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 (± 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 ± 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 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 $\pm$ 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 $\frac{BOD}{ThOD} \times 100$	$D_{1(a1)}$ $D_{2(a2)}$ 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

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

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

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

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

Standard Methods for the Examination of Water and Wastewater, 12th ed, Am. Pub. Hlth. Ass., Am. Wat. Poll. Control Fed., Oxygen Demand, P 65 (1965).

Wagner R. (1976). Vom Wasser, 46, 139.

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

Kelkenberg H. (1975). Z. Wasser und Abwasserforschung, 8, 146 (1975).

Gerike P. (1984). The biodegradability testing of poorly water soluble compounds. Chemosphere, 13 (1), 169.

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 sequential, being carried out by distinct and different bacterial species, it is possible for the concentration of nitrite to increase or 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.

OECD/OCDE

307

Adopted:
24th April 2002

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Aerobic and Anaerobic Transformation in Soil

INTRODUCTION

1. This Test Guideline is based on existing guidelines (1)(2)(3)(4)(5)(6)(7)(8)(9). The method described in this Guideline is designed for evaluating aerobic and anaerobic transformation of chemicals in soil. The experiments are performed to determine (i) the rate of transformation of the test substance, and (ii) the nature and rates of formation and decline of transformation products to which plants and soil organisms may be exposed. Such studies are required for chemicals which are directly applied to soil or which are likely to reach the soil environment. The results of such laboratory studies can also be used to develop sampling and analysis protocols for related field studies.

2. Aerobic and anaerobic studies with one soil type are generally sufficient for the evaluation of transformation pathways (8)(10)(11). Rates of transformation should be determined in at least three additional soils (8)(10).

3. An OECD Workshop on soil and sediment selection, held at Belgirate, Italy in 1995 (10) agreed, in particular, on the number and types of soils for use in this test. The types of soils tested should be representative of the environmental conditions where use or release will occur. For example, chemicals that may be released in subtropical to tropical climates should be tested with Ferrasols or Nitosols (FAO system). The Workshop also made recommendations relating to collection, handling and storage of soil samples, based on the ISO Guidance (12). The use of paddy (rice) soils is also considered in this Guideline.

PRINCIPLE OF THE TEST

4. Soil samples are treated with the test substance and incubated in the dark in biometer-type flasks or in flow-through systems under controlled laboratory conditions (at constant temperature and soil moisture). After appropriate time intervals, soil samples are extracted and analysed for the parent substance and for transformation products. Volatile products are also collected for analysis using appropriate adsorption devices. Using ^{14}C-labelled material, the various mineralisation rates of the test substance can be measured by trapping evolved $^{14}CO_2$ and a mass balance, including the formation of soil bound residues, can be established.

APPLICABILITY OF THE TEST

5. The method is applicable to all chemical substances (non-labelled or radiolabelled) for which an analytical method with sufficient accuracy and sensitivity is available. It is applicable to slightly volatile, non-volatile, water-soluble or water-insoluble compounds. The test should not be applied to chemicals which are highly volatile from soil (e.g. fumigants, organic solvents) and thus cannot be kept in soil under the experimental conditions of this test.

INFORMATION ON THE TEST SUBSTANCE

6. Non-labelled or labelled test substance can be used to measure the rate of transformation. Labelled material is required for studying the pathway of transformation and for establishing a mass balance. ^{14}C-labelling is recommended but the use of other isotopes, such as ^{13}C, ^{15}N, ^{3}H, ^{32}P, may also be informative. As far as possible, the label should be positioned in the most stable part(s) of the molecule[1]. The purity of the test substance should be at least 95 %.

7. Before carrying out a test on aerobic and anaerobic transformation in soil, the following information on the test substance should be available:

(a) solubility in water [OECD Guideline 105] (13);
(b) solubility in organic solvents;
(c) vapour pressure [OECD Guideline 104] (13) and Henry's law constant;
(d) n-octanol/water partition coefficient [OECD Guidelines 107 and 117] (13);
(e) chemical stability in dark (hydrolysis) [OECD Guideline 111] (13);
(f) pK_a if a molecule is liable to protonation or deprotonation [OECD Guideline 112] (13).

8. Other useful information may include data on toxicity of the test substance to soil microorganisms [OECD Guidelines 216 and 217] (13).

9. Analytical methods (including extraction and clean-up methods) for quantification and identification of the test substance and its transformation products should be available.

REFERENCE SUBSTANCES

10. Reference substances should be used for the characterisation and/or identification of transformation products by spectroscopic and chromatographic methods.

DEFINITIONS

11. See Annex 1.

QUALITY CRITERIA

Recovery

12. Extraction and analysis of, at least, duplicate soil samples immediately after the addition of the test substance gives a first indication of the repeatability of the analytical method and of the uniformity of the application procedure for the test substance. Recoveries for later stages of the experiments are given by the respective mass balances. Recoveries should range from 90% to 110% for labelled chemicals (8) and from 70% to 110% for non-labelled chemicals (3).

