INTRODUCTION

1. Matters of general interest concerning the assessment of biodegradability are discussed in "General Procedures and Preparations" and it is advisable to read this before proceeding. For this method, the formula of the test substance and its purity, or relative proportions of major components, should be known so that the ThOD may be calculated. If the ThOD cannot be calculated, the COD should be determined, but falsely high values of percentage biodegradation may be obtained if the test substance is incompletely oxidised in the COD test. Insoluble and volatile substances may be assessed provided precautions are taken.

PRINCIPLE OF THE TEST

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

DESCRIPTION OF THE METHOD

Apparatus

3. Normal laboratory apparatus and:

   (a) Suitable respirometer;
   (b) Temperature control, maintaining ± 1°C or better;
   (c) Membrane-filtration assembly (optional);
   (d) Carbon analyser (optional).

Water

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

Stock solutions for mineral medium

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

Preparation of mineral medium

6. Refer to 301 A (paragraph 6).
Stock solutions of test substances

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

Inoculum

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

Pre-conditioning of inocula

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

Preparation of flasks

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

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

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

13. Introduce the requisite volume of solutions of test and reference substances, respectively, into at least duplicate respirometer flasks. Add to further flasks mineral medium only (for inoculum controls) and, if required, the mixed test/reference substance solution and the sterile solution. If the test substance is poorly soluble, add it directly at this stage on a weight or volume basis or handle it as described in Annex III. Add potassium hydroxide, soda-lime pellets or other absorbent to the CO₂-absorber compartments.

Number of flasks

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

<table>
<thead>
<tr>
<th>Flasks 1 &amp; 2</th>
<th>containing test substance and inoculum (test suspension);</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flasks 3 &amp; 4</td>
<td>containing only inoculum (inoculum blank);</td>
</tr>
<tr>
<td>Flask 5</td>
<td>containing reference compound and inoculum (procedure control); and, preferably and when necessary, also</td>
</tr>
<tr>
<td>Flask 6</td>
<td>containing test substance and sterilising agent (abiotic sterile control);</td>
</tr>
<tr>
<td>Flask 7</td>
<td>containing test substance, reference compound and inoculum (toxicity control).</td>
</tr>
</tbody>
</table>
PROCEDURE

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

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

17. Calculate the oxygen uptake from the readings taken at regular and frequent intervals, using the methods given by the manufacturer of the equipment. At the end of incubation, normally 28 days, measure the pH of the contents of the flasks, especially if oxygen uptakes are low or greater than ThOD NH₃, for N-containing chemicals.

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

DATA AND REPORTING

Treatment of results

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

20. First calculate the BOD (mg O₂/mg test chemical) exerted after each time period by dividing the oxygen uptake (mg) of the test chemical, corrected for that by the blank inoculum control, by the weight of the test chemical used, i.e.:

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

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

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

Validity of results

22. The oxygen uptake of the inoculum blank is normally 20-30 mg O₂/l and should not be greater than 60 mg/l in 28 days. Values higher than 60 mg/l require critical examination of the data and experimental technique. If the pH value is outside the range 6-8.5 and the oxygen consumption by the test substance is less than 60%, the test should be repeated with a lower concentration of test substance.
23. The validity criteria given in "Data and Reporting" (p. 7) apply.

**Test report**

24. The test report should include the information described in "Data and Reporting" (p. 8).
MANOMETRIC RESPIROMETRY TEST
DATA SHEET

1. LABORATORY:

2. DATE AT START OF TEST:

3. TEST SUBSTANCE:

   Name:
   Stock solution concentration: mg/l
   Initial concentration in medium, C₀,: mg/l
   Volume in test flask (V): ml
   ThOD or COD: mg O₂/mg test substance (NH₄, NO₃)

4. INOCULUM:

   Source:
   Treatment given:
   Pre-conditioning treatment, if any:
   Suspended solids concentration in reaction mixture: mg/l
5. **O₂ UPTAKE, BIODEGRADABILITY:**

