1. **INTRODUCTORY INFORMATION**

- **Prerequisites**
  - The test material must be at least soluble at the concentration to be tested, 40 mg dissolved organic carbon/litre (DOC/l).
  - The organic carbon content of the test material must be established.

- **Guidance information**
  - Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level".
  - Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

- **Interpretation of results**

  Because of the stringency of this test a result of less than 70 per cent loss of DOC (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

- **Qualifying statements**

  The method is only applicable to those organic test materials which, at the concentration used in the test,

  - are soluble in water,
  - have negligible vapour pressure,
  - are not inhibitory to bacteria, and
  - do not significantly adsorb on glass surfaces.

This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Programme (1978-1980).

Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.
Recommendations

- Test Chemicals giving a result of greater than 70 per cent loss of DOC (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 per cent.

- If the limits of sensitivity of organic carbon analysers are improved, the use of lower test concentrations may be an advantage, particularly for toxic compounds.

Standard documents

This test is based on "Norme Experimentale AFNOR T 90-302".

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

The method described in this Test Guideline allows the evaluation in aqueous medium of so-called "total" biodegradability under experimental conditions which are easy to establish. These conventional conditions do not necessarily correspond in all cases to the optimal conditions which would result in the maximal value of biodegradability.

The method is applicable to those organic products, alone or in mixtures, which are:

- soluble in water at the concentration used under the conditions of the test,
- non-volatile or at least have a negligible vapour pressure under the conditions of the test,
- at the concentration foreseen in the test, not inhibitory with respect to the bacteria responsible for the biodegradation.

Reference substances

In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. Aniline, sodium acetate or sodium benzoate may be used for this purpose; they must exhibit DOC removal of
70 per cent within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

In this specific test glucose is used especially for the inhibition test and may be used also to check the activity of the inoculum.

- **Principle of the test method**

  The biodegradation of organic products dissolved in water by chemico-organotrophic micro-organisms using the products as the sole source of carbon and energy is observed. These products are studied at a concentration such that the initial content of organic carbon is 40 mg/l. The organic carbon remaining in solution after 3, 7, 14, 28 (and 42) days is measured and the corresponding level of biodegradation calculated. The biodegradability is evaluated on the basis of this level.

- **Quality criteria**

  This is an evaluation method and not a method for the determination of biodegradability.

**Reproducibility**

To date no OECD ring test has been performed especially for reproducibility, but some general ring tests have been made with the CEFIC and with AFNOR, which have some relevance.

**Sensitivity**

This method is sensitive to an order of magnitude of ± 10 per cent.

**Specificity**

This Test Guideline is applicable to organic products soluble in the test medium at a concentration equal at least to one containing 40 mg/l as carbon.

**Possibility of standardisation**

This method may be standardised in the course of work by ISO.
Possibility of automation

Automation seems possible.

B. DESCRIPTION OF THE TEST PROCEDURE

• Preparations

Site

The incubation must be carried out in the absence of any intense lighting, in an incubator maintained at 25 ± 1°C and free from toxic vapours.

Reagents

The chemical products used must be of recognised analytical purity.

Distilled water must not contain more than 2 mg/l of organic carbon. In any case the organic carbon content of the distilled water must not exceed 10 per cent of the organic carbon level introduced by the test substance.

Test medium: Prepare the test medium as indicated below, using sterile material.

For one litre of solution, dissolve the following in distilled water:

- Ammonium sulphate \((NH_4)_2SO_4\) ................. 0.300 g
- Ammonium nitrate \(NH_4NO_3\) .................. 0.150 g
- Monopotassium phosphate \(KH_2PO_4\) .......... 0.300 g
- Disodium phosphate \(Na_2HPO_4 \cdot 12H_2O\) .... 2.000 g
- Magnesium sulphate \(MgSO_4 \cdot 7H_2O\) ........ 0.050 g
- Calcium chloride \(CaCl_2 \cdot 2H_2O\) ............. 0.050 g
- Yeast extract .............................. 0.005 g

The pH is 7.5 ± 0.1

Add 1 ml of trace element solution of the following composition:
Ferrous sulphate FeSO₄·7H₂O .............. 0.100 g
Manganese sulphate MnSO₄·H₂O ............ 0.100 g
Potassium molybdate K₂MoO₄ ............... 0.025 g
Sodium tetraborate Na₂B₄O₇·10H₂O ....... 0.025 g
Cobalt nitrate Co(NO₃)₂·6H₂O ............. 0.025 g
Copper chloride CuCl₂·2H₂O ............... 0.025 g
Zinc chloride ZnCl₂ .......................... 0.025 g
Ammonium metavanadate NH₄VO₃ .......... 0.010 g
Distilled water to .......................... 100 ml

The trace element solution can be kept for one month at a temperature between +1 and +4°C.

Make up to the volume stated (1 litre) and mix. The medium must be used within 12 hours.

**Apparatus**

Usual laboratory items, and

- Apparatus for the assay of organic carbon
- Spectrophotometer
- Centrifuge, 4000 ms⁻²
- Shaker allowing adequate aeration and shaking
- Apparatus for the assay of dissolved oxygen
- pH meter
- 500-ml wide-neck conical flasks, sterile
- Apparatus for sterile filtration

The glassware must be thoroughly cleaned and in particular free from all traces of organic or toxic matter.

**Procedure**

**Preparation of the test solutions**

Prepare the following solutions:

1) Solution of tested product in the test medium in such a way as to obtain a concentration of 40 mg/l of organic carbon.

2) Solution of glucose in the test medium in such a way as to obtain a concentration of 40 mg/l of organic carbon.
3) Solution containing in the test medium the concentrations of product and glucose used for the preparation of solutions.

4) An adequate volume of test medium should be available.

Mix the four solutions individually and sterilise by filtration through a membrane filter of 0.22 μm porosity.

**Preparation of the inoculum**

Take an adequate volume of a mixture of three samples from polluted surface water (effluent from town sewage works) free from major specific pollutants. The bacterial count for each sample must be at least 10^2 bacteria/ml.

The samples must be used for the inoculation within a period of 12 hours, including transportation, and must not remain for more than 6 hours without aeration.

Filter through paper to eliminate the larger insoluble particles, collect the filtrate and pass through a membrane filter of pore size 0.22 μm. Wash with any isotonic solution. Take up the bacteria deposited on the membrane filter in a small volume of solution or with any other isotonic solution. Mix well. Measure the absorbance at 620 nm and from this deduce the concentration of bacteria in relation to a standard curve obtained previously by means of solid medium counts of *Pseudomonas fluorescens* strain ATCC 15453. Add the volume of solution required to adjust the concentration of bacteria to 5 ± 3 x 10^7/ml. Use the inoculum within the next hour.

**Actual test performance**

All the necessary manipulations must be carried out by sterile methods. Divide solutions into the test flasks (previously sterilised) according to the following scheme:

- Flask No 1 (test) ......... 150 ml solution 1
- Flask No 2 (test) ......... 150 ml solution 1
- Flask No 3 (test) .......... 150 ml solution 1
- Flask No 4 (sterile control) ...... 150 ml solution 1
- Flask No 5 (glucose control) ...... 150 ml solution 2
- Flask No 6 (control of inhibitory action) .......... 150 ml solution 3
- Flask No 7 (inoculum control) .... 150 ml solution 4
Seed flasks 1, 2, 3, 5, 6 and 7 with 1.5 ml of inoculum and mix well by manual shaking.

Take an aliquot of 3-5 ml from each flask. Centrifuge the aliquots at 4000 ms⁻² for 15 minutes*, keeping the temperature below 26°C. Collect the supernatants for assays of organic carbon at time 0.

Place the flasks on the shaker and leave them there throughout the test period; the shaking must be such that the validity clause is fulfilled.

In the same way as for the assay of organic carbon at time 0, carry out this assay on flasks 1, 2, 3, 5, 6 and 7 after 3, 7, 14, 28 (and 42) days of incubation. However, if the reduction in carbon content reaches 95 per cent of the initial content in flasks 1, 2 and 3, consider the test as ended.

The test can be finished before the 28th day in the case where a plateau is observed before the 28th day. In the case where a degradation has obviously started on day 28 but has not reached a plateau on day 28, it is considered good practice to extend the experiment for 1 or 2 weeks longer.

At the end of the test carry out an assay of organic carbon in flask 4 in the same manner as at time 0.

If the assays of organic carbon contents have to be deferred, keep the supernatant at 4°C in the dark in hermetically sealed glass flasks; the maximum acceptable duration of preservation is 24 hours. If the analysis cannot be carried out within 24 hours, then freeze at a temperature below -18°C.

To compensate for loss of water due to evaporation, before each sampling verify the volume of medium in the flask and, if necessary fill up with distilled water sterilised by filtration through a membrane of 0.22 μm pore size to restore the volume measured after the previous sampling.

* In certain cases, and especially in presence of relatively low specific gravity insoluble fractions, it may be necessary to centrifuge for a longer time or with a higher acceleration.
3. DATA AND REPORTING

- Treatment of the results

Calculation:

Determine the percentage elimination of soluble organic carbon for each sampling, using the following formula:

\[
\% \text{ elimination of carbon at time } t = \frac{(C_0 - C_{0i}) - (C_t - C_{ti})}{(C_0 - C_{0i})} \times 100
\]

where:

- \(C_0\) = the mean level of organic carbon at time 0 in flasks 1, 2 and 3 (or the organic carbon content at time 0 in one of the flasks 4, 5 or 6).
- \(C_{0i}\) = the organic carbon level in flask 7 at time 0.
- \(C_t\) = the mean level of organic carbon at time \(t\) in flasks 1, 2 and 3 (or the organic carbon level at time \(t\) in one of the flasks 4, 5 or 6).
- \(C_{ti}\) = the organic carbon level in flask 7 at time \(t\).

The level of biodegradation is the percentage elimination of the organic carbon.

Set up a table of biodegradation levels as a function of time. Draw the curve of the change in biodegradation level as a function of time when a large enough number of significant points is available.

Note: The comparison of the percentage eliminations of carbon in flasks 1, 2 and 3, on the one hand, and in flask 4, on the other hand, allows the causes of the degradation observed to be differentiated: the physical-chemical mechanisms in flask 4 and the physical-chemical plus biological mechanisms in flasks 1, 2 and 3.

- Validity of the results

If one of the following conditions 1 to 3 is not fulfilled, begin the test anew. In the case of condition 1, the level of shaking must be increased in the new test.

1) The concentration of dissolved oxygen on day 3 in flask 5 must be at least 5 mg/l.

2) The level of glucose degradation in flask 5 must be at least 80 per cent by day 7.
3) At the end of the test, flask 4 must still be sterile. Check this by seeding into a tube of liquid culture medium* and incubating at 25°C for 5 days. The level of glucose biodegradation in flask 6 must, by day 7, be at least 75 per cent of that observed in flask 5. If this limit is not reached, consider that the product subjected to the test presents an inhibitory effect towards the bacteria present and that the method is not applicable to it at the concentration laid down by this standard.

Interpretation of results
See Section 1.

Test report
Mention the following points in particular:
- motivation for any rejection of the test;
- extent of the disappearance of the product in flask 4 at the end of the test;
- any inhibition phenomena observed.

