ENVIRONMENT AGENCY

The assessment of aerobic biodegradability by measurement of dissolved and/or gaseous inorganic carbon in sealed vessels (2005)

Methods for the Examination of Waters and Associated Materials

The assessment of aerobic biodegradability by measurement of dissolved and/or gaseous inorganic carbon in sealed vessels (2005)

Methods for the Examination of Waters and Associated Materials

This booklet describes a method for estimating the biodegradability of organic substances under aerobic conditions.

Whilst this booklet reports details of the materials actually used, this does not constitute an endorsement of these products. Equivalent products are available and it should be understood that the performance characteristics of the method might differ when other materials are used. It is left to users to evaluate this method in their own laboratories. Only limited performance data are provided.

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results encompassing at least ten degrees of freedom from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available. An indication of the status of the method is shown at the front of this publication on whether the method has undergone full performance testing.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with this method are listed at the back of the booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advance notice of forthcoming publications, or obtain details of the index of methods, then contact the Secretary on the Agency's internet web-page (http://www.environment-agency.gov.uk/nls) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary
December 2004

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

The assessment of aerobic biodegradability by measurement of dissolved and/or gaseous inorganic carbon in sealed vessels

1 Introduction

This method describes procedures for estimating the extent of ready, ultimate biodegradability of organic compounds under aerobic conditions. Like the modified Sturm procedure[1] this method is based on the determination of inorganic carbon or carbon dioxide produced during biodegradation, and therefore provides unequivocal evidence of biodegradation. Unlike the Sturm test, in which the inorganic carbon is collected in alkaline solution outside of the reaction vessel, this method enables inorganic carbon or carbon dioxide to be determined following analysis of samples taken from the liquid phase and/or head-space gas. This is facilitated by the addition of concentrated alkali or acid to a selected number of culture vessels on each sampling occasion. After analysis, the vessels are then discarded.

The general procedures, media and inocula described in this booklet are based on those described elsewhere[1], as revised by the OECD[2] in harmonising the various tests for ready biodegradability. Thus, the use of this method enables data to be generated using these procedures to be regarded as being equivalent to those obtained using the OECD procedures. In the small number of comparisons so far carried out using the modified Sturm test and this method, results have been obtained that are not significantly different. This method is simple, inexpensive to perform, and takes up relatively little space. This enables a large number of replicate tests to be carried out, which facilitates a more representative statistical treatment of the data to be made. A further advantage of this method over the Sturm test is that the rate of production of inorganic carbon or carbon dioxide by the micro-organisms in the test solution is reflected in the measurements[3] made, since, in the Sturm test, carbon dioxide tends not to be flushed out adequately at the pH of the test solution, and accumulates in the medium. In addition, this method is applicable to insoluble substances; also, since the system is sealed, this procedure, unlike that described in the modified Sturm test, is suitable for the study of the biodegradability of volatile compounds.

The test method may also be used to provide information on the extent of primary biodegradation and/or the extent of metabolite formation by using a specific analytical method to determine the concentration of the test substance/metabolite on appropriate sampling occasions. The effect of concentrated alkali or acid on the chemical species to be quantified should be assessed before commencing the biodegradation test if this analysis is to be performed on aliquots removed from the vessels used to determine the level of carbon dioxide. Otherwise, dedicated cultures for specific analysis may be established and sampled accordingly. The extent of primary biodegradation/ metabolite formation should be compared to the concentration of the test substance determined in abiotic control vessels (i.e. sterile culture medium plus the test substance).

Currently, there is no procedure for assessing the inherent biodegradability of volatile and insoluble materials. This method can be modified to suit this purpose by: -

a) extending the test duration; or
b) using pre-exposed or acclimatised inocula; or
c) using higher population densities of micro-organisms; or
d) using a combination of procedures described in a, b and c.

As described in the Sturm test and other respirometric methods[1], the theoretical amount of inorganic carbon or carbon dioxide (i.e. 100 % of the theoretical amount that could be produced) will not normally be reached even with readily biodegradable substances. This is because only a proportion of the test compound is utilised by the micro-organisms for respiration (energy) while some is incorporated into new cells. These new cells are not then further degraded within the duration of the test (normally 28 days).