Type of respirometer:

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>n₁</th>
<th>n₂</th>
<th>n₃</th>
<th>n₄</th>
<th>nₓ</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>O₂ uptake by test chemical (mg)</th>
<th>a₁</th>
<th>a₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ uptake by blank (mg)</td>
<td>b₁</td>
<td>b₂</td>
</tr>
<tr>
<td>Corrected O₂ uptake (mg)</td>
<td>(a₁ - bₘ)</td>
<td>(a₂ - bₘ)</td>
</tr>
</tbody>
</table>
| BOD (mgO₂/mg test substance)    | \[
\frac{a₁ - bₘ}{C₀V} \\
\frac{a₂ - bₘ}{C₀V}
\] |

% degradation D

\[
\frac{BOD}{ThOD} \times 100
\]

mean*  

* D₁ and D₂ should not be averaged if there is a considerable difference.

Note: Similar formats may be used for the reference compound and toxicity control.
6. **CORRECTION FOR NITRIFICATION (see Annex V)**

<table>
<thead>
<tr>
<th></th>
<th>Time of incubation (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>(i) Concentration of nitrate (mgN/1)</td>
<td></td>
</tr>
<tr>
<td>(ii) oxygen equivalent (4.57 x N x V) (mg)</td>
<td>–</td>
</tr>
<tr>
<td>(iii) concentration of nitrite (mgN/1)</td>
<td></td>
</tr>
<tr>
<td>(iv) oxygen equivalent (3.43 x N x V) (mg)</td>
<td>–</td>
</tr>
<tr>
<td>(ii+iv) total oxygen equivalent</td>
<td>–</td>
</tr>
</tbody>
</table>

7. **CARBON ANALYSIS (optional)**

Carbon analyser:

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Test chemical (mg/l)</th>
<th>Blank (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(C₀)</td>
<td>(C₀₀)</td>
</tr>
<tr>
<td>28*</td>
<td>(Cₜ)</td>
<td>(C₀₀)</td>
</tr>
</tbody>
</table>

* or at end of incubation

\[
\% \text{ DOC removed} = \left[ 1 - \frac{C_τ - C₀₀}{C₀ - C₀₀} \right] \times 100
\]

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

<table>
<thead>
<tr>
<th></th>
<th>residual amount of test substance at end of test</th>
<th>% primary degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile control</td>
<td>S₀</td>
<td></td>
</tr>
<tr>
<td>Inoculated test medium</td>
<td>Sₐ</td>
<td>( \frac{S₀ - Sₐ}{S₀} \times 100 )</td>
</tr>
</tbody>
</table>
9. **ABiotic Degradation**

\[ a = O_2 \text{ consumption in sterile flasks at end of test (mg)} \]

\[
O_2 \text{ consumption per mg test chemical} = \frac{a}{C_0V}
\]

\[
\% \text{ abiotic degradation} = \frac{a}{C_0V \times ThOD} \times 100
\]
ABBREVIATIONS AND DEFINITIONS

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

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

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

DOC: Dissolved organic carbon is the organic carbon present in solution or that which passes through a 0.45 micrometre filter or remains in the supernatant after centrifuging at approx. 4000 g (about 40,000 m sec⁻²) for 15 min.

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

ThCO₂: Theoretical carbon dioxide (mg) is the quantity of carbon dioxide calculated to be produced from the known or measured carbon content of the test compound when fully mineralized; also expressed as mg carbon dioxide evolved per mg test compound.

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

IC: Inorganic carbon

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

**Primary Biodegradation:** The alteration in the chemical structure of a substance, brought about by biological action, resulting in the loss of a specific property of that substance.

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

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

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

**Treatability:** Is the amenability of compounds to removal during biological wastewater treatment without adversely affecting the normal operation of the treatment processes. Generally readily biodegradable compounds are treatable but this is not the case for all inherently biodegradable compounds. Abiotic processes may also operate.
**Lag phase:** Is the period from inoculation in a die-away test until the degradation percentage has increased to about 10%. The lag time is often variable and poorly reproducible.