[1] For example, if the test substance contains one ring, labelling on this ring is required; if the test substance contains two or more rings, separate studies may be needed to evaluate the fate of each labelled ring and to obtain suitable information on formation of transformation products.

Repeatability and sensitivity of analytical method

13. Repeatability of the analytical method (excluding the initial extraction efficiency) to quantify test substance and transformation products can be checked by duplicate analysis of the same extract of the soil, incubated long enough for formation of transformation products.

14. The limit of detection (LOD) of the analytical method for the test substance and for the transformation products should be at least 0.01 $mg \cdot kg^{-1}$ soil (as test substance) or 1% of applied dose whichever is lower. The limit of quantification (LOQ) should also be specified.

Accuracy of transformation data

15. Regression analysis of the concentrations of the test substance as a function of time gives the appropriate information on the reliability of the transformation curve and allows the calculation of the confidence limits for half-lives (in the case of pseudo first order kinetics) or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values.

DESCRIPTION OF THE TEST METHOD

Equipment and chemical reagents

16. Incubation systems consist of static closed systems or suitable flow-through systems (7)(14). Examples of suitable flow-through soil incubation apparatus and biometer-type flask are shown in Figures 1 and 2, respectively. Both types of incubation systems have advantages and limitations (7)(14).

17. Standard laboratory equipment is required and especially the following:

- Analytical instruments such as GLC, HPLC, TLC-equipment, including the appropriate detection systems for analysing radiolabelled or non-labelled substances or inverse isotopes dilution method;
- Instruments for identification purposes (e.g. MS, GC-MS, HPLC-MS, NMR, etc.);
- Liquid scintillation counter;
- Oxidiser for combustion of radioactive material;
- Centrifuge;
- Extraction apparatus (for example, centrifuge tubes for cold extraction and Soxhlet apparatus for continuous extraction under reflux);
- Instrumentation for concentrating solutions and extracts (e.g. rotating evaporator);
- Water bath;
- Mechanical mixing device (e.g. kneading machine, rotating mixer);

18. Chemical reagents used include, for example:

NaOH, analytical grade, 2 $mol \cdot dm^{-3}$, or other appropriate base (e.g. KOH, ethanolamine);
H_2SO_4, analytical grade, 0.05 $mol \cdot dm^{-3}$;
Ethylene glycol, analytical grade;
Solid absorption materials such as soda lime and polyurethane plugs;
Organic solvents, analytical grade, such as acetone, methanol, etc.;
Scintillation liquid.

Test substance application

19. For addition to and distribution in soil, the test substance can be dissolved in water (deionised or distilled) or, when necessary, in minimum amounts of acetone or other organic solvents (6) in which the test substance is sufficiently soluble and stable. However, the amount of solvent selected should not have a significant influence on soil microbial activity (see paragraphs 8 and 44). The use of solvents which inhibit microbial activity, such as chloroform, dichloromethane and other halogenated solvents, should be avoided.

20. The test substance can also be added as a solid, e.g. mixed in quartz sand (6) or in a small sub-sample of the test soil which has been air-dried and sterilised. If the test substance is added using a solvent the solvent should be allowed to evaporate before the spiked sub-sample is added to the original non-sterile soil sample.

21. For general chemicals, whose major route of entry into soil is through sewage sludge/farming application, the test substance should be first added to sludge which is then introduced into the soil sample (see paragraph 41).

22. The use of formulated products is not routinely recommended. However, e.g. for poorly soluble test substances, the use of formulated material may be an appropriate alternative.

Soils

Soil selection

23. To determine the transformation pathway, a representative soil can be used; a sandy loam or silty loam or loam or loamy sand [according to FAO and USDA classification (15)] with a pH of 5.5-8.0, an organic carbon content of 0.5 - 2.5% and a microbial biomass of at least 1% of total organic carbon is recommended (10).

24. For transformation rate studies at least three additional soils should be used representing a range of relevant soils. Those soils should vary in their organic carbon content, pH, clay content and microbial biomass (10).

25. All soils should be characterised, at least, for texture (% sand, % silt, % clay) [according to FAO and USDA classification (15)], pH, cation exchange capacity, organic carbon, bulk density, water retention characteristic[2] and microbial biomass (for aerobic studies only). Additional information on soil properties may be useful in interpreting the results. For determination of the soil characteristics the methods recommended in references (16)(17)(18)(19)(20) can be used. Microbial biomass should be determined by using the substrate-induced respiration (SIR) method (21)(22) or alternative methods (17).

Collection, handling, and storage of soils

26. Detailed information on the history of the field site from where the test soil is collected should be available. Details include exact location, vegetation cover, treatments with chemicals, treatments with organic and inorganic fertilisers, additions of biological materials or other contamination. If soils have

[2] Water retention characteristic of a soil can be measured as field capacity, as water holding capacity or as water suction tension (pF). For explanations see Annex 2. It should be reported in the test report whether water retention characteristics and bulk density of soils were determined in undisturbed field samples or in disturbed (processed) samples.