

## C.4. DETERMINATION OF 'READY' BIODEGRADABILITY

### PART I. GENERAL CONSIDERATIONS

#### I.1. INTRODUCTION

Six test methods are described that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium:

- (a) Dissolved Organic Carbon (DOC) Die-Away (Method C.4-A)
- (b) Modified OECD Screening -DOC Die-Away (Method C.4-B)
- (c) Carbon dioxide (CO<sub>2</sub>) Evolution (Modified Sturm Test) (Method C.4-C)
- (d) Manometric Respirometry (Method C.4-D)
- (e) Closed Bottle (Method C.4-E)
- (f) MITI (Ministry of International Trade and Industry -Japan) (Method C.4-F)

General and common considerations to all six tests are given in Part I of the method. Items specific for individual methods are given in Parts II to VII. The annexes contain definitions, formulas and guidance material.

An OECD inter-laboratory comparison exercise, done in 1988, has shown that the methods give consistent results. However, depending on the physical characteristics of the substance to be tested, one or other of the methods may be preferred.

#### I.2. SELECTION OF THE APPROPRIATE METHOD

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<sub>2</sub>, DOC, TOC, COD (see Annexes I and II).

Test chemicals 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. 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 substances which are: |          |           |
|---------------------------|---|---------------------------------------|----------|-----------|
|                           |   | poorly soluble                        | volatile | adsorbing |
| DOC Die-Away              | Dissolved organic carbon                    | —                                     | —        | + / —     |
| Mod. OECD Die-Away        | Dissolved organic carbon                    | —                                     | —        | + / —     |
| CO <sub>2</sub> Evolution | Respirometry: CO <sub>2</sub> evolution     | +                                     | —        | +         |
| Manometric Respirometry   | Manometric respirometry: oxygen consumption | +                                     | + / —    | +         |
| Closed Bottle             | Respirometry: dissolved oxygen              | + / —                                 | +        | +         |
| MITI                      | Respirometry: oxygen consumption            | +                                     | + / —    | +         |

Information on the purity or the relative proportions of major components of the test material is required to interpret the results obtained, especially when the results are low or marginal.

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

### 1.3. REFERENCE SUBSTANCES

In order to check the procedure, reference chemicals which meet the criteria for ready biodegradability are tested by setting up an appropriate flask in parallel to the normal test runs.

Suitable chemicals are aniline (freshly distilled), sodium acetate and sodium benzoate. These reference chemicals all degrade in these methods even when no inoculum is deliberately added.

It was suggested that a reference chemical 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 substance before it can be accepted as a reference substance.

In the respirometric tests, nitrogen-containing compounds may affect the oxygen uptake because of nitrification (see Annexes II and V).

### 1.4. PRINCIPLE OF THE TEST METHODS

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 to the amount of DOC due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blank tests with inoculum but without test substance, although the endogenous activity of cells in the presence of the substance will not exactly match that in the endogenous control. A reference substance is run in parallel to check the operation of the procedures.

In general, degradation is followed by the determination of parameters, such as DOC, CO<sub>2</sub> 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 the 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 (obligatory in the MITI test).

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 3 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 day 28.

### 1.5. QUALITY CRITERIA

#### 1.5.1. Reproducibility

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 common experience 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.

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### 1.5.2. Validity of the test

A test is considered valid if the difference of extremes of replicate values of the removal of test chemical at the plateau, at the end of the test or at the end of the 10-day window, as appropriate, is less than 20% and if the percentage degradation of the reference substance has reached the level for ready biodegradability by 14 days. 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.

If in a toxicity test, containing both the test substance and a reference chemical, less than 35% degradation (based on DOC) or less than 25 % (based on ThOD or ThCO<sub>2</sub>) occurred in 14 days, the test chemicals can be assumed to be inhibitory (see also Annex IV). The test series should be repeated, if possible using a lower concentration of test chemical and/or a higher concentration of inoculum, but not greater than 30 mg solids/litre.

### 1.6. GENERAL PROCEDURES AND PREPARATIONS

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

**Table 2: Test conditions**

| Test   | DOC Die-Away   | CO <sub>2</sub> Evolution       | Manometric Respirometry | Modified OECD Screeing                            | Closed Bottle  | MITI (I)   |
|--|--|---------------------------------|-------------------------|---|--|--|
| Concentration of Test Substance as mg/l<br>mg DOC/l<br>mg ThOD/l | 10-40  | 10-20                           | 100<br>50-100           | 10-40   | 2-10<br>5-10   | 100  |
| Concentration of Inoculum (in cells/l, approximatively)          | ≤ 30 mg/l SS<br>or ≤ 100 ml effluent/l<br>(10 <sup>7</sup> - 10 <sup>8</sup> ) |                                 |                         | 0,5 ml secondary effluent/l<br>(10 <sup>5</sup> ) | ≤ 5 ml of effluent/l<br>(10 <sup>4</sup> - 10 <sup>6</sup> ) | 30 mg/l SS<br>(10 <sup>7</sup> - 10 <sup>8</sup> ) |
| Concentration of elements in mineral medium (in mg/l)            |  |                                 |                         |   |  |  |
| P  | 116  |                                 |                         |   | 11,6   | 29   |
| N  | 1,3  |                                 |                         |   | 0,13   | 1,3  |
| Na   | 86   |                                 |                         |   | 8,6  | 17,2   |
| K  | 122  |                                 |                         |   | 12,2   | 36,5   |
| Mg   | 2,2  |                                 |                         |   | 2,2  | 6,6  |
| Ca   | 9,9  |                                 |                         |   | 9,9  | 29,7   |
| Fe   | 0,05-0,1   |                                 |                         |   | 0,05-0,1   | 0,15   |
| pH   | 7,4 ± 0,2  |                                 |                         |   |  | preferably 7,0                                     |
| Temperature  | 22 ± 2 °C  |                                 |                         |   |  | 25 ± 1 °C  |
| DOC = Dissolved organic Carbon                                   |  | ThOD =Theoretical Oxygen Demand |                         | SS = Suspended Solids                             |  |  |

#### I.6.1. Dilution water

Deionized or distilled water, free from inhibitory concentrations of toxic substances ( e.g. Cu <sup>++</sup> 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 to eliminate high blank values. Contamination may result from inherent impurities and also from the ion-exchange resins and lysed material from bacterial and algae. For each series of tests use only one batch of water, checked beforehand by DOC analysis. Such a check is not necessary for the closed bottle test, but the oxygen consumption of the water must be low.

#### I.6.2. Stock solutions of mineral components

To make up the test solutions, stock solutions of appropriate concentrations of mineral components are made up. The following stock solutions may be used (with different dilution factors) for the methods DOC Die-Away, Modified OECD Screening, CO<sub>2</sub> Evolution, Manometric Respirometry, Closed Bottle test.

The dilution factors and, for the MITI test, the specific preparation of the mineral medium are given under the headings of the specific tests.

Stock solutions:

Prepare the following stock solutions, using analytical grade reagents.

|  |         |
|--|---------|
| (a) Monopotassium dihydrogen orthophosphate, KH <sub>2</sub> PO <sub>4</sub>                         | 8,50 g  |
| Dipotassium monohydrogen orthophosphate, K <sub>2</sub> HPO <sub>4</sub>                             | 21,75 g |
| Disodium monohydrogen orthophosphate dihydrate Na <sub>2</sub> HPO <sub>4</sub> . 2 H <sub>2</sub> O | 33,40 g |
| Ammonium chloride, NH <sub>4</sub> Cl  | 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 <sub>2</sub>                    | 27,50 g |
| or Calcium chloride dihydrate, CaCl <sub>2</sub> , 2 H <sub>2</sub> O | 36,40 g |

Dissolve in water and make up to 1 litre

|   |         |
|---|---------|
| (c) Magnesium sulphate heptahydrate, MgSO <sub>4</sub> . 7 H <sub>2</sub> O | 22,50 g |
|---|---------|

Dissolve in water and make up to 1 litre.

|  |        |
|--|--------|
| (d) Iron (III) chloride hexahydrate, FeCl <sub>3</sub> . 6H <sub>2</sub> O | 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 conc. HCL or 0,4 g ethylenediaminetetra-acetic acid disodium salt (EDTA) par litre.