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

**10-d window:** The 10 days immediately following the attainment of 10% biodegradation.
When a chemical is subjected to ready biodegradability testing and appears to be non-biodegradable, the following procedure is recommended if a distinction between inhibition and inertness is desired (Reynolds et al., 1987).

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

2. To assess the toxicity of chemicals studied in ready biodegradability tests, the application of one or a combination of the inhibition of sludge respiration rate (OECD Guideline 209; ISO Standard 8192), BOD and/or growth inhibition methods would seem appropriate.

3. If inhibition due to toxicity is to be avoided, it is suggested that the test substance concentrations used in ready biodegradability testing should be less than 1/10 of the EC$_{50}$ values (or less than EC$_{20}$ values) obtained in toxicity testing. Compounds with an EC$_{50}$ value greater than 300 mg/l are not likely to have toxic effects in ready biodegradability testing.

4. EC$_{50}$ values of less than 20 mg/l are likely to pose serious problems for the subsequent testing. Low test concentrations should be employed, necessitating the use of the stringent and sensitive Closed Bottle test or the use of C$^{14}$-labelled material. Alternatively, an inoculum previously exposed to the test substance may permit higher test substance concentrations to be used. In the latter case, however, the specific criterion of the ready biodegradability test is lost.

**LITERATURE**

ANNEX III

EVALUATION OF THE BIODEGRADABILITY OF POORLY SOLUBLE COMPOUNDS

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

1. While homogeneous liquids will seldom present sampling problems, it is recommended that solid materials be homogenised by appropriate means to avoid errors due to non-homogeneity. Special care must be taken when representative samples of a few milligrams are required from mixtures of chemicals or substances with large amounts of impurities.

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

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

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

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

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

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

LITERATURE


ANNEX IV
CALCULATION AND DETERMINATION OF SUITABLE SUMMARY PARAMETERS

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

1. Carbon Content

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

2. Theoretical Oxygen Demand (ThOD)

The theoretical oxygen demand (ThOD) may be calculated if the elemental composition is determined or known. For the compound:

\[ C_{c}H_{h}Cl_{cl}N_{n}Na_{na}O_{o}P_{p}S_{s} \]

the ThOD, without nitrification, would be:

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

with nitrification:

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

where MW = molecular weight

3. Chemical Oxygen Demand (COD)

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

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

4. Dissolved Organic Carbon (DOC)

Dissolved organic carbon (DOC) is, by definition, the organic carbon of any chemical or mixture in water passing through a 0.45 μm filter.
Samples from the test vessels are withdrawn and filtered immediately in the filtration apparatus using an appropriate membrane filter. The first 20 ml (amount can be reduced when using small filters) of the filtrate are discarded. Volumes of 10-20 ml or lower, if injected (volume depending on the amount required for carbon analyser) are retained for carbon analysis. The DOC concentration is determined by means of an organic carbon analyser which is capable of accurately measuring a carbon concentration equivalent to or lower than 10% of the initial DOC concentration used in the test.

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

**Remarks:**

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

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

Centrifugation at 4000 g (about 40,000 m sec⁻²) for 15 min. may be used for differentiation of TOC versus DOC instead of filtration. The method is not reliable at initial concentrations of < 10 mg DOC/l since either not all bacteria are removed or carbon as part of the bacterial plasma is redissolved.

**LITERATURE**


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


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

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

\[
\text{NH}_4^+ + 2\text{O}_2 \rightarrow \text{NO}_3^- + 2\text{H}^+ + \text{H}_2\text{O}.
\]

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

\[
\text{NH}_4^+ + \frac{3}{2} \text{O}_2 \rightarrow \text{NO}_2^- + 2\text{H}^+ + \text{H}_2\text{O}
\]

\[
\text{NO}_2^- + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_3^-.
\]

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

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

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

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