Biodegradability: Express the biodegradability as the highest level of biodegradation noted during the period of 28 days. The course of the degradation should be displayed graphically in a diagram.

* Dehydrated yeast extract 3 g
Pancreatic caseine peptone 6 g
Water 1000 ml

Dissolve the components or the dehydrated complete medium in boiling water. If necessary, adjust the pH in such a way that after sterilisation it is 7.2 ± 0.2 at 20°C.
1. INTRODUCTORY INFORMATION

- **Prerequisites**
  - The total organic carbon content of the test material should be calculated or, if this is not possible, analysed to enable the theoretical yield of CO₂ to be calculated.

- **Guidance information**
  - Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level".
  - Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

- **Interpretation of results**
  - Because of the stringency of this test a result of less than 60 per cent yield of CO₂ (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

- **Qualifying statements**
  - The method is only applicable to those organic test materials which, at the concentration used in the test,
    - have negligible vapour pressure,
    - are not inhibitory to bacteria, and
    - do not significantly adsorb to glass surfaces.

This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Programme (1978-1980).

Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.
Evolution of a significant amount of CO₂ from the blank flask during the test would indicate contamination of the medium, glassware or air supply. A total CO₂ evolution in the blank at the end of the test exceeding 50 mg CO₂ per 3 litres medium should be considered as invalidating the test.

- **Recommendations**

Test chemicals giving a result of greater than 60 per cent yield of CO₂ (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 per cent.

If the test material is not soluble at the test concentration, special measures, such as the use of ultra-sound dispersion, may have to be employed to achieve a good dispersion of the test material.

- **Standard documents**

This Test Guideline is based on biodegradability test methods described in references (1) and (2); see Section 4, Literature. Various improvements have been made in the test protocol, the latest ones being the object of the paper cited in reference (3).

2. **METHOD**

A. **INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST**

This Test Guideline has been developed for screening readily biodegradable chemicals. When a broader understanding of the biodegradability of a compound is required (particularly for new chemicals never discharged into the environment) environmentally relevant results may be obtained by operating the same Test Guideline but with inoculum preadapted to the test compound (1) (2) (3).

A high biodegradation result in this test provides the evidence that the test compound is highly biodegradable in aerobic systems.

On the contrary, a low biodegradation result may have other causes than poor biodegradability of the test compound. Inhibition effects of the inoculum by the toxicity of the compound at the test concentration is often a cause for low biodegradation. In such cases
the result is meaningless and further work is needed to assess the biodegradability of the test compound in systems and at concentrations where inhibition effects are overcome.

An estimate of the expected environmental concentration will help to put toxicity effects into perspective and determine test concentrations.

The proportion of carbon incorporated into cellular material to carbon released as CO₂ will vary depending on the organic substrate, on the particular micro-organism(s) carrying out the conversion and on the environmental conditions under which the conversion takes place. In principle, this uncertainty is a drawback in the interpretation of the results from this test.

However, it has been observed that under the conditions of this test with a test compound concentration (sole source of carbon) ranging from 5 to 20 mg/l, a majority of substrate carbon is used to drive catabolic processes and a minimum is used to generate new cells (3). Under these conditions, the measure of CO₂ evolution becomes an accurate measure of the rate and extent of catabolism of a given material.

- Definitions and units

The amount of CO₂ produced by the test compound during the test is measured and expressed as percent of the theoretical CO₂ it should have produced (TCO₂) calculated from the carbon content of the test compound. Biodegradability is therefore expressed as percentage TCO₂.

- Reference substances

In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate or sodium benzoate may be used for this purpose. They must give a yield >60 per cent CO₂ within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.
Principle of the test method

A chemically defined liquid medium, essentially free of other organic carbon sources, is spiked with the test material and inoculated with sewage microorganisms. The CO₂ released is trapped as BaCO₃.

After reference to suitable blank controls, the total amount of CO₂ produced by the test compound is determined for the test period and calculated as the percentage of total CO₂ that the test material could have theoretically produced based on its carbon composition.

Quality criteria

Reproducibility

In the absence of toxicity effects of the test compound on the inoculum, the reproducibility is around ± 5 per cent, relative. When toxicity effects start occurring, the reproducibility becomes poor.

Sensitivity

The endogeneous CO₂ production of the inoculum as measured in the blank flask (no test material) is the main reason why the test cannot use test compound concentrations lower than 5 mg/l. (When the test is adapted to handle ¹⁴C labelled test compounds, test compound concentrations can be very much lower.)

Specificity

This test is adequate for soluble and insoluble organic materials. The test material must not be volatile. The purity of the test material should be high since organic impurities will complicate the interpretation of the CO₂ production data.

Test results are meaningful only if, at the test concentration, the material has no toxic effect on the inoculum.

Possibility of standardisation

This possibility exists. The major difficulty is to standardise the inoculum in such a way that inter-laboratory reproducibility is ensured. The selection
of test organisms and their handling in the laboratory as described below. (Test organisms) is one way to ensure "healthy" inoculum with a multiplicity of micro-organism species. Procedures using preadaptation of the inoculum to the test compound usually reach higher reproducibility.

Possibility of automation

None at present.

B. DESCRIPTION OF THE TEST PROCEDURE

• Preparations

Apparatus equipment

CO₂ scrubbing apparatus: For a series of 12 carboys (3 test materials):

- Four one-litre plastic bottles, filled with 700 ml 10 N NaOH.
- One one-litre Erlenmeyer flask containing 700 ml 0.025 N Ba(OH)₂ solution.
- One empty one-litre Erlenmeyer to prevent liquid carry-over.

These bottles are connected in series, using Tygon tubing, to a pressurised air source, and air is sparged through the scrubbing solutions at a constant rate.

For each additional set of 4 carboys, add 1 additional one-litre plastic bottle filled with 700 ml 10 N NaOH.

CO₂ production apparatus

Four 5-litre disposable amber carboys for each test material.

Stoppers, flexible tubing, plastic tubing.

"French squares" (i.e. 100 ml barium hydroxide absorber bottles) or similar containers.
Analytical equipment

(Carbon analyser: optional if sample is well characterised.)

Analytical balance.

100 ml-buret.

Chemicals/reagents/materials

Stock solutions for test medium

a) Ferric chloride solution -
   dissolve 0.25 g FeCl₃·6H₂O in 1 litre
distilled water.

b) Magnesium sulfate solution -
   dissolve 22.5 g MgSO₄·7H₂O in 1 litre
distilled water.

c) Calcium chloride solution -
   dissolve 27.5 g anhydrous CaCl₂ in 1 litre
distilled water.

d) Phosphate buffer solution -
   dissolve: 8.5 g KH₂PO₄, 33.4 g Na₂HPO₄·7H₂O and 1.7 g NH₄Cl in
   1 litre distilled water.

e) Ammonium sulfate solution -
   dissolve 40 g (NH₄)₂SO₄ in 1 litre distilled
   water.

Test medium

The test medium will contain per litre of high quality
water* the following reagents:

- 1 ml of above magnesium sulfate solution
- 1 ml of above calcium chloride solution
- 2 ml of above phosphate buffer solution
- 4 ml of above ferric chloride solution
- 1 ml of above ammonium sulfate solution

* High quality water, free of toxic substances
(copper, in particular), with low carbon content
(< 2.0 mg/litre TOC) and with a resistivity
> 18 megohms.cm. (Distilled water must never
contain more than 10 per cent of organic carbon
introduced by the test material.)
Barium hydroxide, 0.025 N

Dissolve 4.0 g Ba(OH)₂.8H₂O per litre high quality water. Filter through filter paper and seal the clear solution to prevent absorption of CO₂ from the air. It is wise to prepare more than 5 litres at a time when running series.

Test materials

Basic physical-chemical data regarding water solubility, impurities solvent and percentage active must be specified by the sample submitter.

An initial stock solution is prepared from the test material by weighing out a homogeneous aliquot of the sample. The sample is dissolved in high quality water to give a solution of test material of 1000 mg/l. Stock solutions are made up on the basis of percentage of active compound in the test material. If the percentage of active compound is unknown, stock solutions are made up to concentration of 1000 mg/l on a weight basis. To obtain a homogeneous sample, it may be necessary to mix well, at the same time avoiding foaming which will tend to concentrate the active ingredient disproportionately. For solid samples, it may be necessary to melt and mix the entire contents of the sample bottle before taking the aliquot. This portion of the procedure is extremely important since the calculations of percentage of biodegradation depend directly on having added the correct amount of carbon to the test system.

The pH of the stock solution need not be adjusted unless it falls outside the range of 3-10, since the phosphate buffer in the test medium will control it. If the pH lies outside this range, adjust an aliquot of the stock solution to pH 7.0 (± 1.0) with 1 N HCl or NaOH, making sure that the solution is being vigorously mixed during the addition of acid or base.

To confirm the nominal concentration of organic carbon of the test compound, the stock solution (or the neutralised aliquot) may be analysed for total organic carbon (TOC). A TOC analysis is also required for the control stock solution.
If a test material is insoluble in water, add the appropriate amount of test material directly to the carboy on a weight or volume basis. Total organic carbon analysis cannot be performed on insoluble test materials.

Test organisms

The source of test organisms is activated sludge freshly sampled from a well-operated municipal sewage treatment plant. This sewage treatment should receive no or minimal effluents from industry.

Upon arrival at the laboratory, the activated sludge is aerated for 4 hours. 500 ml of the mixed liquor is sampled and homogenised for 2 minutes at medium speed in a Waring blender. It is then settled for 1/2 hour.

If the supernatant still contains high levels of sludge solids at the end of 30 minutes, it may be settled for an additional 30-60 minutes or adapted to laboratory conditions to obtain better settleability.

The supernatant is decanted to provide sufficient volume for a 1 per cent inoculum for each CO₂ test flask. Avoid carry-over of sludge solids which would interfere with the measurement of CO₂ production.

Although optional, it is useful to perform viable counts on the supernatant fraction to determine microbial numbers. This inoculum should normally contain 10⁶ to 20 x 10⁶ colony forming units per ml. It should be used on the day it is prepared. The CO₂ production test then proceeds as follows.

- **Test conditions**
  1) Since a 1 per cent inoculum is used in the CO₂ test, it is necessary to make dilutions in the CO₂ test medium.

This is most easily achieved as follows:

a) To each of the 5-litre test carboys, add 2470 ml of high quality water.

b) To each of the 5-litre test carboys, add 3 ml each of the ammonium sulfate, magnesium sulfate, and calcium chloride stock solutions; add 6 ml of the phosphate buffer stock solution and 12 ml of the ferric chloride solution.
c) To each of the 5-litre test carboys, add 30 ml of the activated sludge inoculum.

d) These additions now total 2527 ml in each carboy.

2) This mixture is aerated with CO₂-free air for 24 hours, to purge the system of carbon dioxide (see CO₂ scrubbing apparatus, above).

3) After the aeration period, three CO₂ absorber bottles are filled with 100 ml 0.025 N Ba(OH)₂ and connected in series to the exit air line of each test carboy.