#### I.6.3. Stock solutions of chemicals

For example, dissolve 1-10 g, as appropriate, of test or reference chemical in deionized water and make up to 1 litre when the solubility exceeds 1 g/l. Otherwise, prepare stock solutions in the mineral medium or add the chemical direct to the mineral medium. For the handling of less soluble chemicals, see Annex III, but in the MITI test (Method C.4-F), neither solvents nor emulsifying agents are to be used.

#### I.6.4. Inocula

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, CO<sub>2</sub> Evolution and Manometric Respirometry tests, 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 have been found to give higher scattering of results. For the Modified OECD Screening and the Closed Bottle tests 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

test the inoculum is derived from a mixture of sources and is described under the heading of this specific test.

#### I.6.4.1. Inoculum from activated sludges

Collect a sample of activated sludge freshly 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.

Alternatively, settle or centrifuge (e.g. at 1 100 g for 10 min.) after removal of any coarse particles. 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 and aerate until required.

Sludge should be taken from a properly working conventional 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.

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.

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

#### I.6.4.2. Other sources of inoculum

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

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.

#### I.6.5. Pre-conditioning of inocula

Inocula may be pre-conditioned to the experimental conditions, but not pre-adapted to the test chemical. 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 inoculum.

#### I.6.6. Abiotic controls

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 micrometre) 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 chemical 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.

#### I.6.7. Number of flasks

The number of flasks in a typical run is described under the headings of each tests.

The following type of flask may be used:

Test suspension: containing test substance and inoculum

Inoculum blank: containing only inoculum

Procedure control: containing reference substance and inoculum

Abiotic sterile control: sterile, containing test substance (see 1.6.6)

Adsorption control: containing test substance, inoculum and sterilising agent

Toxicity control: containing test substance, reference substance and inoculum

It is mandatory that determination in test suspension and inoculum blank is made in parallel. It is advisable to make the determinations in the other flasks in parallel as well.

This may, however, not always be possible. Ensure that sufficient samples or readings are taken to allow the percentage removal in the 10-day window to be assessed.

#### I.7. DATA AND EVALUATION

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 tests. The course of degradation is displayed graphically and the 10-day window is indicated. Calculate and report the percentage removal achieved at the end of the 10-day window and the value at the plateau or at the end of the test, whichever is appropriate.

In respirometric tests nitrogen-containing compounds may affect the oxygen uptake because of nitrification (see Annexes II and V).

##### I.7.1. Degradation measured by means of DOC determination

The percentage degradation  $D_t$  at each time a sample was taken should be calculated separately for the flasks containing test substance using mean values of duplicate DOC measurements in order that the validity of the test can be assessed (see 1.5.2.). It is calculated using the following equation:

$$\text{ThOD}_{\text{NH}_4} = \frac{16[2c + 1/2(h - cl - 3n) + 3s + 5/2p + 1/2na - o]}{\text{MW}} \text{ mg/mg}$$

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),

$C_{bo}$  = mean starting concentration of DOC in blank inoculated mineral medium (mg DOC/l),

$C_{bt}$  = mean concentration of DOC blank inoculated mineral medium at time t (mg DOC/l).

All concentrations are measured experimentally.

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### 1.7.2. Degradation measured by means of specific analysis

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

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

where:

$D_t$  = % degradation at time t, normally 28 days,

$S_a$  = residual amount of test substance in inoculated medium at end of test (mg),

$S_b$  = residual amount of test substance in the blank test with water/medium to which only the test substance was added (mg).

### 1.7.3. Abiotic degradation

When an abiotic sterile control is used, calculate the percentage abiotic degradation using

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

where

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

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

## I.8. REPORTING

The test report shall, if possible, contain the following:

- test and reference chemicals, and their purity;
- test conditions;
- inoculum: nature and sampling site(s), concentration and any pre-conditioning treatment;
- proportion and nature of industrial waste present in sewage if known;
- test duration and temperature;
- in the case of poorly soluble test chemicals, treatment given;
- test method applied; scientific reasons and explanation should be given for any change of procedure;
- data sheet;
- 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, 10-day window and slope should be clearly indicated (Annex I). If the test has complied with the validity criteria, the mean of the degradation percentages of the flasks containing test substance may be used for the graph.
- percentage removal after 10-day window, and at plateau or at end of the test.

## **PART II. DOC DIE-AWAY TEST (Method C.4-A)**

### **II.1. PRINCIPLE OF THE METHOD**

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 diffused light at  $22 \pm 2$  °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. The degree of primary biodegradation may also be calculated from supplemental chemical analysis made at the beginning and end of incubation.

### **II.2. DESCRIPTION OF THE METHOD**

#### **II.2.1. Apparatus**

- (a) Conical flasks, e. g. 250 ml to 2 l, depending on the volume needed for DOC analysis;
- (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;
- (f) Centrifuge.

#### **II.2.2. Preparation of mineral medium**

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 l with dilution water .

#### **II.2.3. Preparation and pre-conditioning of inoculum**

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

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

#### **II.2.4. Preparation of flasks**

As an example, introduce 800 ml portions of mineral medium into 2 l conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10-40 mg DOC/l. Check the pH values and adjust, if necessary, to 7.4. Inoculate the flasks with activated sludge or other source of inocula (see I.6.4.), 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 chemical.

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

Also, if required, set up a further, sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6.).



Additionally, if the test chemical is suspected of being significantly adsorbed on to 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). Set up a flask containing the test substance, inoculum and sterilizing agent.

Make up the volumes in all flasks to 11 with mineral medium and, after mixing, take a sample from each flask to determine the initial concentration of DOC (see Annex II.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.

#### II.2.5. Number of flasks in typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Adsorption control

Flask 8: Toxicity control

See also I.6.7.

#### II.2.6. Performance of the test

Throughout the test, determine the concentrations of DOC in each flask in duplicate at known time intervals, sufficiently frequently to be able to determine the start of the 10-day window and the percentage removal at the end of the 10-day window. Take only the minimal volume of test suspension necessary for each determination.

Before sampling make good evaporation losses from the flasks by adding dilution water (I.6.1) in the required amount if necessary. Mix the culture medium thoroughly before withdrawing a sample and ensure that material adhering to the walls of the vessels is dissolved or suspended before sampling. Membrane-filter or centrifuge (see Annex II.4) immediately after the sample 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.

### II.3. DATA AND REPORTING

#### II.3.1. Treatment of results

Calculate the percentage degradation at time t as given under I.7.1. (DOC determination) and, optionally, under I.7.2. (specific analysis).

Record all results on the data sheets provided.

#### II.3.2. Validity of results

See I.5.2.

#### II.3.3. Reporting

See I.8.

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## II.4. DATA SHEET

An example of a data sheet is given hereafter.

### DOC DIE-AWAY TEST

1. LABORATORY
2. DATE AT START OF TEST
3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/l as chemical

Initial concentration in medium, to: mg/l as chemical

### 4. INOCULUM

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/l

### 5. CARBON DETERMINATIONS

Carbon analyser:

|   | Flask nr |                              | DOC after n days (mg/l) |                |                |                |                |
|---|----------|------------------------------|-------------------------|----------------|----------------|----------------|----------------|
|   |          |                              | 0                       | n <sub>1</sub> | n <sub>2</sub> | n <sub>3</sub> | n <sub>x</sub> |
| Test chemical plus inoculum                 | 1        | a <sub>1</sub>               |                         |                |                |                |                |
|   |          | a <sub>2</sub>               |                         |                |                |                |                |
|   |          | a, mean<br>C <sub>a(t)</sub> |                         |                |                |                |                |
|   | 2        | b <sub>1</sub>               |                         |                |                |                |                |
|   |          | b <sub>2</sub>               |                         |                |                |                |                |
|   |          | b, mean<br>C <sub>b(t)</sub> |                         |                |                |                |                |
| Blank inoculum without test chemical        | 3        | c <sub>1</sub>               |                         |                |                |                |                |
|   |          | c <sub>2</sub>               |                         |                |                |                |                |
|   |          | c, mean<br>C <sub>c(t)</sub> |                         |                |                |                |                |
|   | 4        | d <sub>1</sub>               |                         |                |                |                |                |
|   |          | d <sub>2</sub>               |                         |                |                |                |                |
|   |          | d, mean<br>C <sub>d(t)</sub> |                         |                |                |                |                |
| $C_{bl(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$ |          |                              |                         |                |                |                |                |

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## 6. EVALUATION OF RAW DATA

| Flask nr |   | % degradation after n days |                |                |                |                |
|----------|---|----------------------------|----------------|----------------|----------------|----------------|
|          |   | 0                          | n <sub>1</sub> | n <sub>2</sub> | n <sub>3</sub> | n <sub>x</sub> |
| 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 (*) | $D = \frac{D_1 - D_2}{2}$   | 0                          |                |                |                |                |

(\*) D<sub>1</sub> and D<sub>2</sub> should not be averaged if there is a considerable difference.