The benefit of using apparatus employed for anaerobic biodegradable assessments[4, 5] was recognised[6, 7] in the application towards the measurement of carbon dioxide in aerobic biodegradable assessments. Thus, three procedures are described for measuring the amount of inorganic carbon or carbon dioxide produced in the test. Section 8.5.1 describes a head-space technique following acidification to a pH value of less than 3.0. Section 8.5.2 describes a two-phase analysis without further treatment and section 8.5.3 describes procedures for converting carbon dioxide to carbonate by the addition of sodium hydroxide and analysing the liquid phase. Since the procedures may generate slightly different results due to the fact that different conditions are used and different determinations undertaken, only one procedure should be used within a single test study.

2 Performance characteristics of the method

2.1	Property determined	Ready, ultimate biodegradability under aerobic conditions, expressed as inorganic carbon or carbon dioxide produced as a percentage of the theoretical production.
2.2	Type of sample	Single organic compounds, soluble or insoluble, which are not inhibitory to micro-organisms at the concentration used (i.e. 2 - 20 mg of carbon per litre of the test mixture). Volatile compounds may also be tested. If a mixture of compounds is tested, an unequivocal interpretation of the data cannot be made, as it is impossible to assign the inorganic carbon or carbon dioxide to specific compounds within the test mixture.
2.3	Basis of method	Determination of the amount of inorganic carbon or carbon dioxide produced when the test compound is incubated with micro-organisms under enclosed aerobic conditions.
2.4	Precision	See table 1.
2.5	Time required for test	Normally, 28 days. Operator time approximately 30 hours for 5 substances over 28 days.

3 Principle

A known volume of test mixture, usually 100 ml, (containing an inoculum of micro-organisms from a mixed population, for example activated sludge or secondary sewage effluent) is added to a series of vessels. The test compound (at a concentration between 2 - 20 mg of carbon per litre of test mixture) is dissolved or suspended in mineral salts

medium and the vessels sealed and incubated (for up to 28 days) at a constant temperature (± 2 °C) between 20 and 25 °C. Blank control vessels containing no added test compound are similarly prepared and tested. Each vessel is filled (to about two-thirds of its volume) with test mixture (the same volume in each vessel). This allows a large excess of available oxygen in the head-space gas over that theoretically required for converting all of the carbon within the test compound to inorganic carbon or carbon dioxide. After suitable periods of time, a number of the vessels are analysed to determine the inorganic carbon or carbon dioxide in the head-space gas (vapour phase) and/or in the liquid phase, as appropriate. A number of the blank control vessels are similarly analysed. The amount of inorganic carbon or carbon dioxide produced in the test vessels containing the test compound in excess of the amount produced in the blank control vessels is compared with the theoretical amount calculated from the carbon content of the test compound. From this comparison an estimate of the ultimate biodegradability of the test compound can be calculated.

For test compounds that are sufficiently soluble in water the reduction in the amount of dissolved organic carbon (DOC) in the filtered test mixtures may also be followed. This is carried out by preparing additional test vessels and determining the DOC in filtered samples at the beginning of the test and again at the end of the test. In addition, the activity of the inoculum should be checked. This is carried out using additional test vessels where the test compound is replaced by a known readily biodegradable reference compound. The inhibitory or toxic effect of the test compound to the bacteria used may be assessed using test vessels containing a mixture of test compound and reference compound.

4 Interferences

Test compounds or their intermediate breakdown products that at the concentration used have an inhibitory or toxic effect on the biological system will give falsely low results. If it is suspected that a toxic or inhibitory effect is occurring, or has occurred, the procedure should be terminated. The test should then be repeated with a lower concentration of test compound.

5 Hazards

Since potentially pathogenic micro-organisms may be present in the inoculum, precautions should be taken to avoid risk of infection. Test compounds may be toxic and should be regarded as being potentially hazardous. Precautions should therefore be taken to avoid skin and clothing contact and inhalation of dusts and vapours.

6 Reagents

Reagents should be of a high analytical grade quality or similar.

6.1 Distilled or de-ionised water.

6.2 Mineral salts medium.

6.2.1 Calcium chloride dihydrate solution. Dissolve 36.4 ± 0.1 g of calcium chloride dihydrate in water (6.1) and make to 1 litre with water (6.1).

6.2.2 Magnesium sulphate heptahydrate solution. Dissolve 22.5 ± 0.1 g of magnesium sulphate heptahydrate in water (6.1) and make to 1 litre with water (6.1).