4) Test material is added to two of the four carboys to begin the testing period. Each material is tested at two concentrations: 10 and 20 mg/l. The amount of test material stock solution required in the carboy is calculated as follows:

\[ \text{ml of stock solution per carboy} = \frac{B \times C}{A} \]

where
- \( B \) is test compound concentration in test carboy (mg/l),
- \( A \) is test compound concentration in stock solution (mg/l),
- \( C \) is final volume of test medium in test carboy (ml).

5) Sufficient stock solution to reach the desired test concentration, as calculated above, plus enough distilled water to make 473 ml (stock solution + high quality water) are added to the appropriate carboys. To the third carboy, used as blank control and containing no test material, 473 ml of high quality water are added. The final volume of each carboy is now 3000 ml.

6) A control substance at a concentration of 20 mg/l is added to the last of the four carboys (see section on Reference substances, above).

**Performance of test**

1) The test is started by bubbling CO₂-free air through the solution at a rate of 50-100 ml/min per carboy (approximately 1-2 bubbles/second). The CO₂ produced in each carboy reacts with the barium hydroxide and is precipitated out as barium carbonate; the amount of CO₂ produced is determined by titrating the remaining Ba(OH)₂ with 0.05 N standardised HCl (see below). Periodically (every 2 or 3 days), the
CO₂ absorber nearest the carboy is removed for titration. The remaining two absorbers are each moved one place closer to the carboy, and a new absorber filled with 100 ml of fresh 0.025 N Ba(OH)₂ is placed at the far end of the series. Titrations are made as needed (before any BaCO₃ precipitate is evident in the second trap), approximately every other day for the first 10 days, and the every fifth day until the 28th day.

For water-insoluble test materials, incorporated dry into the CO₂ test carboy, agitation can be done with a magnetic stirrer. For foaming chemicals, CO₂-free air bubbling can be replaced by overhead aeration and magnetic stirring.

2) On the 26th day, the pH of the carboy contents is measured again, and then 1 ml of concentrated HCl is added to each of the test carboys to drive off inorganic carbonate. The carboys are aerated overnight, and samples are removed from each carboy for dissolved organic carbon (DOC) analysis. The final titration is made on day 28.

3) Titrations of the 100-ml Ba(OH)₂ solution are made after removing the bottles closest to the carboys. The Ba(OH)₂ is titrated with 0.05 N HCl, using phenolphthalein as an indicator.

4) The test is run at room temperature and temperature is recorded during the test period.

3. DATA AND REPORTING

- Treatment of results

TCO₂ - The theoretical amount of CO₂ that can be generated by a test material, or TCO₂, is calculated as follows:

$$\text{TCO}_2 = \frac{\text{No of carbons in test material} \times \text{mol. wt. of CO}_2}{\text{mol. wt. of active test material}}$$

For instance, dextrose (C₆H₁₂O₆) contains 6 carbon, therefore TCO₂ of dextrose =

$$\frac{6 \times 44}{180} = 1.467 \text{ mg CO}_2/\text{mg dextrose}$$
For mixtures, the TCO$_2$ of the total active material is a weighted average of the TCO$_2$'s of the individual components.

**Amount of CO$_2$ produced**

The first step in calculating the amount of CO$_2$ produced is to correct the test material carboys for endogeneous CO$_2$ production. The control carboy serves as a "seed blank" to correct for CO$_2$ which may be produced through endogeneous respiration of the bacteria. The amount of CO$_2$ produced by a test material is determined by the difference (in ml of titrant) between the experimental and blank Ba(OH)$_2$ traps.

For example: Blank: 48.0 ml HCl titrated  
Experimental: 45.0 ml HCl titrated  
Test Material: 3.0 ml HCl titrated

The next step is to convert ml HCl titrated into mg of CO$_2$ produced. When CO$_2$ enters the absorber bottles, it reacts in the following manner:

Ba(OH)$_2$ + CO$_2$ $\rightarrow$ BaCO$_3$ $\downarrow$ + H$_2$O

The BaCO$_3$ formed is insoluble and precipitates. The amount of Ba(OH)$_2$ remaining in solution is determined by titration of the 100 ml with HCl according to the following equation:

Ba(OH)$_2$ + 2 HCl $\rightarrow$ BaCl$_2$ + 2H$_2$O

From the above two equations, it can be seen that 1 mmol of CO$_2$ is produced for every 2 mmol of HCl titrated. This means that the number of mmol of CO$_2$ produced:

$$\text{mmol CO}_2 = \frac{\text{mmol HCl}}{2}$$

The normality of HCl used is 0.05 N. Substituting for mmol gives:

$$\text{mmol CO}_2 = \frac{(0.05 \text{ N}) \times (\text{ml of HCl})}{2}$$

To convert to mg CO$_2$, the value must be multiplied by the molecular weight of CO$_2$ which is 44:

$$\text{mg CO}_2 = \frac{(0.05 \times \text{ml titrated})}{2} \times 44 = 1.1 \times \text{ml of HCl titrated}$$
Thus, to convert ml of HCl to mg CO₂, the former is multiplied by 1.1.

**Percentage of theoretical CO₂**

The percentage of theoretical CO₂ produced is calculated from the following formula:

\[
\% \text{ TCO}_2 = \frac{\text{mg CO}_2 \text{ produced}}{(\text{mg test material added in test})(\text{mg CO}_2/\text{mg test material})} \times 100
\]

\[
= \frac{\text{mg CO}_2 \text{ produced}}{(\text{mg test material added in test})(\text{TCO}_2)} \times 100
\]

Example: Blank 48.0 ml 0.05 N HCl titrated.
Dextrose 45.0 ml 0.05 N HCl titrated.
Difference 3.0 ml 0.05 N HCl titrated.

When multiplied by 1.1, 3.0 is equivalent to 3.3 mg CO₂ produced.

Since dextrose has a TCO₂ of 1.47 mg CO₂/mg dextrose, and since 60 mg of dextrose were placed in the test (i.e. 3 litres x 20 mg/l), the percentage of theoretical CO₂ that was degraded on the particular day was:

\[
% \text{TCO}_2 = \frac{3.3 \text{ mg CO}_2 \text{ produced}}{(60 \text{ mg dextrose})(1.47 \text{ mg CO}_2/\text{mg dextrose})} \times 100 = 3.74
\]

**Test report**

- The test material should be well identified (source, physical-chemical data, organic carbon content, purity, etc.).

- The test concentration at start should be reported with end results.

- Indicate date and location where test organisms were sampled.

- COD and TOC analyses on the stock solution of the test compound should be reported.

- Temperature range recorded during the test period must be noted.
- If measured as suggested above (Test organisms), report number of micro-organisms per ml (colony forming units - CFU/ml).

- For each titration, calculate percentage TC02 evolved. Plot cumulative percentage TC02 versus time until the end of the test. Not only the end result is important, but also the length of the lag phase and the slope (rate).

- If the curve reaches a plateau before 28 days, the test can be ended and the plateau value considered as final.

- If the curve shows that biodegradation started before day 28 but the plateau is not reached at day 28, then the test should be prolonged until the plateau is reached.

- If a more rigorous mathematical treatment of the data is desired, the cumulative CO2 versus time data can be fit into a non-linear regression model to generate rate constants for mineralisation and a final extent of degradation at infinite time (asymptote) (3).

Interpretation of results

See Section 1.

4. LITERATURE


1. **INTRODUCTIONARY INFORMATION**

- **Prerequisites**
  - An analytical method must be available for determining the concentration of the test material in the test solution.
  - The empirical formula of the test material is required so that the theoretical oxygen demand (TOD) may be calculated.

- **Guidance information**
  - Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level".
  - Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

- **Interpretation of results**

  Because of the stringency of this test, a result of less than 60 per cent of BOD* (70 per cent LPC**) (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability. The possibility that nitrogen-containing compounds may affect the results should be considered.

- **Qualifying statements**

  The method is only applicable to those organic test materials which, at the concentration used in the test,
  - have negligible vapour pressure,
  - are not inhibitory to bacteria, and
  - do not reach and react with the CO₂ adsorbant.

* biochemical oxygen demand
** loss of parent compound

Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.
This test has been found suitable by the OECD Expert Group "Degradation/Accumulation" for determining the "ready biodegradability of organic chemicals under aerobic conditions". It has been tested in the OECD Laboratory Intercomparison Test Programme (1978-1980).

**Recommendations**

- Test chemicals giving a result of greater than 60 per cent BOD, 70 per cent LFC, (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 per cent.

- If the test material is not soluble at the test concentration, special measures, such as the use of ultrasound dispersion, may have to be employed to achieve a good dispersion of the test material.

**Standard Documents**

This Test Guideline was based upon the Order prescribing the items of test, relating to new chemical substances of the Chemical Substance Control Law (Japanese Law No. 117, 1973). [Order of the Japanese Prime Minister, the Minister of Health and Welfare, and the Minister of International Trade and Industry, No. 1 (July 13, 1974)]

2. **Method**

A. **Introduction; Purpose, Scope, Relevance, Application and Limits of Test**

Purpose: Measurement of biochemical oxygen demand and the analysis of residual chemicals, intermediates necessary in the evaluation of the biodegradability of chemical substances.

Appropriate chemicals: non-volatile and water soluble (more than 100 ppm) organic chemicals.

Applicable chemicals: slightly water soluble chemicals
- volatile chemicals* (C_{water}/C_{air} > 1**)

Non-applicable chemicals: volatile chemical substances (C_{water}/C_{air} < 1).

* Modified BOD meter should be used

** C = concentration
Definitions and units

Definitions

Percentage degradation = \( \frac{\text{BOD}_B}{\text{TOD}} \times 100\% \)

or

Percentage degradation = \( \frac{\text{Sb}-\text{Sa}}{\text{Sb}} \times 100(\%) \)

\( \text{BOD} \): Biochemical oxygen demand (experimental) (mg) of the test compound measured on the BOD curve

\( B \): Oxygen consumption (experimental) (mg) of basal culture medium to which the inoculum is added measured on the BOD curve

\( \text{TOD} \): Theoretical oxygen demand (theoretical) (mg) required when the test compound is completely oxidised

\( \text{Sa} \): Residual amount (experimental) (mg) of the test compound after completion of the biodegradability test

\( \text{Sb} \): Residual amount (experimental) (mg) of the test compound in the blank test with water to which only the test compound has been added

Reference substances

In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate or sodium benzoate may be used for this purpose.

Aniline: If the percentage of degradation of aniline calculated from the oxygen consumption does not exceed 40 per cent after 7 days or 65 per cent after 14 days the test is regarded as invalid.
Principle of the test method

This test method is based on the following conditions:

(i) test chemicals as sole organic carbon sources, and

(ii) no adaptation of micro-organisms to test chemicals.

An automated closed system oxygen consumption measuring apparatus (BOD meter) is used. Chemicals to be tested are inoculated with micro-organisms in the testing vessels. During the test period, biochemical oxygen demand is measured continuously by the BOD meter. Biodegradability is calculated on the basis of BOD and supplemental chemical analysis is undertaken, such as measuring dissolved organic carbon concentration, concentration of residual chemicals, etc.