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

## 7. ABIOTIC CONTROL (optional)

|                                     | Time (days)       |                   |
|-------------------------------------|-------------------|-------------------|
|                                     | 0                 | t                 |
| DOC conc. (mg/l) in sterile control | C <sub>s(o)</sub> | C <sub>s(t)</sub> |

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

## 8. SPECIFIC CHEMICAL ANALYSIS (optional)

|                        | residual amount of test chemical at end of test (mg/l) | % primary degradation |  |  |
|------------------------|--|-----------------------|--|--|
| Sterile control        | S <sub>b</sub>   |                       |  |  |
| Inoculated test medium | S <sub>a</sub>   |                       |  |  |

## PART III. MODIFIED OECD SCREENING TEST (Method C.4-B)

### III.1. PRINCIPLE OF THE METHOD

A measured volume of mineral medium containing a known concentration of the test substance (10-40 mg DOC/litre) 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-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. The degree of primary biodegradation may also be calculated from supplemental chemical analysis made at the beginning and end of incubation.

### III.2. DESCRIPTION OF THE METHOD

#### III.2.1. Apparatus

- (a) Conical flasks, e.g. 250 ml to 2 litres, depending on the volume needed for DOC analysis;
- (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;
- (f) Centrifuge.

#### III.2.2. Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 80 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 litre with dilution water.

This method uses only 0,5 ml effluent/litre 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:

Trace element solution:

|   |          |
|---|----------|
| Manganese sulfate tetrahydrate, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ | 39,9 mg  |
| Boric acid, $\text{H}_3\text{BO}_3$                                       | 57,2 mg  |
| Zinc sulfate heptahydrate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$      | 42,8 mg  |
| Ammonium heptamolybdate $(\text{NH}_4)_6 \text{Mo}_7\text{O}_{24}$        | 34,7 mg  |
| Fe-chelate ( $\text{FeCl}_3$ ethylenediamine-tetra-acetic acid)           | 100,0 mg |

Dissolve in, and make up to 1000 ml with dilution water

Vitamin solution:

|               |         |
|---------------|---------|
| Yeast extract | 15,0 mg |
|---------------|---------|

Dissolve the yeast extract in 100 ml water. Sterilise by passage through a 0,2 micron membrane, or make up freshly.

#### III.2.3. Preparation and pre-conditioning of inoculum

The inoculum is derived from the secondary effluent of a treatment plant or laboratory scale unit receiving predominantly domestic sewage. See I.6.4.2. and I.6.5.

0,5 ml per litre of mineral medium is used.

#### III.2.4. Preparation of flasks

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/litre. Check the pH value and adjust, if necessary, to 7,4. Inoculate the flasks with sewage effluent at 0,5 ml/litre (see I.6.4.2.). Also prepare inoculum controls in the mineral medium but without test or reference chemical.

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

Also, if required, set up a further, sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6.).

Additionally, if the test chemical is suspected of being significantly adsorbed on to 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). Set up a flask containing the test substance, inoculum and sterilizing agent.

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 (see Annex II.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.

#### III.2.5. Number of flasks in typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

and preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Adsorption control

Flask 8: Toxicity control

See also 1.6.7.

#### III.2.6. Performance of the test

Throughout the test, determine the concentrations of DOC in each flask in duplicate at known time intervals, sufficiently frequently to be able to determine the start of the 10-day window and the percentage removal at the end of the 10-day window. Take only the minimal volume of test suspension necessary for each determination.

Before sampling make good evaporation losses from the flasks by adding dilution water (I.6.1) in the required amount if necessary. Mix the culture medium thoroughly before withdrawing a sample and ensure that material adhering to the walls of the vessels is dissolved or suspended before sampling. Membrane-filter or centrifuge (see Annex II.4) immediately after the sample 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.

### III.3. DATA AND REPORTING

#### III.3.1. Treatment of results

Calculate the percentage degradation at time  $t$  as given under I.7.1. (DOC determination) and, optionally, under I.7.2. (specific analysis).

Record all results on the data sheets provided.

#### III.3.2. Validity of results

See I.5.2.

#### III.3.3. Reporting

See I.8.

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### III.4. DATA SHEET

An example of a data sheet is given hereafter .

#### MODIFIED OECD SCREENING TEST

1. LABORATORY
2. DATE AT START OF TEST
3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre as chemical

Initial concentration in medium, to: mg/litre as chemical

#### 4. INOCULUM

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/l

#### 5. CARBON DETERMINATIONS

Carbon analyser:

|                                      | Flask nr                                   |                              | DOC after n days (mg/l) |                |                |                |                |  |
|--------------------------------------|--|------------------------------|-------------------------|----------------|----------------|----------------|----------------|--|
|                                      |  |                              | 0                       | n <sub>1</sub> | n <sub>2</sub> | n <sub>3</sub> | n <sub>x</sub> |  |
| Test chemical plus inoculum          | 1  | a <sub>1</sub>               |                         |                |                |                |                |  |
|                                      |  | a <sub>2</sub>               |                         |                |                |                |                |  |
|                                      |  | a, mean<br>C <sub>a(t)</sub> |                         |                |                |                |                |  |
|                                      | 2  | b <sub>1</sub>               |                         |                |                |                |                |  |
|                                      |  | b <sub>2</sub>               |                         |                |                |                |                |  |
|                                      |  | b, mean<br>C <sub>b(t)</sub> |                         |                |                |                |                |  |
| Blank inoculum without test chemical | 3  | c <sub>1</sub>               |                         |                |                |                |                |  |
|                                      |  | c <sub>2</sub>               |                         |                |                |                |                |  |
|                                      |  | c, mean<br>C <sub>c(t)</sub> |                         |                |                |                |                |  |
|                                      | 4  | d <sub>1</sub>               |                         |                |                |                |                |  |
|                                      |  | d <sub>2</sub>               |                         |                |                |                |                |  |
|                                      |  | d, mean<br>C <sub>d(t)</sub> |                         |                |                |                |                |  |
|                                      | $C_{b(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$ |                              |                         |                |                |                |                |  |

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6. EVALUATION OF RAW DATA

| Flask nr |   | % degradation after n days |                |                |                |                |
|----------|---|----------------------------|----------------|----------------|----------------|----------------|
|          |   | 0                          | n <sub>1</sub> | n <sub>2</sub> | n <sub>3</sub> | n <sub>x</sub> |
| 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 (*) | $D = \frac{D_1 - D_2}{2}$   | 0                          |                |                |                |                |

(\*) D<sub>1</sub> and D<sub>2</sub> should not be averaged if there is a considerable difference.

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

7. ABIOTIC CONTROL (optional)

|  | Time (days)       |                   |
|--|-------------------|-------------------|
|  | 0                 | t                 |
| DOC conc.(mg/litre)<br>sterile control | C <sub>s(o)</sub> | C <sub>s(t)</sub> |

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

8. SPECIFIC CHEMICAL ANALYSIS (optional)

|                           | residual amount<br>of test chemical<br>at end of test | % primary degradation              |
|---------------------------|---|------------------------------------|
| Steril control            | S <sub>b</sub>  |                                    |
| Inoculated test<br>medium | S <sub>a</sub>  | $\frac{S_b - S_a}{S_b} \times 100$ |

## **PART IV. CO<sub>2</sub> EVOLUTION TEST (Method C.4-C)**

### **IV.1. PRINCIPLE OF THE METHOD**

A measured volume of inoculated mineral medium containing a known concentration of the test chemical (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, which is trapped in barium or sodium hydroxide and which is measured by titration of the residual hydroxide or as inorganic carbon. The amount of carbon dioxide produced from the test chemical (corrected for that derived from the blank inoculum) is expressed as a percentage of ThCO<sub>2</sub>. The degree of biodegradation may also be calculated from supplemental DOC analysis made at the beginning and end of incubation.