6.2.3 Iron(III) chloride hexahydrate solution. Dissolve 0.25 ± 0.01 g of iron(III) chloride hexahydrate in water (6.1) and make to 1 litre with water (6.1). To prevent precipitation, a drop (approximately 0.05 ml) of concentrated hydrochloric acid may be added to the solution.

6.2.4 Phosphate buffer solution. Dissolve the following compounds, i.e. 8.5 ± 0.1 g of potassium dihydrogen orthophosphate, 21.75 ± 0.10 g of dipotassium hydrogen orthophosphate, 33.4 ± 0.1 g of disodium hydrogen orthophosphate dihydrate and 0.50 ± 0.01 g of ammonium chloride, in water (6.1) and make to 1 litre with water (6.1). The pH of this solution should be 7.4 ± 0.2. If not, adjust with hydrochloric acid (6.9) or sodium hydroxide solution (6.8) as appropriate.

Store the solutions (6.2.1 - 6.2.4) in the dark, at 5 ± 3 ^{o}C (in a refrigerator) and discard the solutions when the first signs of sediment, turbidity or biological growth occur.

6.2.5 Complete medium. To prepare the complete medium, add 10.0 ± 0.1 ml of phosphate buffer solution (6.2.4) to approximately 800 ml of water (6.1) and mix well. Add 1.00 ± 0.05 ml of each of the solutions 6.2.1 - 6.2.3 to the dilute buffer solution and mix well. Make to 1 litre with water (6.1) and mix well.

6.3 Carbon dioxide-free air. A carbon dioxide-free mixture of nitrogen and oxygen. (Air may be bubbled through sodium hydroxide solution (1.0 mol l^{-1}) and then through barium hydroxide solution (0.0125 mol l^{-1}). The absence of a precipitate in the barium hydroxide solution confirms the absence of carbon dioxide in the gas).

6.4 Carbon dioxide-free water. Acidify water (6.1) to a pH value of less than 3 with phosphoric acid solution (6.7) and aerate (sparge) the solution using carbon dioxide-free air (6.3) for approximately 1 hour. Adjust the pH of the acidified water to its original value with sodium hydroxide solution (6.10). This solution should be used immediately after preparation.

6.5 Test compound stock solutions. The concentration of the test compound under study in the final test mixture should be in the range of 2 - 20 mg of carbon per litre of test mixture. Normally, the test is undertaken at a concentration of 10 mg of carbon per litre of test mixture. For soluble test compounds, the preparation of a stock solution will be necessary. For some liquid test compounds, it may be necessary to add the appropriate amount of test compound directly to the test vessel. Insoluble test compounds may require some form of pre-treatment[8] and may need to be added directly to the test vessels.

6.5.1 Soluble test compounds. Prepare a stock solution (of the test compound under study) in water (6.1) at a concentration of 10- to 100-times the final concentration used in the test mixture. Adjust the pH of the stock solution to 7.0 ± 0.2 with dilute hydrochloric acid (6.9) or sodium hydroxide solution (6.10) as appropriate. For sparingly soluble test compounds, prepare (if possible) a solution of the compound at a concentration of 10 mg of carbon per litre of test mixture.

6.5.2 Liquid test compounds (soluble and insoluble). The appropriate amount of liquid test compounds (soluble and insoluble) may be added directly to the test vessel. For volatile liquid test compounds, the test material may be added directly to the test vessel

through the seal. A high precision liquid delivery syringe (7.4) or equivalent system should be used.

6.5.3 Sparingly soluble, insoluble solids and viscous liquids. One of four procedures may be used whereby the appropriate amount of sparingly soluble, insoluble or viscous test compound may be added to the test vessel. These are[8]:

(i) direct addition of weighed amounts;
(ii) ultrasonic dispersion before addition;
(iii) dispersion with the aid of an emulsifying agent before addition; or
(iv) adsorption onto an inert medium or support, followed by direct addition of weighed amounts.

6.5.4 Other compounds. It may also be possible to prepare a saturated solution of some test compounds in water (6.1) and to add an appropriate amount of the saturated solution to the test vessel. The solubility of the test compound (at saturation) would need to be known or determined.