Quality criteria

Reproducibility

Generally good, especially so for chemicals of water solubility greater than 0.1 g/l.

Sensitivity

(A) Oxygen consumption: Detection limit = 1 mg (oxygen consumption by micro-organisms)

(B) Chemical analysis: Depends on the sensitivity of analytical methods.

Specificity

Applicable to every kind of chemical, for which C_{water}/C_{air} \geq 1. For volatile chemicals a "modified BOD meter", composed of capillary tubing and normal BOD meter, should be used (See Annex 1).

Possibility of automation

By using a BOD meter (See Annex 1) oxygen consumption by micro-organisms (in a closed system) is recorded automatically.
B. DESCRIPTION OF THE TEST PROCEDURE

Preparations

Apparatus

BOD meter equipped with 6 bottles (300 ml each):

- Bottle 1 and 2: deionised water*, 300 ml + test chemical, 30 mg
- Bottle 3 and 4: basal culture medium, 300 ml + activated sludge, 9 mg (dry base) + test chemical, 30 mg
- Bottle 5: basal culture medium, 300 ml + activated sludge, 9 mg (dry base) + aniline, 30 mg
- Bottle 6: basal culture medium, 300 ml + activated sludge, 9 mg (dry base)

Pretreatment of test chemical

Where the test compound is not soluble in water up to the test concentration, the test compound pulverised as finely as possible is employed.

Where the test compound is volatile, test chemicals should be well cooled to prevent evaporation.

If necessary, identification of the test sample should be undertaken.

Basal culture medium

To each 3 ml of solution A, solution B, solution C and solution D, water is added to make up to 1000 ml. (Deionised water is used throughout.)

Solution A: 21.75 g of dipotassium hydrogen phosphate, 8.5 g of potassium acid phosphate, 44.6 g of dibasic sodium phosphate dodecahydrate and 1.7 g of ammonium chloride are dissolved in water and the volume is made up to 1000 ml. (The pH of the solution is 7.2.)

* Distilled water must never contain more than 10 per cent of organic carbon introduced by the test substance.
Solution B: 22.5 g of magnesium sulphate heptahydrate is dissolved in water and the volume is made up to 1000 ml.

Solution C: 27.5 g of calcium chloride is dissolved in water and the volume is made up to 1000 ml.

Solution D: 0.25 g of ferric chloride hexahydrate is dissolved in water and the volume is made up to 1000 ml.

Activated sludge

Sludge sampling sites: Sludge sampling is made, in principle, at not less than 10 places throughout the country, chiefly in those areas where a variety of chemical substances may be considered to be consumed and discarded.

For example, standard activated sludge of the Japanese Chemical Testing Center is taken up from the following places and mixed:

- City sewage plant: 3 plants located in the northern, central and southern part of Japan.
- Industry sewage plant: One plant used for the waste water treatment of chemical industries.
- River: 3 rivers located in the northern, central and southern part of Japan.
- Lake: One lake located in the middle of Japan.
- Sea: 2 inland seas of Japan.

Frequency of sludge sampling: Sludge sampling should be made, in principle, four times a year, in March, June, September and December.

Sludge sampling methods:

- City sewage: 1 litre of return sludge at a sewage disposal plant.
- Rivers, lakes and marshes or sea: 1 litre of surface water and 1 litre of surface soil on the beach which is in contact with atmosphere.

Preparation: The sludge samples collected from the sampling sites are mixed by stirring in a single container, and the mixture is allowed to stand. The floating foreign matters are removed, and the supernatant is filtered with No. 2 filter paper. The filtrate is adjusted to pH 7.0 ± 1.0 with sodium hydroxide or phosphoric acid, transferred into a culture tank and aerated.
Culture: Thirty minutes after stopping the aeration of the solution obtained above, approximately 1/3 of the whole volume of the supernatant is removed. An equal volume of 0.1 per cent synthetic sewage is added to the remaining portion of the supernatant and the mixture is aerated again. This procedure is repeated once every day. The culturing is carried out at 25 ± 2°C.

Control: For the control of the culturing step, the following items are checked and necessary adjustments are made.

- Appearance of supernatant: The supernatant of active sludge should be clear.

- Precipitability of active sludge: The active sludge, being in large flocks, must have high precipitability.

- State of formation of active sludge: In the case where growth of flocks is not observed, either the volume of 0.1 per cent synthetic sewage to be added or the frequency of addition of synthetic sewage is increased.

- The pH of the supernatant is 7.0 ± 1.0.

- Temperature: The temperature for cultivation of active sludge is 25 ± 2°C.

- Amount of aeration: In replacing the supernatant with the synthetic sewage, the solution in the culturing tank must be sufficiently aerated to maintain the dissolved oxygen concentration of the solution above 5 ppm.

- Microflora of activated sludge: When the active sludge is microscopically observed (at 100~400 x magnification), a number of protozoa of different species together with cloudy flocks must be seen.

- Mixing of fresh and old activated sludge: In order to maintain fresh and old activated sludges at the same activity, the filtrate of the supernatant of an activated sludge in use in the test is mixed with an equal volume of the filtrate of the supernatant of an activated sludge freshly collected and the mixture is cultured.

* 0.1 per cent synthetic sewage: 1 g of glucose, 1 g of peptones and 1 g of monopotassium phosphate are dissolved in 1 litre of water and the solution is adjusted to pH 7.0 ± 1.0 with sodium hydroxide.
Checking of activity of activated sludge: Activity of activated sludge should be checked periodically, at least once every three months, with standard substances by applying the test method provided below. Where fresh and old activated sludge samples are mixed, especially, careful checking must be done in relation to the old activated sludge.

Example of preparation of activated sludge samples and period of use:

```
 Culture Period of use

 Mar.  Apr.  May  
 Mixing, culture Period of use

 Mixing, culture Period of use

 Mixing, culture Period of use
```

(The same pattern of preparation and use follows.)

Addition of test compound and preparation for test

The following test vessels are provided and adjusted to the test temperature.

1) A test vessel containing the basal culture medium, to which is added 100 ppm (W/V) of test compound: the pH of this solution is adjusted to 7 before the inoculation of active sludge, if necessary.

2) A test vessel for the control blank test, containing only the basal culture medium.

3) A test vessel containing water to which is added 100 ppm (W/V) of the test compound.

4) A test vessel containing basal culture medium to which is added 100 ppm (W/V) of aniline or any other of the control substances.

Inoculation of active sludge

Inoculum is added to the test vessels 1, 2 and 4 above so that the suspended matter defined in the Japanese Industrial Standards described in Annex 2 is contained at a concentration of 30 ppm (V/V).
Test conditions
- Concentration of test chemicals: 100 ppm (W/V)
- Concentration of activated sludge: 30 ppm (W/V)
- Test temperature: 25°C ± 2°C
- Period: 14 days
- Perform in darkness. Every day, the temperature, the change in colour of the culturing vessel should be checked.
- Stir vigorously with mechanical stirrer.

Performance of the test

The BOD curve is obtained continuously and automatically for 14 days.

After the 14-day testing period, pH, residual chemicals, intermediates in the testing vessels are analysed.

Figure 1: Degradation curve of a readily degradable compound.

a = adaptation period
l = period of logarithmic growth
m = maximum degradation rate
r = required level of degradation
t = time window
The test chemicals in the testing vessel without activated sludge are also analysed in order to confirm whether there is any change in the test chemical during the testing period or any loss of the original test chemical by evaporation or adsorption by the walls of test vessels, etc.

° Analytical means

If the test compound is soluble in water, the residual amount of total organic carbon is also determined.

(a) Where a total organic carbon analyser is used:

10 ml of the tested solution is sampled from the test vessel and centrifuged at 3000 g for five minutes. The residual amount of the total organic carbon in the supernatant is determined on a total organic carbon analyser.

(b) Where other analysers are used:

The total content of a test vessel is extracted with a suitable solvent for the test compound, and, after proper pretreatment such as concentration, the residual amount of the test compound is determined on an analysing instrument (gas chromatography, absorptiometry, mass spectrometry, atomic absorption spectrophotometry, etc.).

For volatile chemicals, the temperature control bath of the BOD meter should be cooled to 10°C and this temperature held for at least 30 min, in order to prevent evaporation. The analytical procedures (a) and (b) should then be started.

3. DATA AND REPORTING

° Treatment of results

(a) Method for calculating the percentage degradation from the oxygen consumption.

Percentage degradation = \( \frac{\text{BOD}_B}{\text{TOD}} \times 100(\%) \)

See Definitions and units, above, for clarification of symbols.
(b) Method for calculating the percentage degradation from the result of direct analysis

\[ \text{Percentage degradation} = \frac{S_b - S_a}{S_b} \times 100 \text{ (%)} \]

See Definitions and units, above, for clarification of symbols.

**Evaluation of results**

The following calculations are to be made:

- Calculation of theoretical oxygen demand

<table>
<thead>
<tr>
<th>Element</th>
<th>Oxidised form</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>CO₂</td>
</tr>
<tr>
<td>H</td>
<td>H₂O</td>
</tr>
<tr>
<td>N</td>
<td>NO₂</td>
</tr>
<tr>
<td>S</td>
<td>SO₂</td>
</tr>
<tr>
<td>X(halogen)</td>
<td>X</td>
</tr>
</tbody>
</table>

- Recovery rate of analytical procedure

**Test report**

The test report should include the following points:

- Information on the test chemicals

  Name, structural formula, molecular weight, purity, kind of impurities, physical chemical properties of test chemical, identification data of test chemical.

- Test conditions

  Activated sludge: sludge sampling site and concentration
  Test chemical: concentration
  Test period
  Test temperature

- Analytical procedure

  Pretreatment
  Analytical conditions of instrument
  Recovery rate of analysis
  Identification of intermediate
Results

BOD curves and instrument name
BOD (mg)
B (mg)
Sb (mg)
TOD (mg)

Percentage of degradation by BOD

Percentage of degradation by chemical analysis.

Chromatograms or spectra of test chemicals obtained and used for the purpose of analysis.

Remarks

Interpretation of results

For the purpose of comparison with reference substances, the biodegradability of the test compound is categorised based on the relative degree of degradability compared to that of aniline.

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

If the recovery rate of Sb is found to be in the order of 10 per cent or less, the test is also regarded as invalid.

See also Section 1.

4. LITERATURE

(1) Biodegradability and bioaccumulation test of chemical substances (C-5/78/JAP), 1978.

(2) The chemical substances control law in Japan (Chemical Products Safety Division, Basic Industries Bureau, MITI) (C-2/78/JAP), 1978.

(3) The biodegradability and bioaccumulation of new and existing chemical substances, 5,8 (C-3/78/JAP), 1978.
5. ANNEX

1. PRINCIPLE OF CLOSED SYSTEM OXYGEN CONSUMPTION MEASURING APPARATUS

The coulometer is an instrument for measurement of the oxygen consumption of micro-organisms using electro-chemical analysis process (coulometry).

The following is a block diagram:

(For Modified BOD meter*, hatched part of tube should be replaced with capillary tube)

The specimen contained in cultivating bottle (1) is stirred by means of a magnetic stirrer (2). When the reaction progresses, the dissolved oxygen in liquid will be consumed. Oxygen (O₂) in the space in the cultivating bottle is dissolved in liquid, resulting in generation of CO₂ in its place.