### **IV.2. DESCRIPTION OF THE METHOD**

#### **IV.2.1. Apparatus**

- (a) Flasks, 2-5 litres, each fitted with an aeration tube reaching nearly the bottom of the vessel and an outlet;
- (b) magnetic stirrers, when assessing poorly soluble chemicals;
- (c) Gas-absorption bottles;
- (d) Device for controlling and measuring airflow;
- (e) Apparatus for carbon dioxide scrubbing, for preparation of air which is free from carbon dioxide; alternatively, a mixture of CO<sub>2</sub>-free oxygen and CO<sub>2</sub>-free nitrogen, from gas cylinders, in the correct proportions (20% O<sub>2</sub>: 80% N<sub>2</sub>) 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).

#### **IV .2.2. Preparation of mineral medium**

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 l with dilution water.

#### **IV .2.3. Preparation and pre-conditioning of inoculum**

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

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

#### **IV .2.4. Preparation of flasks**

As an example the following volumes and weights indicate the values for 5-litre flasks containing 3 l 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 2400 ml mineral medium. Add an appropriate volume of the prepared activated sludge (see I.6.4.1. and I.6.5.) to give a concentration of suspended solids of not more than 30 mg/l in the final 3 l of inoculated mixture. Alternatively first dilute the prepared sludge to give a suspension of 500-1000 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 I.6.4.2.).

Aerate these inoculated mixtures with CO<sub>2</sub>-free air overnight to purge the system of carbon dioxide.

Add the test material and reference substance, separately, as known volume 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.

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

Also, if required, use a sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6.). Sterilise by the addition of a toxic substance at an appropriate concentration.

Make up the volumes of suspensions in all flasks to 3 l by the addition of mineral medium previously aerated with CO<sub>2</sub>-free air. Optionally, samples may be withdrawn for analysis of DOC (see Annex II.4.) and/or specific analysis. Connect the absorption bottles to the air outlets of the flasks.

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, which is sufficient to absorb the total quantity of carbon dioxide evolved when the test chemical 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.

#### IV.2.5. Number of flasks in a typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

and, preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Toxicity control

See also I.6.7.

#### IV.2.6. Performance of the test

Start the test by bubbling CO<sub>2</sub>-free air through the suspensions at a rate of 30-100 ml/min. Take samples of the carbon dioxide absorbent periodically for analysis of the CO<sub>2</sub>-content. During the first ten days it is recommended that analyses should be made every second or third day and then every fifth day until the 28<sup>th</sup> day so that the 10-day window period can be identified.

On the 28<sup>th</sup> day, withdraw samples (optionally) for DOC and/or specific analysis, measure the pH of the suspensions and add 1 ml of concentrated hydrochloric acid to each flask; aerate the flasks overnight to drive off the carbon dioxide present in the test suspensions. On day 29 make the last analysis of evolved carbon dioxide.

On the days of measurement of CO<sub>2</sub>, disconnect the barium hydroxide absorber closest to the flask and titrate the hydroxide solution with HCl 0,05 M using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the flask 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 NaOH as absorbent, withdraw with a syringe a small sample (depending on the characteristics of the carbon analyser used) of the sodium hydroxide solution in the absorber nearer to the flask. 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 to correct for any carryover of carbon dioxide.

### IV.3. DATA AND REPORTING

#### IV.3.1. Treatment of results

The amount of CO<sub>2</sub> trapped in an absorber when titrated is given by:

$$\text{mgCO}_2 = (100 \times C_B - 0,5 \times V \times C_A) \times 44$$

where:

V = volume of HCl used for titration of the 100 ml in the absorber (ml),

C<sub>B</sub> = concentration of the barium hydroxide solution (M),

C<sub>A</sub> = concentration of the hydrochloric acid solution (M),

if C<sub>B</sub> is 0,0125 M and C<sub>A</sub> is 0,05 M, the titration for 100 ml barium hydroxide is 50 ml and the weight of CO<sub>2</sub> is given by:

$$\frac{0.05}{2} \times 44 \times \text{ml HCl titrated} = 1.1 \times \text{ml HCl}$$

Thus, in this case, to convert volume of HCl titrated to mg CO<sub>2</sub> produced the factor is 1,1.

Calculate the weights of CO<sub>2</sub> produced from the inoculum alone and from the inoculum plus test chemical using the respective titration values and the difference is the weight of CO<sub>2</sub> produced from the test chemical alone.

For example, if the inoculum alone gives a titration of 48 ml and inoculum plus test chemical gives 45 ml,

$$\text{CO}_2 \text{ from inoculum} = 1,1 \times (50-48) = 2,2 \text{ mg}$$

$$\text{CO}_2 \text{ from inoculum plus test chemical} = 1,1 \times (50-45) = 5,5 \text{ mg}$$

and thus the weight of CO<sub>2</sub> produced from the test chemical is 3,3 mg.

The percentage biodegradation is calculated from:

$$\% \text{ degradation} = (\text{mg CO}_2 \text{ produced} \times 100) / (\text{ThCO}_2 \times \text{mg test chemical added})$$

or,

$$\% \text{ degradation} = (\text{mg CO}_2 \text{ produced} \times 100) / (\text{mg TO added in test} \times 3.67)$$

3,67 being the conversion factor (44/12) for carbon to carbon dioxide.

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Obtain the percentage degradation after any time interval by adding the percentage of ThCO<sub>2</sub> values calculated for each of the days, up to that time, on which it was measured.

For sodium hydroxide absorbers, calculate the amount of carbon dioxide produced, expressed as IC (mg), by multiplying the concentration of IC in the absorbent by the volume of the absorbent.

Calculate the percentage degradation from :

$$\% \text{ of ThCO}_2 = (\text{mg IC flask} - \text{mg IC blank} \times 100) / (\text{MG TOC added as test chemical})$$

Calculate DOC removals (optional) as described under I.7. Record these and all other results on the data sheets provided.

#### IV.3.2. Validity of results

The IC content of the test chemical suspension in the mineral medium at the beginning of the test must be less than 5 % of the TC, and the total CO<sub>2</sub> 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<sub>2</sub>/litre are obtained, the data and experimental technique should be examined critically.

See also I.5.2.

#### IV.3.3. Reporting

See I.8.

### IV.4. DATA SHEET

An example of a data sheet is given hereafter .

#### CARBON DIOXIDE EVOLUTION TEST

##### 1. LABORATORY

##### 2. DATE AT START OF TEST

##### 3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre as chemical

Initial conc. in medium: mg/litre as chemical

Total C added to flask: mg C

ThCO<sub>2</sub>: mg CO<sub>2</sub>

##### 4. INOCULUM

Source:

Treatment given:

Pre-conditioning if any:

Concentration of suspended solids in reaction mixture: mg/litre

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| Time (day)     | CO <sub>2</sub> formed Test (mg) |   | CO <sub>2</sub> formed blank (mg) |   | CO <sub>2</sub> formed cumulative (mg) (test minus blank mean) |   | ThCO <sub>2</sub> $\frac{\text{cumulative CO}_2}{\text{ThCO}_2} \times 100$ |   |      |
|----------------|----------------------------------|---|-----------------------------------|---|--|---|---|---|------|
|                | 1                                | 2 | 3                                 | 4 | 1  | 2 | 1   | 2 | mean |
|                | mean                             |   | mean                              |   |  |   |   |   |      |
| 0              |                                  |   |                                   |   |  |   |   |   |      |
| n <sub>1</sub> |                                  |   |                                   |   |  |   |   |   |      |
| n <sub>2</sub> |                                  |   |                                   |   |  |   |   |   |      |
| n <sub>3</sub> |                                  |   |                                   |   |  |   |   |   |      |
|                |                                  |   |                                   |   |  |   |   |   |      |
|                |                                  |   |                                   |   |  |   |   |   |      |
| 28             |                                  |   |                                   |   |  |   |   |   |      |