6.6 Reference compound solution. Prepare a stock solution of a reference compound (for example, containing 1 g of carbon per litre) in water (6.1). Compounds that have been used and found satisfactory are glucose, sodium benzoate and aniline. Insoluble reference compounds include hexadecane and anthraquinone. (Using this example and the volume given in 8.1.1.1 produces a test mixture containing 20 mg of carbon per litre of test mixture).

6.7 ortho phosphoric acid solution. (7N H_3PO_4). Cautiously add 230 ± 1 g of concentrated ortho phosphoric acid to about 800 ml of water (6.1). Mix well and make to 1 litre with water (6.1). Mix well.

6.8 Sodium hydroxide solution (7N NaOH). Cautiously add 280 ± 1 g of sodium hydroxide to about 800 ml of water (6.1). Cool, mix well and make to 1 litre with water (6.1). Mix well.

6.9 Dilute hydrochloric acid. Cautiously add 172 ± 1 ml of concentrated hydrochloric acid to about 800 ml of water (6.1). Mix well and make to 1 litre with water (6.1). Mix well.

6.10 Sodium hydroxide solution (carbon dioxide-free). Cautiously add 40 ± 1 g of sodium hydroxide to about 800 ml of carbon dioxide-free water (6.4). Mix well and make to 1 litre with carbon dioxide-free water (6.4). Mix well. This solution should be prepared immediately before use.

6.11 Gas mixtures of known amounts of carbon dioxide in nitrogen. For example in the range 0.2 - 1 % v/v carbon dioxide in nitrogen. These mixtures are commercially available.

6.12 Anhydrous sodium carbonate solution in carbon dioxide-free water. Prepare a series of solutions containing 0.5 - 10 mg of inorganic carbon per litre of solution[9].

6.13 Standard sodium hydrogen carbonate solution in carbon dioxide-free water. Prepare a series of solutions containing 0.5 - 10 mg of inorganic carbon per litre of solution[9].

6.14 Inocula.

6.14.1 Secondary sewage effluent. An inoculum of freshly collected secondary sewage effluent should be taken from a sewage-treatment works that receives predominantly domestic sewage. The inoculum should be maintained under aerobic conditions and used on the day of collection. Filter the effluent through a coarse filter, for example Whatman No.1 or equivalent, to remove gross particulate matter. On a separate portion of the suspension, measure the pH of the filtrate. Adjust the pH of the filtrate to 6.5 ± 0.2, using phosphoric acid solution (6.7). Bubble carbon dioxide-free gas (6.3) through the filtrate for about one hour to reduce the concentration of inorganic carbon in the filtrate. Restore the pH of the suspension to its original value with carbon dioxide-free sodium hydroxide solution (6.10).

It has been shown that when 100 ml of filtered effluent per litre of test mixture is used as an inoculum, the amount of inorganic carbon determined in blank control vessels (in 28 days) ranges between 0.4 - 1.3 mg of carbon per litre of test mixture. This was shown to be equivalent to less than 10 % of the inorganic carbon determined with biodegradable reference test compounds at original concentrations of 10 mg of carbon per litre of test mixture.

6.14.2 Activated sludge. Activated sludge should be collected from a sewage treatment works that receives predominantly domestic sewage. If necessary, coarse particulate matter should be removed by filtration of the sludge through a sieve, and the sieved sludge kept under aerobic conditions. The inoculum in the blank control vessels should generate as little inorganic carbon or carbon dioxide as possible; hence the sludge may need further treatment before use. For example, settle or centrifuge the sludge, for example at 1100 g for 10 minutes and discard the supernatant liquid. Wash the solids with the mineral salts medium (6.2.5). Discard the washings. Re-suspend the settled or centrifuged solids in the medium (6.2.5) to yield a concentration of about 3 g of suspended solids per litre of medium. Alternatively or additionally, aerate the sludge overnight.

To reduce the blank control value still further, it may be necessary to acclimatise the sludge to the medium (6.2.5) but not to the test compound. For example, aerate the sludge (at a suspended solids concentration of about 30 mg l^{-1}) in the medium for up to one week.

In the biodegradability tests, the inoculum comprises 4 - 30 mg of dry matter per litre of test mixture. Activated sludge inocula may give rise to higher blank control carbon dioxide or inorganic carbon values than those obtained with effluent inocula. It has been reported[6] that 4 mg l^{-1} of inoculum is sufficient to reduce blank values without sacrificing activity to degrade readily biodegradable chemicals within 28 days.