* The BOD-meter is produced by Ohkura Electric Co., Ltd. 1-11-16, Shibuya, Shibuya-ku, Tokyo, Japan. (For volatile chemicals, a capillary tube should be installed between each testing vessel and electrolytic bottle.)
As this CO₂ is absorbed by soda lime (3), the partial pressure of oxygen in the space and the total pressure decrease.

The drop in pressure is detected and converted into an electric signal by means of an electrode type manometer (4) and is amplified by an amplifier (5) for operating a relay circuit (6), resulting in operation of a synchronous motor (8). Simultaneously, by constant current, electrolytic oxygen is generated from sulphuric acid copper solution contained in an electrolytic bottle (7).

This oxygen is supplied to the cultivating bottle and restoration of pressure is detected by means of the manometer, resulting in switching off of the relay circuit and stopping the electrolytic and synchronous motor.

The upper space in the cultivating bottle is always kept under a constant pressure of oxygen and the quantity of oxygen consumed in the cultivating bottle is proportional to the quantity of electrolytic oxygen. As this quantity of electrolytic oxygen is proportional to electrolytic time, there is a constant electrolysis current. Accordingly, the revolution angle of a synchronous motor (9) is converted to a mV signal by means of the interlocking potentiometer, resulting in an indicator quantity of consumed oxygen at the recorder (10).

2. SUSPENDED MATTER

(From Japanese Industrial Standards K0102 - 10.2)

Susparated matter is that material which can be separated by filtration or by means of a centrifugal separator. It can be determined by any of the methods described below. When the test water is difficult to filter the centrifugal separation method should be applied; when the test water contains an extremely large quantity of suspended matter, the Büchner funnel method should be used.

Test water is taken from the waste water passed through a 2 mm mesh sieve. At least 5 mg of the filtrate are necessary for the determination.
A. FILTRATION THROUGH FILTER PAPER

* Sintered glass filter method

Apparatus
Sintered glass filter: A crucible-type sintered glass filter "T&G" or a Büchner funnel-type sintered glass filter 302.

Procedure
Prepare two sintered glass filters of the same type and of approximately the same weight; lay six sheets of filter paper in them and pour water through several times so that they adhere by suction. Then transfer the filters to an air oven and dry them for two hours at 105-110°C. Allow them to cool in a desiccator, and weigh. (When a chemical balance is used, the lighter filter may be used as a supplementary weight.) Pour a suitable amount of the test water into the heavier filter*, filter it by suction, and wash the wall of the filter several times with the filtrate, in order to remove substances adhering to the wall. Next, pour the filtrate into the lighter filter several times and filter it by suction. Dry the two filters in the air oven for two hours at 105-110°C, and allow them to cool in a desiccator. Weigh each filter (when a chemical balance is used, the lighter filter may be used as a supplementary weight), obtain the difference in weight before and after the filtration, and calculate the quantity of the suspended matter in ppm according to the following formula:

\[ S = \frac{(a - b) \times 1000}{V} \]

Where,
- \( S \): Suspended matter (ppm)
- \( a \): Difference in weight before and after the filtration of test water (mg)
- \( b \): Difference in weight before and after the filtration of the filtrate (mg) (when a chemical balance is used, \( b = 0 \))
- \( V \): Amount of test water (ml)

* Take sufficient test water to give a weight of suspended matter of not more than 5 mg after drying. Ordinarily, 200 ml of the test water is enough. However, if the test water is difficult to filter, 10 ml from each test water sample must be added from the 10 ml measuring cylinder during the filter process.
Remarks

(1) To determine the ignition loss of volatile suspended matter, a test should be carried out in accordance with the Glass Fibre paper method (3) below, or, after washing the suspended matter together with filter paper into a crucible or an evaporating dish, dry and ignite in muffle furnace.

(2) When the soluble evaporated residue is less than 5000 ppm, correction (for difference in weight of the filtrate before and after filtration) may be omitted. However, when a chemical balance is used, the lighter filter should be used as a supplemental weight so the filtration of the filtrate can be carried out at the same time.

Even when a direct reading balance is used the weight varies with the hygroscopic properties of substances contained in the test water, and with other conditions, so it is desirable that a correction be performed by obtaining the blank test value of the filter through which the filtrate is passed. In the case of the test water containing fats and oils, grease, wax, etc., a portion of these substances should be determined as the suspended matter.

When the determination of the suspended matter exclusive of oils and fats is required, pour 10 ml volumes of n-hexane several times through the filter which has been dried and weighed after filtration and wash out the fats and oils. Then dry the filter and weigh.

(3) Glass fibre paper method (GFP Method): Fix an appropriate GFP* of known weight, which has been dried at 105 to 110°C for 2 hours after washing, on a suitable supporting plate. Add the amount of test water to give a weight of the suspended matter after drying of over 5 mg. After filtration by suction, return a portion of the filtrate to the container holding the original test water. Wash down the suspended matter adhering to the walls of container and filter again on GFP by suction. Repeat this operation several times. Detach GFP from the filter and transfer it onto a water glass. Then operate as described for the Büchner funnel method below and determine ppm of the suspended matter.

After determination of the suspended matter determine the ignition residue of the suspended matter, if necessary, according to the operation described in the section on Filtration through asbestos layer, below.

* Whatman GF/B or equivalent.
Büchner funnel method

This method is applicable to samples containing a large quantity of suspended matter such as sludge.

Apparatus

Perforated plate: Stainless steel (SUS 27 or 28), approximately 0.5 mm in thickness, 50 mm or 90 mm in diameter. It is shaped like a watch glass with a slightly bent edge. Small holes about 0.5 mm in diameter are bored at suitable intervals all over its flat surface.

Rubber packing: A rubber ring 2 to 3 mm in thickness, 10 mm to 90 mm in diameter and about 10 mm in width, can be put in a Büchner funnel and can be used for filtration by suction, with the perforated plate on it.

Büchner funnel: 50 mm or 90 mm

Testing procedure

Prepare two perforated plates. Put rubber packing in Büchner funnel and place the perforated plate on it. Position the filter paper (grade 6), pour water on the filter paper several times and suck. Remove the filter paper with the perforated plates and dry at a temperature of 105 to 110°C for 2 to 3 hours. Allow to cool in a desiccator and weigh to constant weight. (When chemical balance is used, the lighter perforated plate is used as supplemental weight.)

Next put the heavier perforated plate together with the filter paper in the funnel and filter 200 to 400 ml of the test water by suction. Pour the filtrate into the lighter plate with filter paper several times and continue as for first plate.

Obtain the difference in weight before and after this operation, and calculate ppm of the suspended substances contained in the test water by the following formula:

\[
S = \frac{1000}{v} (a - b)
\]

where,
- \(S\): Suspended substances (ppm)
- \(a\): Difference in weight before and after filtration of the test water (mg)
- \(b\): Difference in the weight before and after filtration of the filtrate (mg) (when chemical balance is used \(b=0\))
- \(v\): Test water (ml)

See also Remarks 1-3, above.
B. FILTRATION THROUGH ASBESTOS LAYER

Apparatus
Gooch crucible, 25 to 35 ml

Reagents
Suspension of asbestos: add water to 15 g asbestos and, after removing fine portion by decantation several times, add water to make 1 litre.

Procedure
Prepare two Gooch crucibles (same shape and approximately same weight). After drying, pour about 20 ml of the well-stirred asbestos suspension to obtain a layer of asbestos about 3 mm thick (about 0.3 g)* and suck gently.

Next put the Gooch crucibles into the air oven. After drying for two hours at a temperature of 105 to 110°C, allow to cool to constant weight in the desiccator and measure the weight of each crucible (when chemical balance is used, the lighter crucible is used as the supplemental weight). Attach the heavier crucible to the suction bottle and pour in enough test water to give a weight of suspended matter of more than 5 mg after drying and gently filter by suction. At this time, repeat the filtration of the initial portion of the filtrate.

Next pour a small amount of filtrate into the lighter crucible several times using suction, then dry in the air oven for two hours at 105 to 110°C, and allow to cool in a desiccator. Weigh the crucible and obtain the difference in weight using the crucible as a supplemental weight) and calculate ppm of the suspended matter by the following formula.

\[ S = (a - b) \times \frac{1000}{V} \]

(see above for clarification of symbols).

Remark: The test water should be sampled as specified in glass filter method. When the soluble volatile residue is less than 5000 ppm, refer to remark 2 in the same section.

* When half the amount of asbestos solution is poured out put in the perforated plate and pour the other half of the solution.
C. CENTRIFUGATION METHOD

This method is applicable to samples which are very difficult to filter due to their content of suspended matter.

Apparatus

Centrifugal separator about 2000 rpm. Precipitation tube 50 to 100 ml.

Procedure

Pour into the precipitation tube enough test water to give more than 5 mg suspended matter.

After weighing each tube, centrifuge at about 2000 rpm for 20 minutes and precipitate the suspended matter in the test water. Remove the supernatant liquid by decantation*.

Add 10 ml of the water to the precipitate, centrifuge again and remove the supernatant liquid by decantation.

Transfer the precipitate into an evaporating dish which has been previously heated to constant weight at 105 to 110°C and evaporate to dryness on the steam bath. After drying in the drier at 105 to 110°C for 2 hours, allow to cool in a desiccator and weigh. (When a chemical balance is used, an evaporating dish of the same shape should be used as a supplemental weight after the blank test for it has been performed.) Obtain the difference in weight before and after this operation. Calculate ppm of the suspended matter by the following formula:

\[ S = a \times \frac{1000}{V} \]

(see above for clarification of symbols).

Remark: There should be a certain degree of difference in density between the dispersed phase and the dispersion medium to make centrifugal separation possible. When a particle of 1 mg is centrifuged at an angular velocity of \( w \) rad/sec at a position of \( r \) cm from the centre of rotation, the centrifugal force which a particle receives is as follows:

* When the determination of soluble evaporated residue is to be performed, keep the supernatant liquid.
Supposing that the mass of the dispersion medium expelled by a particle is 1 mg

then \[ F = (m - m')w^2r \]

Supposing that the specific centrifugal force is RCF and rotational frequency per minute is \( N \) (rpm)

then \[ RCF = \frac{F}{(m - m')g} = \frac{w^2r}{g} = 0.00001118 \text{ rN}^2 \]

From the above equation, it is clear that the centrifugal force near the surface differs from that at the bottom portion of the liquid. For instance, when \( N = 2000 \) rpm and the distance between the surface of the liquid in the precipitation tube and the center of rotation is 5 cm (\( r = 5 \) cm), RCF is 223 g; when the distance between the bottom of the precipitation tube and the central axis of rotation is 13 cm, RCF becomes 581 g. Therefore, the RCF value near the surface and that at the bottom should both be reported.

Depth of the liquid layer = 

\( \frac{(\text{RCF at the bottom}) - (\text{RCF at the surface})}{(\text{RCF at the bottom})} \times \text{distance from the bottom} \)

In this test, a centrifugal separator whose bottom is 13 cm from the central rotation axis at a rotational frequency of 2000 rpm is regarded as standard.