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

#### 6. CARBON ANALYSIS (optional)

Carbon analyser:

| Time (day)                  | Blank mg/l        | Test chemical mg/l |
|-----------------------------|-------------------|--------------------|
| 0                           | C <sub>b(o)</sub> | C <sub>o</sub>     |
| 28 (*)                      | C <sub>b(t)</sub> | C <sub>t</sub>     |
| (*) or at end of incubation |                   |                    |

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

#### 7. ABIOTIC DEGRADATION (optional)

$$\% \text{ abiotic degradation} = \frac{\text{CO}_2 \text{ formation in sterile flask after 28 day (mg)}}{\text{ThCO}_2 \text{ (mg)}} \times 100$$

## PART V. MANOMETRIC RESPIROMETRY TEST (Method C.4-D)

### V.1. PRINCIPLE OF THE METHOD

A measured volume of inoculated mineral medium, containing a known concentration of test chemical (100 mg/litre of the test substance, to give at least 50-100 mg ThOD/litre) as the nominal sole source of organic carbon, is stirred in a closed flask at a constant temperature ( $\pm 1^\circ \text{C}$  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 test chemical (corrected for uptake by blank inoculum, run in parallel) is expressed as a percentage of ThOD or COD. Optionally, primary biodegradation may also be calculated from supplemental specific analysis made at the beginning and end of incubation, and ultimate biodegradation by DOC analysis.

### V.2. DESCRIPTION OF THE METHOD

#### V.2.1. Apparatus

- (a) suitable respirometer;
- (b) temperature control, maintaining  $\pm 1^\circ \text{C}$  or better;
- (c) membrane-filtration assembly (optional);
- (d) carbon analyser (optional).

#### V.2.2. Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 litre with dilution water.

#### V.2.3. Preparation and pre-conditioning of inoculum

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

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

#### V.2.4. Preparation of flasks

Prepare solutions of the test and reference chemicals, in separate batches, in mineral medium equivalent to a concentration, normally, of 100 mg chemical/litre (giving at least 50-100 mg ThOD/litre), 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 II.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 (see below).

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

If measurement of the physico-chemical uptake of oxygen is required, prepare a solution of the test chemical at, normally, 100 mg ThOD/litre which has been sterilised by the addition of a suitable toxic substance (see I.6.6.).

Introduce the requisite volume of solutions of test and reference chemicals, respectively, into at least duplicate flasks. Add to further flasks mineral medium only (for inoculum controls) and, if required, the mixed test/ reference chemical solution and the sterile solution.

If the test chemical 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<sub>2</sub>-absorber compartments.

#### IV.2.5. Number of flasks in a typical run

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

preferably, and when necessary:

Flask 6: Sterile control

Flask 7: Toxicity control

See also I.6.7.

#### V.2.6. Performance of the test

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/litre. Assemble the equipment, start the stirrer and check for air-tightness, 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.

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 ThODNH<sub>4</sub> (for nitrogen-containing compounds).

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

### V.3. DATA AND REPORTING

#### V.3.1. Treatment of results

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

$$\begin{aligned} \text{BOD} &= (\text{mg O}_2 \text{ uptake by test chemical} - \text{mg O}_2 \text{ uptake by blank}) / (\text{mg test chemical in flask}) \\ &= \text{mg O}_2 \text{ per mg test chemical.} \end{aligned}$$

calculate the percentage biodegradation either from:

$$\% \text{ biodegradation} = \% \text{ ThOD} = \text{BOD (mg O}_2\text{/mg chemical)} \times 100 / \text{ThOD (mg O}_2 \text{ chemical)}$$

or from

$$\% \text{ COD} = \text{BOD (mg O}_2\text{/mg chemical)} \times 100 / \text{COD (mg O}_2 \text{ chemical)}$$

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

For test substances containing nitrogen, use the appropriate ThOD ( $\text{NH}_4$  or  $\text{NO}_3$ ) according to what is known or expected about the occurrence of nitrification (Annex II.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 (Annex V).

When optional determinations of organic carbon and/or specific chemical are made, calculate the percentage degradation, as described under I.7.

Record all results on the data sheets attached.

#### V.3.2. Validity of results

The oxygen uptake of the inoculum blank is normally 20-30 mg  $\text{O}_2$ /litre and should not be greater than 60 mg/litre in 28 days. Values higher than 60 mg/litre require critical examination of the data and experimental techniques. If the pH value is outside the range 6-8,5 and the oxygen consumption by the test chemical is less than 60% , the test should be repeated with a lower concentration of test chemical.

See also I.5.2.

#### V.3.3. Reporting

See I.8.

### V.4. DATA SHEET

An example of a data sheet is given hereafter .

#### MANOMETRIC RESPIROMETRY TEST

1. LABORATORY
2. DATE AT START OF TEST
3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre

Initial concentration in medium,  $C_0$ : mg/litre

Volume in test flask (V): ml

ThOD or COD: mg  $\text{O}_2$ /mg test substance ( $\text{NH}_4$  or  $\text{NO}_3$ )

#### 4. INOCULUM

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/l

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5. OXYGEN UPTAKE: BIODEGRADABILITY

|  |                                    | Zeit (Tage) |  |   |  |    |  |    |  |    |  |  |  |  |
|--|------------------------------------|-------------|--|---|--|----|--|----|--|----|--|--|--|--|
|  |                                    | 0           |  | 7 |  | 14 |  | 21 |  | 28 |  |  |  |  |
| O <sub>2</sub> upt. (mg) test chemical | 1                                  |             |  |   |  |    |  |    |  |    |  |  |  |  |
|  | 2                                  |             |  |   |  |    |  |    |  |    |  |  |  |  |
|  | a, mean                            |             |  |   |  |    |  |    |  |    |  |  |  |  |
| O <sub>2</sub> upt. (mg) blank         | 3                                  |             |  |   |  |    |  |    |  |    |  |  |  |  |
|  | 4                                  |             |  |   |  |    |  |    |  |    |  |  |  |  |
|  | b, mean                            |             |  |   |  |    |  |    |  |    |  |  |  |  |
| Corrected BOD (mg)                     | (a <sub>1</sub> - b <sub>m</sub> ) |             |  |   |  |    |  |    |  |    |  |  |  |  |
|  | (a <sub>2</sub> - b <sub>m</sub> ) |             |  |   |  |    |  |    |  |    |  |  |  |  |
| BOD per mg test chemical               | $\frac{(a_1 - b)}{C_0 V}$          |             |  |   |  |    |  |    |  |    |  |  |  |  |
|  | $\frac{(a_2 - b)}{C_0 V}$          |             |  |   |  |    |  |    |  |    |  |  |  |  |
| % degradation                          | D <sub>1</sub> (a <sub>1</sub> )   |             |  |   |  |    |  |    |  |    |  |  |  |  |
|  | D <sub>2</sub> (a <sub>2</sub> )   |             |  |   |  |    |  |    |  |    |  |  |  |  |
| $\frac{BOD}{ThOD} \times 100$          | Mean *                             |             |  |   |  |    |  |    |  |    |  |  |  |  |

V = volume of medium in test flask  
 \* D<sub>1</sub> and D<sub>2</sub> should not be averaged if there is a considerable difference.  
 N.B.: Similar formats may be used for the reference chemical and the toxicity controls.

