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	Mean
	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 O_2/mg chemical) of the test substance. For poorly degradable compounds and those with a low ThOD, 5-10 mg/l can be used. In some cases, it would be advisable to run parallel series of test substance at two different concentrations, for example, 2 and 5 mg/l. Normally, calculate the ThOD on the basis of formation of ammonium salts but, if nitrification is expected or known to occur, calculate on the basis of the formation of nitrate $ThOD_{NO3}$ (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:

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

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

or

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

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/l)					
(ii)	Change in nitrate conc. (mg N/l)	-				
(iii)	Oxygen equivalent (mg/l)	-				
(iv)	Concentration of nitrite (mg N/l)					
(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
$(m_b - a_1)^{(1)}$				
$(m_b - a_2)^{(1)}$				
$\% Da_1 = \frac{(m_b - a_1)^{(1)}}{test\ substance\ (mg/l) \times ThOD} \times 100$				
$\% Da_2 = \frac{(mb - a_2)^{(1)}}{test\ substance\ (mg/l) \times ThOD} \times 100$				
$\% D_{mean}{}^{*} = \frac{Da_1 + Da_2}{2}$				

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

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(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 ± 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	$C_{s(o)}$	$C_{s(t)}$

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