- **Calculation of suspended matter from the difference in weight of evaporated residue**

Calculate the suspended matter from the difference between the total evaporated residue and the soluble evaporated residue.

\[ A = B - C \]

where,  
\[ A = \text{suspended matter (ppm)} \]  
\[ B = \text{total evaporated residue (ppm)} \]  
\[ C = \text{soluble evaporated residue (ppm)} \]
3. **SUSPENDED MATTER FORMED AT pH 7**

(From Japanese Industrial Standards K0102 - 10.3)

For suspended matter formed when the test water is neutralised to pH 7 ± 0.5.

**Reagents**

- NaOH (sodium hydroxide) solution (4 to 24 w/v %)
- Acetic acid, diluted 1:2 to 1:16, acid:water

**Procedure**

Place enough test water to give more than 5 mg suspended matter in a beaker and neutralise it with sodium hydroxide solution or with diluted acetic acid, according to the acidity/alkalinity of the test water, taking care to minimise the increase in the volume of the solution during neutralisation. Then proceed according to procedures in the methods above to obtain amount of suspended matter at pH 7 and calculate ppm of the suspended matter formed at pH 7 by the following formula.

\[ A = B - C \] (See above for clarification of symbols.)

**Remarks:**

(1) Depending on the kind of waste water, the weight of suspended matter may decrease when it is neutralised. In such cases, the weight of suspended matter should be reported as suspended matter formed at pH 7.

(2) Suspended matter formed at pH 7 may be determined with the supernatant liquid or filtrate after removing the suspended matter. This method is applicable to test water which contains a relatively small amount of suspended matter but which forms a large amount of precipitate after neutralisation (without changing the first suspended matter) or to waste water which forms a relatively small amount of precipitate. The method should not be applied to waste water which is apt to cause the formation of a complex precipitate or a dissolution reaction by neutralisation.
1. **INTRODUCTORY INFORMATION**

- **Prerequisites**
  
  The empirical formula of the test material is required so that the theoretical oxygen demand (TOD) may be calculated. If this is unknown, the chemical oxygen demand (COD) of the test material may serve as the reference point (6).

- **Guidance information**
  
  Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level".

- Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

- **Interpretation of results**

  Because of the stringency of this test, a measured BOD* which is less than 60 per cent of TOD (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability. The possibility that nitrogen-containing compounds may affect the results should be considered.

- **Qualifying statements**

  - The method is only applicable to those organic test materials which, at the concentration used in the test, are not inhibitory to bacteria.

  - This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Programme (1978-1980).

---

*Biochemical oxygen demand*
Recommendations

- Test chemicals giving a measured BOD which is greater than 60 per cent of the TOD (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 per cent.

- If the test material is not soluble at the test concentration, special measures, such as the use of ultrasound dispersion, may have to be employed to achieve a good dispersion of test material.

Standard documents

This Test Guideline is based on a paper found in reference 8, Section 4, Literature.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

The purpose of the method is the measurement of the biodegradability of organic compounds in an aerobic, aqueous medium at a test concentration of 2 (standard concentration) to 10 mg/l of active material. Most data elaborated with this test pertain to water-soluble compounds; however, volatile compounds and those of low water solubility may also be tested, at least in principle.

Definitions and units

The degradation is stated as the biochemical oxygen demand (BOD) within 28 days as a percentage of either the theoretical oxygen demand (TOD) or the chemical oxygen demand (COD).

For example:

\[
\text{% biodegradability} = \frac{\text{mg } O_2/\text{mg active substance}}{\text{TOD}} \times 100
\]

or

\[
= \frac{\text{mg } O_2/\text{mg active substance}}{\text{COD}} \times 100
\]

Where

TOD = theoretical oxygen demand (calculation, see Data and reporting, below)

COD = chemical oxygen demand determined experimentally.
Reference substances

In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate, sodium benzoate, sodium n-dodecylsulfate and the sodium salt of linear alkylbenzenesulfonic acid (8, 9) may be used for this purpose. They must exhibit a BOD which is > 60 per cent of their TOD within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

Principle of the test method

A predetermined amount of the compound is dissolved in an inorganic medium (mineral nutrient solution), providing a usual concentration of 2 mg active substance per litre (AS/l). The solution is inoculated with a small number of micro-organisms from a mixed population and kept in closed bottles in the dark in a constant temperature bath at 20 ± 1°C. The degradation is followed by oxygen analyses over a 28-day period. A control with inoculum, but without test material is run parallel for the determination of oxygen blanks.

Quality criteria

Reproducibility

The reproducibility of the method is appropriate for a screening test for "ready biodegradability", which has solely an acceptance function, but is not sufficient for a final decision regarding biodegradibility.

Sensitivity

A starting concentration of 2 mg AS/l usually allows the determination of 95 per cent degradation. Compounds with a low TOD may require higher starting concentrations.

Specificity

The test is applicable for the biodegradability evaluation of organic compounds. Most experience has been gathered with water soluble compounds; however, the test lends itself, at least in principle, to the biodegradability evaluation of volatile and insoluble compounds.
Possibility of standardisation
The test is suited for standardisation.

Possibility of automation
Although the whole test cannot be automated, parts of it, e.g., the analysis, may. The procedure is well suited however, for being operated with whole series of test materials.

B. DESCRIPTION OF THE TEST PROCEDURE
   * Preparations

Reagents
Distilled or ion exchange water: containing not more than 0.01 mg Cu/I, air saturated. Volume according to the need of the day, e.g. 50 l, at room temperature, as close as possible to 20°C, aerated strongly for 20 minutes with compressed air. Generally, the water is ready for use after standing for 20 hours at 20°C. Oxygen is determined for control purposes. The concentration at 20°C should be about 9 mg O₂/l. All transfer and filling operations of the air saturated water must be conducted bubble-free by siphon.

Standard dilution water for BOD determinations according to D65 of ENV (Ref. 1)

Solution 1: 8.5g of KH₂PO₄, 21.75g of K₂HPO₄, 33.3g of Na₂HPO₄·2H₂O, and 1.7g of NH₄Cl are dissolved in 500 ml of distilled water. The solution is made up to 1 l. The pH ought to be 7.2.

Solution 2: 22.5 g of MgSO₄·7 H₂O/l
Solution 3: 27.5 g of CaCl₂/l
Solution 4: 0.25 g of FeCl₃·6 H₂O/l.

1 ml each of solutions 1 to 4 are added to 1 litre of aerated distilled water.

Materials
- Calibrated 250-300 ml BOD bottles with glass stoppers. (Uncalibrated narrow neck 250 ml bottles with glass stoppers whose volumes have been determined may be used instead without causing any appreciable error.) All glassware is cleaned according to the H23 procedure (reference 2).
- Several 2, 3 and 5-1 bottles with self-made litre marks for the preparation of the experiments and for the filling of the 800 bottles.
- Pipettes of 1 to 10 ml volume.
- Funnels and coarse filter paper.
- Baby bottles for the preparation and shaking of the inoculum.
- Water bath for keeping the bottles at constant temperature under the exclusion of light.

Inoculation

The following inocula may be used:

An aqueous suspension of unfertilised garden soil: 100g of unfertilised garden soil (soil from a greenhouse which is at constant temperature throughout the year is especially advantageous) are dispersed in chlorine-free tap water (1 litre). After 30 minutes the suspension is filtered through a coarse filter and the first 200 ml of the filtrate are discarded. The main part of the filtrate serves for the inoculation (1 drop from a pointed pipette per litre of final volume, see Procedure, below). The inoculum is prepared immediately before the experiment. It must be aerated if it is to be kept for several hours. The number of bacteria may be determined according to the pour plate method or with nutrient pad sets. There should be no more than $10^3$ to $10^5$ bacteria per millilitre of final volume.

Effluent from an activated sludge plant or trickling filter: Inoculation should preferably be made using a secondary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and application. To prepare the inoculum the sample is filtered through a coarse filter, the first 200 ml being discarded. The rest of the filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

Effluent from a strongly aerated laboratory activated sludge plant: The inoculum is prepared as described in previous section.

Composite inoculum: Equal volumes of the three inoculum samples are combined, mixed well and the final inoculum drawn from this mixture. The suitability of the inoculum is checked by means of a control substance.
Procedure

Direct comparisons are always necessary in biological experiments. Therefore, groups of parallel bottles are prepared for the determination of the BOD of the test and control substances in simultaneous experimental series. If chemical analyses are performed simultaneously, a sufficient number of bottles — including the controls for the inoculum and the blank — have to be prepared [e.g., 7 or 15 parallel bottles are prepared for one test material for the 0, 5, 15 and 28-day tests after a sufficient volume of water has been prepared in large bottles (see Reagents above)].

These large bottles are first filled to about one third of their volume with distilled water by hose. Then the individual salt stock solutions are pipetted into these bottles according to the final volume, and the respective test or control materials are added in such amounts that final concentrations of 2 and sometimes 5 or 10 mg/l (higher concentrations only in case of poorly degradable compounds and those with a low T00) are attained (see general consideration, below). Subsequently, the experimental solution is inoculated with 1 drop (from a pointed pipette) per litre of final volume, and the blank is inoculated similarly. Finally, the solutions are made up to volume with a hose which reaches down to the bottom of the flask, which achieves adequate mixing.

Subsequently, each prepared solution is filled immediately into the respective group of bottles by hose from the lower quarter (not from the bottom!) of the bottle. Furthermore, the zero controls are analysed or preserved for the later analyses (for the O2-determination by precipitation with MnCl2 and NaOH; for the surfactant determination by preservation with 50 mg/l of HgCl2). The remaining parallels are placed in a water bath at 20°C, kept in the dark, and removed after 5, 15 and 28 days, respectively, from the bath, and analysed. The result is recorded as percentage biodegradability and should be visualised as a diagram.

The test can be finished before the 28th day in the case where a plateau is observed before the 28th day. In the case, where a degradation has obviously started on day 28 but has not reached a plateau on day 28, it is considered good practice to extend the experiment for one or two weeks longer.

General considerations

Each series is accompanied by complete parallel series for the determination of the blank, and the oxygen depletion without inoculation.
The oxygen depletion without inoculation should not exceed 0.2 to 0.3 mg O₂/l after 5 days and 0.4 mg O₂/l after 28 days, respectively; the blank with inoculation should not exceed 0.4 to 0.5 mg O₂/l after 5 days and 0.5 to 0.6 mg O₂/l after 15 to 28 days. Typical values for the reference substances can be found in the literature (8, 9).

Degradability is calculated by (1) subtracting the oxygen depletion result of the blank from that of the test substance and (2) dividing this number by the concentration (W/V) of the test material. This calculation gives the oxygen depletion in mgO₂/mg of active material; this value is transformed to the "% biodegradability" according to Data and reporting (below) for the day under consideration.

If the structure or elemental composition of the test material is unknown, or it is a mixture of unknown organic compounds, the chemical oxygen demand (COD) may serve as a reference instead of the TOD.