6. CORRECTION FOR NITRIFICATION (see Annex V)

| Day   | 0 | 28 | Difference |
|---|---|----|------------|
| (i) Concentration of nitrate (mg N/litre)   |   |    | (N)        |
| (ii) Oxygen equivalent (4,57 × N × V) (mg)  | — | —  |            |
| (iii) Concentration of nitrite (mg N/litre) |   |    | (N)        |
| (iv) Oxygen equivalent (3,43 × N × V) (mg)  | — | —  |            |
| (ii + iv) Total oxygen equivalent           | — | —  |            |

7. CARBON ANALYSIS (optional)

Carbon analyser:

| Time (day) | Blank mg/litre      | Test chemical mg/litre |
|------------|---------------------|------------------------|
| 0          | (C <sub>blo</sub> ) | (C <sub>o</sub> )      |
| 28*        | (C <sub>bt</sub> )  | (C <sub>t</sub> )      |

\* or at end of incubation



$$\% \text{ DOC removed} = \left( 1 - \frac{C_t - C_{\text{blt}}}{C_0 - C_{\text{blo}}} \right) \times 100$$

#### 8. SPECIFIC CHEMICAL (optional)

$S_b$  = concentration in physico-chemical (sterile) control at 28 days

$S_a$  = concentration in inoculated flask at 28 days,

$$\% \text{ biodegradation} = \frac{S_b - S_a}{S_b} \times 100$$

#### 9. ABIOTIC DEGRADATION (optional)

$a$  = oxygen consumption in sterile flasks after 28 days, (mg)

$$\text{oxygen consumption per mg test chemical} = \frac{ax100}{C_0 V}$$

(see sections 1 and 3)

$$\% \text{ abiotic degradation} = \frac{ax100}{C_0 Vx\text{ThOD}}$$

### Part VI. CLOSED BOTTLE TEST (Method C.4-E)

#### VI.1 PRINCIPLE OF THE TEST METHOD

The solution of the test chemical in mineral medium, usually at 25 mg/litre, 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-day period. The amount of oxygen taken up by the test chemical, corrected for uptake by the blank inoculum run in parallel, is expressed as a percentage of ThOD or COD.

#### VI.2 DESCRIPTION OF THE METHOD

##### VI.2.1. Apparatus

- BOD bottles, with glass stoppers, e.g. 250-300 ml;
- Water bath or incubator, for keeping bottles at constant temperature ( $\pm 1$  °C or better) with the exclusion of light;
- Large glass bottles (2-5 litres) for the preparation of media and for filling the BOD bottles;
- Oxygen electrode and meter, or equipment and reagents for Winkler titration.

##### VI.2.2. Preparation of mineral medium

For the preparation of the stock solutions, see I.6.2.

Mix 1 (one) ml of solution (a) to (d) and make up to 1 litre with dilution water.

### VI.2.3. Preparation of the inoculum

The inoculum is normally derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. An alternative source for the inoculum is surface water. 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 (See I.6.4.2. and I.6.5.).

### VI.2.4. Preparation of flasks

Strongly aerate mineral medium for at least 20 min. Carry out each test series with mineral medium derived from the same batch. Generally, the medium is ready for use after standing for 20 h, at the test temperature. Determine the concentration of dissolved oxygen for control purposes; the value should be about 9 mg/litre at 20 °C. Conduct all transfer and filling operations of the air-saturated medium bubble-free, for example, by the use of siphons.

Prepare parallel groups of BOD bottles for the determination of the test and reference chemicals 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 days. To ensure being able to identify the 10-day window, more bottles may be required.

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 chemical and reference chemical to separate large bottles so that the final concentration of the chemicals is normally not greater than 10 mg/litre. Add no chemicals to the blank control medium contained in a further large bottle.

In order to ensure that the inoculum activity is not limited, the concentration of dissolved oxygen must not fall below 0,5 mg/litre in the BOD bottles. This limits the concentration of test chemical to about 2 mg/litre. However, for poorly degradable compounds and those with a low ThOD, 5-10 mg/litre can be used. In some cases, it would be advisable to run parallel series of test chemical at two different concentrations, for example, 2 and 5 mg/litre. 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<sub>NO3</sub>: see Annex II.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).

If the toxicity of the test chemical 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 chemical and reference chemical at final concentrations normally the same as those in the other large bottles.

Inoculate the solutions in the large bottles with secondary effluent (one drop or about 0,05 ml, to 5 ml/litre) or with another source such as river water (see I.6.4.2.). 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.

### VI.2.5. Number of flasks in a typical run

In a typical run the following bottles are used:

at least 10 containing test chemical and inoculum (test suspension),

at least 10 containing only inoculum (inoculum blank),

at least 10, containing reference chemical and inoculum (procedure control),

and, when necessary, 6 bottles containing test chemical, reference chemical and inoculum (toxicity control). However, to ensure being able to identify the 10-day window, about twice as many bottles would be necessary.

#### VI.2.6. Performance of the test

Dispense each prepared solution 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. Tap gently to remove any air bubbles. 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) sulfate and sodium hydroxide (the first Winkler reagent). Store the carefully stoppered bottles, containing the oxygen fixed as brown manganese (III) hydrated oxide, in the dark at 10-20 °C for no longer than 24 hours 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. 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-day window, whereas sampling every 3-4 days should allow the 10-day window to be identified, which would require about twice as many bottles.

For N-containing test substances, corrections for uptake of oxygen by any nitrification occurring should be made. To do this, use the O<sub>2</sub>-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).

### VI.3. DATA AND REPORTING

#### VI.3.1. Treatment of results

First calculate the BOD exerted after each time period by subtracting the oxygen depletion (mg O<sub>2</sub>/litre) of the inoculum blank from that exhibited by the test chemical. Divide this corrected depletion by the concentration (mg/litre) of the test chemical, to obtain the specific BOD as mg oxygen per mg test chemical. Calculate the percentage biodegradability by dividing the specific BOD by the specific ThOD (calculated according to Annex II.2) or COD (determined by analysis, see Annex II.3), thus:

$$\text{BOD} = (\text{mg O}_2 \text{ uptake by test chemical} - \text{mg O}_2 \text{ uptake by blank}) / \text{mg test chemical in flask} \\ = \text{mg O}_2 \text{ per mg test chemical}$$

$$\% \text{ degradation} = \text{BOD (mg O}_2 / \text{mg test chemical} \times 100) / \text{ThOD (mg O}_2 / \text{mg test chemical)}$$

or

$$\% \text{ degradation} = \text{BOD (mg O}_2 / \text{mg test chemical} \times 100) / \text{COD (mg O}_2 / \text{mg test chemical)}$$

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

For test substances containing nitrogen, use the appropriate ThOD (NH<sub>4</sub> or NO<sub>3</sub>) according to what is known or expected about the occurrence of nitrification (Annex II.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 (Annex V).

#### VI.3.2. Validity of results

Oxygen depletion in the inoculum blank should not exceed 1,5 mg dissolved oxygen/litre 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/litre at any time. Such low oxygen levels are

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valid only if the method of determining dissolved oxygen used is capable of measuring such levels accurately.

See also I.5.2.

### VI.3.3. Reporting

See I.8.

## VI.4. DATA SHEET

An example of a data sheet is given hereafter.

### CLOSED BOTTLE TEST

#### 1. LABORATORY

#### 2. DATE AT START OF TEST

#### 3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/litre

Initial concentration in bottle: mg/litre

ThOD or COD: mg O<sub>2</sub>/mg test substance

#### 4. INOCULUM

Source:

Treatment given:

Pre-conditioning if any:

Concentration in the reaction mixture: mg/litre

#### 5. DO DETERMINATION

Method: Winkler/electrode

### Flask Analyses

|                          | Time of incubation (d)      |                | DO (mg/l) |                |                |  |
|--------------------------|-----------------------------|----------------|-----------|----------------|----------------|--|
|                          |                             |                | 0         | n <sub>1</sub> | n <sub>2</sub> |  |
| Blank (without chemical) | 1                           | C <sub>1</sub> |           |                |                |  |
|                          | 2                           | C <sub>2</sub> |           |                |                |  |
| Mean                     | $m_b = \frac{C_1 + C_2}{2}$ |                |           |                |                |  |
| Test chemical            | 1                           | a <sub>1</sub> |           |                |                |  |
|                          | 2                           | a <sub>2</sub> |           |                |                |  |
| Mean                     | $m_t = \frac{a_1 + a_2}{2}$ |                |           |                |                |  |

