

4. The same criteria apply to solvents as to the emulsifiers.

5. It is not recommended that solid carriers be used for solid test substances but they may be suitable for oily substances.

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

7. Any of the four respirometric tests (301 B, 301 C, 301 D, 301 F) can be used to study the biodegradability of poorly soluble compounds.

LITERATURE

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ANNEX IV

CALCULATION AND DETERMINATION OF SUITABLE SUMMARY PARAMETERS

Depending on the method chosen, certain summary parameters will be required. The following section describes the derivation of these values. The use of these parameters is described in the individual methods.

1. Carbon Content

The carbon content is calculated from the known elemental composition or determined by elemental analysis of the test substance.

2. Theoretical Oxygen Demand (ThOD)

The theoretical oxygen demand (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, would be:

$$ThOD_{NH3} = \frac{16[2c + 1/2(h - cl - 3n) + 3s + 5/2p + 1/2na - o]mg/mg}{MW}$$

with nitrification:

$$ThOD_{NO3} = \frac{16[2c + 1/2(h - cl) + 5/2n + 3s + 5/2p + 1/2na - o]mg/mg}{MW}$$

where MW = molecular weight

3. Chemical Oxygen Demand (COD)

The chemical oxygen demand (COD) of water soluble organic substances is determined by established procedures, e.g. according to the ISO method 6060.

The chemical oxygen demand (COD) is often, and especially in the case of poorly soluble substances, determined advantageously in a variant of the above analysis, i.e., in a closed system with a pressure equaliser (Kelkenberg, 1975). In this modification, compounds which are only with difficulty determined by the conventional method (e.g. acetic acid) may often be successfully quantified. The method also fails, however, in the case of pyridine. If the potassium dichromate concentration is raised from 0.016N (0.0026M) as prescribed by Kelkenberg to 0.25N (0.0416M), the direct weighing-in of 5-10 mg of substance is facilitated which is essential for the COD determination of poorly water soluble substances (Gerike, 1984).

4. Dissolved Organic Carbon (DOC)

Dissolved organic carbon (DOC) is, by definition, the organic carbon of any chemical or mixture in water passing through a 0.45 µm 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 analyser) are retained for carbon analysis. The DOC concentration is determined by means of an organic carbon analyser which is capable of accurately measuring a carbon concentration equivalent to or lower than 10% of the initial DOC concentration used in the test.

Filtered samples which cannot be analysed on the same working day can be preserved by storage in a refrigerator at 4°C. Preserved samples can be retained for 48 hours before analysis, or for longer at -18°C.

Remarks:

Membrane filters are often impregnated with surfactants for hydrophilisation. Thus the filter may contain up to several mg of soluble organic carbon which would interfere in the biodegradability determinations. Surfactants and other soluble organic compounds are removed from the filters by boiling them in deionised water for three periods each of one hour. The filters may then be stored in water for one week. If disposable filter cartridges are used, each lot must be checked to confirm that it does not release soluble organic carbon.

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.

Centrifugation at 4000 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.

LITERATURE

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ANNEX V

CORRECTION FOR OXYGEN UPTAKE FOR INTERFERENCE BY NITRIFICATION

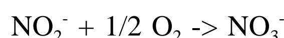
Respirometric methods with oxygen uptake as the analytical procedure may be influenced significantly by the oxygen uptake resulting from ammonium oxidation.

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

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O.$$

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

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



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

The oxygen taken up by 14 g of nitrogen in being oxidised to nitrite is 48 g, i.e. a factor of 3.43.

Since the reactions are <u>sequential</u>, being carried out by distinct and different bacterial species, it is possible for the concentration of nitrite to increase <u>or</u> decrease; in the latter case an equivalent concentration of nitrate would be formed. Thus, the oxygen consumed in the formation of nitrate is 4.57 multiplied by the increase in concentration of nitrate-N, whereas the oxygen associated with the formation of nitrite is 3.43 multiplied by the increase in the concentration of nitrite-N or with the decrease in its concentration the oxygen "loss" is 3.43 multiplied by the decrease in concentration.

Alternatively, if only "total oxidised N" is determined, the oxygen uptake due to nitrification may be taken to be, as a first approximation, 4.57 x increase in oxidised N.

The corrected value for oxygen consumption due to C oxidation is then compared with ThOD NH_3, as calculated in Annex IV.