The concentration of about 9 mg of dissolved oxygen per litre of dilution water at 20°C limits the possible starting concentration of the test material to about 2 mg/l in order to guarantee that oxygen concentration of 4-5 mg/l remains after the oxidation of the test substrate. Substances which are only partially degraded or those which have a low TOD are advantageously tested in parallel experiments at 5 or even 10 mg/l starting concentrations.

Inhibition test

Organic and inorganic materials may easily and simply be tested for inhibitory effects in the closed bottle test:

series 1: 2 mg/l of a well-degraded compound (e.g., fatty alcohol + 10 moles ethylene oxide)
series 2: x mg/l of test material (x is usually 2)
series 3: 2 mg/l of the control compound plus x mg/l of test material

If the BOD values of series 3 correspond to the sum of those of series 1 and 2, the test material does not display inhibitory effects. This control experiment is always necessary if a negative or poor degradability result seems illogical in view of the structure of the test material, i.e., if there are indications that it may be caused by inhibition.
Analytical means
- Oxygen determination according to Winkler (3) or electrometrically, if suitability is assured.
- In case of anionic surfactants: MBAS-determination according to H 23 of DEV (2).
- In case of nonionic surfactants: BIAS*-determination according to Wickbold (4).

3. DATA AND REPORTING

Treatment of results

Calculation of the theoretical oxygen demand (TOD)

The TOD of the compound $C_{6}H_{12}O_{6}$ is calculated according to:

$$\text{TOD}_{NH_{3}} = \frac{16 \left(2 \cdot 6 + \frac{1}{2} \cdot 12 - 6\right)}{\text{mol.wt.}}$$

This calculation implies that C is mineralised to CO$_2$, H to H$_2$O, P to P$_2$O$_5$ and Na to Na$_2$O. Halogen is eliminated as hydrogen halide and nitrogen as ammonia.

Example: Glucose $C_{6}H_{12}O_{6}$, mol.wt. = 180

$$\text{TOD} = \frac{16 (2 \cdot 6 + \frac{1}{2} \cdot 12 - 6)}{180} = 1.07 \text{ mg O}_2/\text{mg active substance}$$

Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolysed.

Sulfur is schematically oxidised to the state of +6. Example: Alkylbenzenesulphonate, $LASC_{18}H_{29}SO_{3}Na$, mol.wt. = 348

$$\text{TOD} = \frac{16 (36 + 22 + 3 + \frac{1}{2} - 3)}{348} = 2.34 \text{ mg O}_2/\text{mg active substance}$$

* bismuth active substance
In the case of nitrogen-containing compounds the nitrogen may be eliminated as ammonia, nitrite, or nitrate corresponding to different theoretical oxygen demands:

\[
\text{TODNO}_2 = \frac{16 \left[ 2 c + \frac{1}{2} (h-cl) + 3 s + \frac{3}{2} n + \frac{5}{2} p + \frac{1}{2} na - o \right]}{\text{mol.wt.}}
\]

\[
\text{TODNO}_3 = \frac{16 \left[ 2 c + \frac{1}{2} (h-cl) + 3 s + \frac{5}{2} n + \frac{5}{2} p + \frac{1}{2} na - o \right]}{\text{mol.wt.}}
\]

Suppose full nitrate formation would have been observed by analysis in case of a secondary amine:

\[
\text{(C}_{12}\text{H}_{25})\text{NH; mol.wt. = 353}
\]

\[
\text{TODNO}_3 = \frac{16 \left( 48 + \frac{51}{2} + \frac{5}{2} \right)}{353} = 3.44 \text{ mg O}_2/\text{mg active substance}
\]

**Calculation of results**

For the calculation of the percentage biodegradability after n days the determined oxygen depletions are divided by the concentration (W/V) of active substance.

Then

\[
\% \text{ biodegradability} = \frac{\text{mg O}_2/\text{mgAS}}{\text{TOD}} \times 100
\]

or

\[
= \frac{\text{mg O}_2/\text{mgAS}}{\text{mgCOD/AS mgAS}} \times 100
\]

**Test report**

The raw data may be listed in and evaluated according to the form sheet in Annex 2.

**Interpretation of results**

See Section 1.
4. LITERATURE


(2) Deutsche Einheitsverfahren zur Wasseruntersuchung: Methode H23, Verlag Chemie, Weinheim 1960

(3) Deutsch Einheitsverfahren zur Wasseruntersuchung: Methode G2, Verlag Chemie, Weinheim 1960

(4) R. Wickbold, Wasser 33, 229, (1966)

(5) R. Wickbold, Tenside Detergents 8, 61 (1971)

(6) Standard Methods for the Examination of Water and Wastewater, 12 ed. American Public Health Association, American Water Works Association, Water Pollution Control Federation, Oxygen Demand, p. 510, (1965)

(7) W.K. Fischer, Fette, Seifen, Anstrichmittel, 65, 37-32 (1963)


(9) P. Gerike and W.K. Fischer, Ecotoxicology & Environmental Safety, 2, No 2, 159-173 (1979)
Scheme for the Bottle Arrangement for the Closed Bottle Test

<table>
<thead>
<tr>
<th>analyses</th>
<th>controls</th>
<th>determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>distilled water salt solutions</td>
<td>distilled water salt solutions</td>
</tr>
<tr>
<td></td>
<td>mineral nutrient solution (control of the oxygen blank)</td>
<td>inoculation blank</td>
</tr>
<tr>
<td></td>
<td>*-det.</td>
<td>*-det.</td>
</tr>
<tr>
<td></td>
<td>O₂-det.</td>
<td>O₂-det.</td>
</tr>
<tr>
<td>immediately</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

( * = specific analysis if available )
2. SAMPLE FORM SHEET FOR THE CLOSED BOTTLE TEST

Exp. no.: 
Date of start of test: 
Test / standard material: 
Theoretical test concentration: 
Inoculum: 
Analysis: (Winkler method or oxygen electrode) 
TOD or COD of test material: 
Temp. of the dilution water after aeration: 
O$_2$-conc. of the distilled (deionised) water after aeration and standing before start of test: 

<table>
<thead>
<tr>
<th>mg AS/l</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>mg O$_2$/mg AS</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>K (°C)</th>
</tr>
</thead>
</table>

mg O$_2$ /l

A: O$_2$ Determinations:

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Flask no.</th>
<th>Analyses</th>
<th>mg O$_2$/l after x days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral nutrient solution without test material and without inoculum</td>
<td>O$_2$-</td>
<td>c$_1$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>c$_2$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>mean = $\frac{c_1 + c_2}{2}$</td>
<td>-</td>
</tr>
<tr>
<td>Mineral nutrient solution without test material but with inoculum</td>
<td>1</td>
<td>c$_3$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>c$_4$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>mean blank</td>
<td>mean blank = $\frac{c_3 + c_4}{2}$</td>
<td>-</td>
</tr>
<tr>
<td>Mineral nutrient solution with test material and with inoculum</td>
<td>1</td>
<td>a$_1$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a$_2$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>mean test</td>
<td>mean test = $\frac{a_1 + a_2}{2}$</td>
<td>-</td>
</tr>
</tbody>
</table>
B: O₂ Depletions (mg BOD/l) after x days

\[ \text{BOD}_x = (m_0 - m_{t_x}) - (m_0 - m_{b_x}) \]

<table>
<thead>
<tr>
<th>mg BOD/l after x days</th>
<th>5</th>
<th>15</th>
<th>28</th>
</tr>
</thead>
</table>

* This difference is important as a check for the validity of the test

C: Evaluation

% biodegradability (I) = \( \frac{\text{mg BOD}_x/l}{\text{mg AS/l.TOD}} \) \cdot 100

or (II) \( \frac{\text{mg BOD}_x/l}{\text{mg AS/l.COD}} \) \cdot 100

<table>
<thead>
<tr>
<th>( \text{(I)} )</th>
<th>after x days</th>
<th>5</th>
<th>15</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{(II)} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1. **INTRODUCTORY INFORMATION**

- **Prerequisites**
  - Water solubility
  - The organic carbon content of the test material must be established.

- **Guidance information**
  - Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level".
  - Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

- **Interpretation of results**

  Because of the stringency of this test a result of less than 70 per cent loss of DOC - dissolved organic carbon - (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

- **Qualifying statements**

  The method is only applicable to those organic test materials which, at the concentration used in the test,
  - are soluble in water (at least 5 to 40 mg dissolved organic carbon/litre)
  - have negligible vapour pressure,
  - are not inhibitory to bacteria, and
  - do not significantly adsorb on glass surfaces.

This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Programme (1978-1980).

Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.
Recommendations

- Test chemicals giving a result of greater than 70 per cent loss of DOC (within 28 days) should be regarded as readily biodegradable. This level is reached within 10 days of biodegradation exceeding 10 per cent.

- If the limits of sensitivity of organic carbon analysers are improved, the use of lower test concentrations may be an advantage, particularly for toxic compounds.

Standard documents


2. Method

A. Introduction, Purpose, Scope, Relevance, Application and Limits of Test

The purpose of the method is the measurement of the ultimate biodegradability of water soluble, non-volatile organic compounds in an aerobic, aqueous medium at a starting test concentration corresponding to 5-40 mg DOC/l (in order to avoid inhibitory effects, it is in the investigator's own interest to choose as low a starting concentration as his analytical capability permits.)

Definitions and Units

Definition of biodegradability:

\[ D_t = \left( 1 - \frac{C_t - C_{blt}}{C_0 - C_{bl_0}} \right) \times 100 \]
where

\[ D_t = \text{degradation in percent DOC-removal at time } t \]

\[ C_0 = \text{starting DOC concentration of the culture medium (mg DOC/l)} \]

\[ C_t = \text{DOC concentration of the culture medium at time } t \text{ (mg DOC/l)} \]

\[ C_{bol} = \text{starting DOC concentration of the blank (mg DOC/l)} \]

\[ C_{bol_t} = \text{DOC concentration of the blank at time } t \text{ (mg DOC/l)} \]

Degradation is stated as the percentage DOC-removal within 28 days with respect to the test material (% DOC-removal).

\[ \text{Reference substances} \]

In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum the use of control substances is desirable. Aniline, sodium acetate or sodium benzoate may be used for this purpose. They must exhibit DOC removal ≥70 per cent within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

\[ \text{Principle of the test method} \]

A predetermined amount of the compound is dissolved in an inorganic medium (mineral nutrient solution, fortified with a trace element and essential vitamin solution), providing a concentration corresponding to 5-40 mg DOC/l. The solution is inoculated with a small number of micro-organisms from a mixed population and aerated at 20-25°C in the dark or at least in diffuse light only. The degradation is followed by DOC analysis over a 28-day period. The procedure is checked by means of a standard.

A control with inoculation, but without either test material or standard, is run parallelly for the determination of DOC blanks.
Reproducibility

The reproducibility of the method is appropriate for a screening test which has solely an acceptance but no rejective function.

Sensitivity

The sensitivity of the method is largely determined by the sensitivity limit of the organic carbon analysis, which is 0.5 mg C/litre at present.

Specificity

This method is applicable for the biodegradability evaluation of water soluble, non-volatile organic compounds.

Possibility of standardisation

The test version with specific analyses for anionic and nonionic surfactants is standardised as the "OECD Screening Test".