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

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6. CORRECTION FOR NITRIFICATION (see Annex V)

| Time of incubation (d)                            | 0 | n <sub>1</sub> | n <sub>2</sub> | n <sub>3</sub> |
|---|---|----------------|----------------|----------------|
| (i) Concentration of nitrate (mg N/litre)         |   |                |                |                |
| (ii) Change in nitrate concentration (mg N/litre) | — |                |                |                |
| (iii) Oxygen equivalent (mg/litre)                | — |                |                |                |
| (iv) Concentration of nitrite (mg N/litre)        |   |                |                |                |
| (v) Change in nitrite concentration (mg N/litre)  | — |                |                |                |
| (vi) Oxygen equivalent (mg/litre)                 | — |                |                |                |
| (iii + vi) Total oxygen equivalent (mg/litre)     | — |                |                |                |

7. DO DEPLETION: % DEGRADATION

|  | Depletion after n days (mg/litre) |                |                |
|--|-----------------------------------|----------------|----------------|
|  | n <sub>1</sub>                    | n <sub>2</sub> | n <sub>3</sub> |
| FLASK 1: (m <sub>to</sub> - m <sub>tx</sub> ) - (m <sub>bo</sub> - m <sub>bx</sub> )   |                                   |                |                |
| FLASK 2: (m <sub>to</sub> - m <sub>tx</sub> ) - (m <sub>bo</sub> - m <sub>bx</sub> )   |                                   |                |                |
| FLASK 1:<br>$\% D_1 = \frac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test} \times \text{ThOD chemical}}$ |                                   |                |                |
| FLASK 2:<br>$\% D_2 = \frac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test} \times \text{ThOD chemical}}$ |                                   |                |                |
| $\% D \text{ mean } * = \frac{D_1 + D_2}{2}$   |                                   |                |                |

(\* ) Do not take mean if there is considerable difference between duplicates.

- m<sub>to</sub> = value in the flask at time 0
- m<sub>tx</sub> = value in the flask at time x
- m<sub>bo</sub> = mean blank value at time 0
- m<sub>bx</sub> = mean blank value at time x

Apply also correction for nitrification from iii + vi in section 6.

8. BLANK DO DEPLETIONS

Oxygen consumption by blank: (m<sub>bo</sub> - m<sub>b28</sub>) mg/litre. This consumption is important for the validity of the test. It should be less than 1,5 mg/litre.

## PART VII. M.I.T.I. TEST (Method C.4F)

### VII.1. PRINCIPLE OF THE METHOD

The oxygen uptake by a stirred solution, or suspension, of the test chemical 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  $25 \pm 1$  °C. Evolved carbon dioxide is absorbed by soda lime. Biodegradability is expressed as the percentage oxygen uptake (corrected for blank uptake) of the theoretical uptake (ThOD). The percentage of primary biodegradability is also calculated from supplemental specific chemical analysis made at the beginning and end of incubation and, optionally, by DOC analysis.

### VII.2. DESCRIPTION OF THE METHOD

#### VII.2.1. Apparatus

- (a) Automatic electrolytic BOD meter or respirometer normally equipped with 6 bottles, 300 ml each and equipped with cups to contain CO<sub>2</sub> absorbent;
- (b) Constant temperature room and/ or water-bath at  $25 \text{ °C} \pm 1 \text{ °C}$  or better;
- (c) Membrane-filtration assembly (optional);
- (d) Carbon analyser (optional).

#### VII.2.2. Preparation of mineral medium

Prepare the following stock solutions, using analytical grade reagents and water (I.6.1.):

- |  |          |
|--|----------|
| (a) Monopotassium dihydrogen ortho phosphate, KH <sub>2</sub> PO <sub>4</sub>                            | 8,50 g   |
| Dipotassium monohydrogen ortho phosphate, K <sub>2</sub> HPO <sub>4</sub>                                | 21,75 g  |
| Disodium monohydrogen ortho phosphate dodecahydrate Na <sub>2</sub> HPO <sub>4</sub> 12 H <sub>2</sub> O | 44,60 g  |
| Ammonium chloride, NH <sub>4</sub> Cl  | 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, MgSO <sub>4</sub> 7 H <sub>2</sub> O                                | 22,50 g  |
| Dissolve in water and make up to 1 litre   |          |
| (c) Calcium chloride anhydrous, CaCl <sub>2</sub>  | 27 ,50 g |
| Dissolve in water and make up to 1 litre   |          |
| (d) Iron (III) chloride hexahydrate, FeCl <sub>3</sub> 6 H <sub>2</sub> O                                | 0,25 g   |
| Dissolve in water and make up to 1 litre   |          |

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

#### VII.2.3. Preparation of inoculum

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, rivers, lakes, seas, collect 11 samples of sludge, surface soil, water, etc. and mix thoroughly together. After removing floating matter and allowing to stand, adjust the supernatant to pH  $7 \pm 1$  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 1/2 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 monopotassium ortho phosphate, to the settled material and recommence aeration. Repeat this procedure once per day. The sludge unit must be operated according to good practice: effluents should be clear, temperature should be kept at  $25 \pm 2^\circ \text{C}$ , pH should be  $7 \pm 1$ , sludge should settle well, 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 irregular 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.

#### VII.2.4. Preparation of flasks

Prepare the following six flasks:

Nr. 1: test chemical in dilution water at 100 mg/l

Nr. 2,3 and 4: test chemical in mineral medium at 100 mg/l

Nr. 5: reference chemical (e.g. aniline) in mineral medium at 100 mg/l

Nr. 6: mineral medium only

Add poorly soluble test chemicals directly on a weight or volume basis or handle as described in Annex III, except that neither solvents nor emulsifying agents should be used. Add the  $\text{CO}_2$  absorbent to all flasks in the special cups provided. Adjust the pH in flasks nr. 2, 3 and 4 to 7,0.

#### VII.2.5. Performance of the test

Inoculate flasks nr. 2, 3 and 4 (test suspensions), nr. 5 (activity control) and nr. 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 flask nr. 1 which serves as an abiotic control. Assemble the equipment, check for airtightness, start the stirrers, and start the measurement of oxygen uptake under conditions of darkness. Daily check the temperature, stirrer and coulometric oxygen uptake recorder, and note any changes in colour of the contents of the flasks. Read the oxygen uptakes for the six flasks directly by an appropriate method, for example, from the six-point chart recorder, which produces a BOD curve. At the end of incubation, normally 28 days, measure the pH of the contents of the flasks and determine the concentration of the residual test chemical and any intermediate and, in the case of water soluble substance, the concentration of DOC (Annex II.4). Take special care in the case of volatile chemicals. If nitrification is anticipated, determine nitrate and nitrite concentration, if possible.

### VII.3. DATA AND REPORTING

#### VII.3.1. Treatment of results

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

$$\text{BOD} = (\text{mg O}_2 \text{ uptake by test chemical} - \text{mg O}_2 \text{ uptake by blank}) / \text{mg test chemical in flask} \\ = \text{mg O}_2 / \text{mg test chemical}.$$

The percentage biodegradation is then obtained from:

% biodegradation = % ThOD = BOD (mg O<sub>2</sub>/mg chemical) x 100 / ThOD (mg O<sub>2</sub>/mg chemical)

For mixtures, calculate the ThOD from the elemental analysis, as for simple compound. Use the appropriate ThOD (ThOD<sub>NH<sub>4</sub></sub> or ThOD<sub>NO<sub>3</sub></sub>) according to whether nitrification is absent or complete (Annex II.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).

Calculate the percentage primary biodegradation from loss of specific (parent) chemical (see 1.7.2).

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

If there has been a loss of test chemical in the flask nr. 1 measuring physico-chemical removal, report this and use the concentration of test chemical (S<sub>b</sub>) after 28 days in this flask to calculate the percentage biodegradation.

When determinations of DOC are made (optional), calculate the percentage ultimate biodegradation from:

$$D_t = \left( 1 - \frac{C_t - C_{bt}}{C_o - C_{bo}} \right) \times 100\%$$

as described under point I.7.1. If there has been a loss of DOC in the flask nr .1, measuring physico-chemical removal, use the DOC concentration in this flask to calculate the percentage biodegradation.

Record all results on the data sheets attached.

### VII.3.2. Validity of results

The oxygen uptake of the inoculum blank is normally 20-30 mg O<sub>2</sub>/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 techniques. If the pH value is outside the range 6-8,5 and the oxygen consumption by the test chemical is less than 60 % , the test should be repeated with a lower concentration of test chemical.

See also I.5.2.

If the percentage degradation of aniline calculated from the oxygen consumption does not exceed 40% after 7 days and 65% after 14 days, the test is regarded as invalid.

### VII.3.3. Reporting

See I.8.

## VII.4. DATA SHEET

An example of a data sheet is given below.

### MITI (I) TEST

#### 1. LABORATORY

#### 2. DATE AT START OF TEST



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### 3. TEST SUBSTANCE

Name:

Stock solution concentration: mg/l as chemical

Initial concentration in medium,  $C_0$ : 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 acclimatization with synthetic sewage = ... mg/l

Volume of activated sludge per litre of final medium = ...ml

Concentration of sludge in final medium = ...mg/l

### 5. OXYGEN UPTAKE: BIODEGRADABILITY

Type of respirometer used:

|  |  | Zeit (Tage) |   |    |    |    |
|--|--|-------------|---|----|----|----|
|  |  | 0           | 7 | 14 | 21 | 28 |
| O <sub>2</sub> upt. (mg) test chemical | a <sub>1</sub>   |             |   |    |    |    |
|  | a <sub>2</sub>   |             |   |    |    |    |
|  | a <sub>3</sub>   |             |   |    |    |    |
| O <sub>2</sub> upt. (mg) blank         | b  |             |   |    |    |    |
| Corrected O <sub>2</sub> upt. (mg)     | (a <sub>1</sub> - b)<br>(a <sub>2</sub> - b)<br>(a <sub>3</sub> - b) |             |   |    |    |    |
| BOD per mg test chemical               | $\frac{(a-b)}{C_0 V}$  | Flask 1     |   |    |    |    |
|  |  | Flask 2     |   |    |    |    |
|  |  | Flask 3     |   |    |    |    |
| % degradation                          | $\frac{BOD}{ThOD} \times 100$  | 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.

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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        |  | —         |  | —             | —    |

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

7. SPECIFIC CHEMICAL ANALYTICAL DATA

|                       | Residual amount of test chemical at end of test | % degradation |
|-----------------------|---|---------------|
| blank test with water | S <sub>b</sub>                                  |               |
| inoculated medium     | S <sub>a1</sub>                                 |               |
|                       | S <sub>a2</sub>                                 |               |
|                       | S <sub>a3</sub>                                 |               |

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

Calculate % degradation for flasks a1, a2 and a3 respectively

8. REMARKS

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

## ANNEX I

### ABBREVIATIONS AND DEFINITIONS

- DO: Dissolved oxygen (mg/l) is the concentration of oxygen dissolved in an aqueous sample.
- BOD: Biochemical oxygen demand (g) is the amount of oxygen consumed by micro-organisms when metabolizing a test compound; also expressed as g oxygen uptake per g test compound. (See method C.5).
- COD: Chemical oxygen demand (g) 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 g oxygen consumed per g test compound. (See method C.6).
- 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 40000 m.s<sup>-2</sup> (± 4000 g) 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 II.2) and is also expressed as mg oxygen required per mg test compound.
- ThCO<sub>2</sub>: 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:

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

#### Ultimate Biodegradation (aerobic):

is 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 recognized 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 not all inherently biodegradable compounds are. Abiotic processes may also operate.

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Lag time

is the time from inoculation, in a die-away test, until the degradation percentage has increased to at least 10%. The lag time is often highly variable and poorly reproducible.

Degradation time

is the time from the end of the lag time till the time that 90% of maximum level of degradation has been reached.

10-day window

is the 10 days immediately following the attainment of 10% degradation.

## ANNEX II

### 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 known or determined by elemental analysis. It is for the compound:



without nitrification,

$$\text{ThOD}_{\text{NH}_4} = \frac{16[2c + 1/2(h - cl - 3n) + 3s + 5/2p + 1/2na - o]}{\text{MW}} \text{ mg/mg}$$

or with nitrification

$$\text{ThOD}_{\text{NO}_3} = \frac{16[2c + 1/2(h - cl) + 5/2n + 3s + 5/2p + 1/2na - o]}{\text{MW}} \text{ mg/mg}$$

#### 3. Chemical Oxygen Demand (COD)

The Chemical oxygen demand (COD) is determined according to method C.6.

#### 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 micrometre 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 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 2-4 °C for 48 h, or below -18 °C for longer periods.

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 40000 m.sec<sup>-2</sup> (4000 g) for 15 min may be used for differentiation of TOC versus DOC instead of filtration. The method is not reliable at initial concentration of < 10 mg DOC/l since either not all bacteria are removed or carbon as part of the bacterial plasma is redissolved.

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### ANNEX III

#### EVALUATION OF THE BIODEGRADABILITY OF POORLY SOLUBLE SUBSTANCES

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

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.

Various forms of agitation during the tests 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.

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.

The same criteria apply to solvents as to the emulsifiers.

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

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

Any of the three respirometric tests CO<sub>2</sub>, BOD, MITI can be used to study the biodegradability of poorly soluble compounds.

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

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#### ANNEX IV

##### 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).

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

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 (activated sludge respiration inhibition test - Dir 87/302/EEC), BOD and/or Growth Inhibition methods would seem appropriate.

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<sub>50</sub> values (or less than EC<sub>20</sub> values) obtained in toxicity testing. Compounds with an EC<sub>50</sub> value of greater than 300 mg/l are not likely to have toxic effects in ready biodegradability testing.

EC<sub>50</sub> 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 <sup>14</sup>C -labelled material. Alternatively, an acclimatised inoculum may permit higher test substance concentrations to be used. In the latter case, however, the specific criterion of the ready biodegradability test is lost.

#### BIBLIOGRAPHY

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

#### ANNEX V

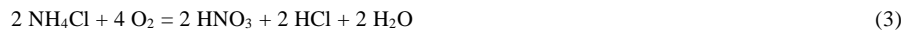
##### CORRECTION FOR OXYGEN UPTAKE FOR INTERFERENCE BY NITRIFICATION

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 nitrification has occurred but is not complete the observed oxygen uptake by the reaction mixture may be corrected for the amount of oxygen used in oxidising ammonium to nitrite and nitrate, if the changes in concentration during incubation of nitrite and nitrate are determined by consideration of the following equations:



Overall:



From equation (1), the oxygen uptake by 28 g of nitrogen contained in ammonium chloride ( $\text{NH}_4\text{Cl}$ ) in being oxidised to nitrite is 96 g, i.e. a factor of 3,43 (96/28). In the same way, from equation (3) the oxygen uptake by 28 g of nitrogen in being oxidised to nitrate is 128 g, i.e. a factor of 4,57 (128/28).

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, whereas the oxygen associated with the formation of nitrite is 3,43 multiplied by the increase in the concentration of nitrite or with the decrease in its concentration the oxygen loss is -3,43 multiplied by the decrease in concentration.

That is:

$$\text{O}_2 \text{ consumed in nitrate formation} = 4,57 \times \text{increase in nitrate concentration} \quad (4)$$

and

$$\text{O}_2 \text{ consumed in nitrite formation} = 3,43 \times \text{increase in nitrite concentration} \quad (5)$$

and

$$\text{O}_2 \text{ lost in nitrite disappearance} = -3,43 \times \text{decrease in nitrate concentration} \quad (6)$$

So that

$$\text{O}_2 \text{ uptake due to nitrification} = \pm 3,43 \times \text{change in nitrite conc.} + 4,57 \times \text{increase in nitrate conc.} \quad (7)$$

and thus

$$\text{O}_2 \text{ uptake due to C oxidation} = \text{total observed uptake} - \text{uptake due to nitrification} \quad (8)$$

Alternatively, if only total oxidized N is determined, the oxygen uptake due to nitrification may be taken to be, as a first approximation, 4,57 x increase in oxidised N.

The corrected value for oxygen consumption due to C oxidation is then compared with ThOD  $\text{NH}_3$ , as calculated in Annex II.