Possibility of automation

Parts of the test, e.g., the analysis, can be automated, although hardly the total procedure.

The procedure is, however, well-suited for being operated with whole series of test materials.

B. DESCRIPTION OF THE TEST PROCEDURE

Preparations

Reagents

Deionised water: Deionised or distilled water free of toxic substances (copper in particular), for general use as a solvent. Water which has been deionised by distillation or ion exchange is suitable. Distilled water will never contain more than 10 per cent of organic carbon introduced by the test material.
The high purity of this test water is necessary in view of the DOC analyses in the concentration range of 0-40 mg/l. Contamination may result from inherent impurities but also from the ion exchange resins and microbial developments (bacteria, algae under the influence of light, etc.). Only one water charge must be used for each test series, which is to be controlled beforehand by DOC analysis. If necessary, suitable water may be obtained by UV irradiation or other means.

Nutrient solution: Mix 1 ml each of the following solutions (a)-(f) and make up to a volume of 1 litre with water, above. (A.R. means analytical reagent).

(a) $KH_2PO_4$  A.R. 8.5 g
   $K_2HPO_4$  A.R. 21.75 g
   $Na_2HPO_4\cdot 2H_2O$  A.R. 33.4 g
   $NH_4Cl$  A.R. 20.0 g

in 1000 ml of water (above) The pH value should be 7.2

(b) 22.5 g of $MgSO_4\cdot 7H_2O$ A.R. dissolved in 1000 ml of water

(c) 27.5 g of $CaCl_2$ A.R. dissolved in 1000 ml of water

(d) 0.25 g of $FeCl_3\cdot 6H_2O$ A.R. dissolved in 1000 ml of water

This solution is prepared freshly immediately before use.

(e) Trace element solution:

$MnSO_4\cdot 4H_2O$  39.9 mg (30.23 mg $MnSO_4\cdot H_2O$)
$H_3BO_3$  57.2 mg
$ZnSO_4\cdot 7H_2O$  42.8 mg
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$  34.7 mg (36.85 mg $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$)

Fe - chelate ($FeCl_3$, EDTA)  100 mg
water (above)  1000 ml

Sterilisation of the trace element stock solution at 120°C (393 K), 2 atm., 20 min.
(f) Vitamin solution

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>water (above)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

The solution is filtered sterile (0.2 μm). Instead of solution (f), 15 mg of yeast extract may be used per 100 ml of water (above).

Control substances: See Reference substances. Aniline must be freshly distilled.

Materials

- Shaking machine accommodating 2 litres. Erlenmeyer flasks either with automatic temperature control or used in a constant temperature room at 20-25°C (293-298 K).
- Narrow neck, 2 litre Erlenmeyer flasks (creased fluted flasks are recommended). The flasks must be carefully cleaned with, e.g., alcoholic hydrochloric acid, before use, rinsed and dried in order to avoid contamination with residues from previous tests. The flasks must also be cleaned before the first use since they may be contaminated.
- Membrane filtration apparatus
- Membrane filters, 0.2 μm
- Carbon analyser

Inoculation

Either of the following three alternatives (1, 2, 3) may be used as inoculum or a composite sample thereof (4).

(1) Inoculum from secondary effluent

The inoculum is gained preferably from a secondary effluent of good quality collected from a treatment plant dealing with a predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.
(2) Inoculum from soil

100 g of soil (fertile, not sterile) are suspended in 1000 ml of chlorine-free drinking water (soils with an extremely large content of clay, sand or organic carbon are unsuitable). After stirring the suspension is allowed to settle for 30 minutes.

The supernatant is filtered through coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and continuously until use. The inoculum must be used on the day of collection.

(3) Inoculum from a surface water

An inoculum is drawn from a suitable surface water.

The sample is filtered through a coarse paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

(4) Composite inoculum

Equal volumes of the 3 inoculum samples are combined, mixed well, and the final inoculum drawn from this mixture. The suitability of the inoculum is checked by means of a control substance.

Procedure

The test materials are evaluated simultaneously in duplicate together with the biodegradability standard and a control test with inoculation but without either test or standard material for the determination of DOC blanks.

The control material must attain $\geq 70$ per cent DOC removal within 28 days at a starting concentration corresponding to 20 mg DOC/l. If $< 70$ per cent DOC removal is not achieved the whole series must be discarded.*

A stock solution of the test material in water (above) is prepared. Enough stock solution is added to the nutrient solution (above) to achieve a carbon concentration of 5-40 mg DOC/l. The starting concentration of the control substance is, however, 20 mg DOC/l.

* This limit is based on present experience with the 19-day version of the test. A revision of this limit or even of the standard might have to be considered after the accumulation of experience with the new 28-day version of the test.
Two reaction vessels are each filled with 900 ml of the nutrient solution and inoculated with 0.5 ml/l of the inoculum. The opening of the vessel is covered (e.g. aluminum foil) in such a way that the exchange of air between the flask and the surrounding atmosphere is not unduly impeded. (Cotton wool is unsuited because of the DOC analysis.) The vessels are then inserted in the shaking machine. The temperature of 20-25°C (293-298 K) must be maintained unchanged during the test, and the vessels should be shielded from light. The air should be free of pollutants and toxic materials (chlorinated solvents, etc.)

In the course of the biodegradation test the DOC concentrations are determined in duplicate at the beginning (day 0), and on the 27th and 28th day. Three additional analyses must be performed at regular time intervals (7th, 14th, and 21st day).

The analyses are registered on the form sheet (below) and evaluated.

Only the necessary volumes of culture medium should be drawn for each determination: however, they must be large enough for the membrane filtration or centrifugation preceding the carbon determination. The latter requires differing volumes for the different instruments. Evaporation losses of the culture medium are to be made up by adding water (above) in the required amounts. The culture medium is to be mixed well before withdrawing a sample. Material adhering to the wall of the vessel must be dissolved or suspended before sampling. The membrane filtration or centrifugation must be done immediately. The filtered or centrifuged samples must be analysed on the same day, otherwise they must be preserved with 0.05 ml of the HgCl₂ solution (above) for each 10 ml of nutrient medium or by storing at 2-4°C. The biodegradability test is valid provided the standard exhibits a degradation rate within the specified range. The test can be finished before the 28th day if complete mineralisation has been accomplished. Where degradation has obviously started on day 28 but did not reach a plateau on day 28, it is considered good practice to extend the experiments for one or two weeks longer.

All steps require great care and cleanliness of the vessels, pipettes, etc. but not sterility.
Analytical means

Membrane filter 0.2 μm, 25 mm diameter. Preparation of the "filters": membrane filters are impregnated with surfactants for hydrophilisation. Thus each filter contains up to several mg of soluble carbon which would interfere in the biodegradability determinations. Therefore, the filters are purified from surfactants and other soluble organic interferences by boiling them in deionised water for three periods each of one hour. These filters may be stored in water (above) for at least one week.

Other membrane filters are suitable if it is assured that they neither release carbon nor adsorb the compound in the filtration step.

If the samples are centrifuged, this must be done at 40,000 msec⁻² (4000 g) for 15 minutes, preferably in a refrigerated centrifuge, in any case <40°C.

(Remark: the differentiation TOC:DOC by centrifugation at very low concentrations does not seem to work well since either not all bacteria are removed or carbon as part of the bacterial plasma is redissolved. At higher test concentrations (> 10 mg C/l) and the same small inoculation the centrifugation error seems to be comparatively small.)

The DOC measurement: The sample withdrawn from the culture medium (about 30 ml) is centrifuged or membrane filtered immediately in the filtration apparatus using the membrane filters prepared as stated above. The first 20 ml of the filtrate are discarded.

The DOC concentration is determined twice in the remaining filtrate (about 10 ml) by means of the TOC/DOC instrument. If the filtrate cannot be analysed on the same day it must be preserved as stated above. The DOC measurements (mg C/l) obtained are registered on the attached data sheet and the DOC concentrations of the culture medium and of the blanks calculated for each sampling time.
3. DATA AND REPORTING

* Treatment of results *

The degradation at time $t$ is calculated from the determinations of the DOC concentrations at the beginning ($C_0$) and at time $t$ ($C_t$) according to

$$D_t = \left[ 1 - \frac{C_t - C_{blt}}{C_0 - C_{bl0}} \right] \times 100$$

where

- $D_t$ = degradation in percent at time $t$
- $C_0$ = measured starting DOC concentration of the inoculated culture medium (mg DOC/l)
- $C_t$ = DOC concentration of the culture medium at time $t$ (mg DOC/l)
- $C_{bl0}$ = starting DOC blank of the mineral nutrient solution with inoculation, but without test material (mg DOC/l)
- $C_{blt}$ = DOC blank of the mineral nutrient solution with inoculation, but without test material at time $t$ (mg DOC/l)

The degradation rates are calculated to the nearest 0.1 per cent. The means of the $D_t$ values are calculated and reported to the nearest full percent. Results ending in 0.5 are rounded up to the nearest whole number. The course of the degradation test is presented graphically in a diagram.

* Test report *

The results may be reported on the data sheet in the Annex.

* Interpretation of results *

The results of the degradation test are valid if the condition is met that in the same test series the control yields 70 per cent DOC-removal.

Because of the stringency of this test a result inferior to the recommended pass level (70 per cent loss of DOC) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish this.
## Annex

**Sample Form Sheet for the Modified OECD Screening Test**

### A: Controls

<table>
<thead>
<tr>
<th>Exp. no.:</th>
<th>Date of start of test:</th>
<th>Test: Standard material:</th>
<th>Theoretical test conc.: mg DOC/L</th>
<th>Inoculum:</th>
<th>Carbon analyzer:</th>
</tr>
</thead>
</table>

| Stock solution of the test material [(1000 mg/L, dilution...1/1000 ml of nutrient solution)] | mg/L | TOC* | DOC** |

### B: Carbon determinations

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Flask no.</th>
<th>Analyses</th>
<th>-</th>
<th></th>
<th>Theor. DOC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral nutrient solution with test material and with inoculum</td>
<td>1</td>
<td>b1</td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b2</td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m1 = (b1 + b2)</td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>b1</td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b2</td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m2 = (b1 + b2)</td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td>Mineral nutrient solution without Blank</td>
<td></td>
<td></td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td>Test material but with inoculum</td>
<td></td>
<td></td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m3 = (C1 + C2)</td>
<td>/</td>
<td></td>
<td>0 (C0)</td>
</tr>
</tbody>
</table>

* Disagreement between DOC and TOC values points towards insufficient solubility of the test material.
** All DOC values determined after membrane filtration.
C: Evaluation of raw data:

<table>
<thead>
<tr>
<th>Flasks no.</th>
<th>% DOC removal after x days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>mean</td>
<td></td>
</tr>
</tbody>
</table>

\[ D_1 = \left( 1 - \frac{m_{1T} - m_{3T}}{m_1 - m_3} \right) \times 100 \]

\[ D_2 = \left( 1 - \frac{m_{2T} - m_{3T}}{m_2 - m_3} \right) \times 100 \]

\[ D = \frac{D_1 + D_2}{2} \text{ for day x} \]