If activated sludge contains 50 % organic carbon, then sludge (at a suspended solids concentration of 30 mg l^{-1} of test mixture) may produce a theoretical maximum of (30x50/100) x (44/12) mg of carbon dioxide, i.e. 55.5 mg of carbon dioxide per litre of test mixture.

In the OECD modified Sturm test, one of the conditions of validation is that the yield of carbon dioxide should not exceed 17 mg l^{-1} in 28 days. In the revised OECD method, the limit is modified to 30 - 40 mg l^{-1}. (The value of 17 mg l^{-1} represents a decay rate of sludge of about 1 % per day).

If a test compound biodegrades to the extent of 60 %, then a concentration of 10 mg of carbon per litre of test mixture will generate (60/100) x 10 x (44/12) mg of carbon dioxide, i.e. 22.0 mg of carbon dioxide per litre of test mixture. Thus, in some cases where a blank control of 17 mg l^{-1} is determined, as much as (17 x 100) / (17 + 22) mg of carbon dioxide, i.e. approximately 44 %, of the carbon dioxide produced is derived from the blank control. In other cases where a blank control of 40 mg l^{-1} is determined, as much as (40 x 100) / (40 + 22) mg of carbon dioxide, i.e. approximately 64 %, of the carbon dioxide produced is derived from the blank control. Both of these proportions are much higher than the proportion of carbon dioxide produced with effluent inocula, i.e. less than 10 %, see section 6.14.1.

6.14.3 Other inocula

While other sources of bacteria may be used, it should be emphasised that if the procedure described in this method is used as a test for "ready biodegradability", micro-organisms previously exposed or acclimatised to the test compound should not be used. Such exposed inocula may however be used for "inherent biodegradability" assessments.

7 Apparatus

7.1 Carbon analyser. Capable of measuring inorganic carbon (and optionally, organic carbon) within the range 0.2 - 20 mg l^{-1} in the aqueous phase and up to 1.5 μg in the gaseous phase. Alternatively, a gas chromatograph for determining carbon dioxide in the gaseous phase may be used (head-space analysis).

7.2 Test vessels. Gas-tight glass vessels (for example serum bottles) of 160 ml capacity (nominal 125 ml) with butyl rubber septa and aluminium crimp seals. Other gas-tight systems may also be used. Rinse the vessels with water (6.1) and clean thoroughly, for example, using an ultrasonic bath. Finally, rinse with water (6.1) and allow the vessels to dry. The volume of each test vessel (V_V ml) should be known. If not, the volume should be determined, for example by measuring the volume (or weighing the amount) of water used to completely fill each vessel. Those test vessels, the volumes of which differ significantly (for example ± 1%) from the mean volume of all test vessels used in the test, should be discarded. The volume of test mixture (V_L ml) in the test vessel should be about twice the corresponding volume of head-space gas (V_H ml).

7.3 Orbital shaker. This system should be maintained at a constant temperature, i.e. (± 2° C) between 20 and 25 °C.

7.4 Syringes. These should be capable of high precision delivery.

7.5 Glass bottles. These should be capable of holding up to 5 litres of test mixture.

7.6 Filtration system (optional). Membrane filters of suitable porosity (nominal pore size between 0.2 - 0.45 μm). These filters should not adsorb organic compounds from or release organic carbon to the filtrate.

8 Analytical procedure

The procedures described enable 5 litres of test mixture to be prepared. For alternative volumes, the amount of components should be changed accordingly.

Step	Procedure	Notes
8.1	**Test and reference compounds**	
8.1.1	*Soluble compounds*	
8.1.1.1	Add approximately 4300 ml (note a) of water (6.1) to a clean 5-litre bottle (7.5). To the bottle, add, in sequence with appropriate mixing, 50 ± 1 ml of phosphate buffer solution (6.2.4), 5.0 ± 0.1 ml of calcium chloride solution (6.2.1), 5.0 ± 0.1 ml of magnesium chloride solution (6.2.2), 5.0 ± 0.1 ml of iron(III) chloride solution (6.2.3), 500 ± 10 ml of effluent inoculum (6.14.1) (note b) and 100 ± 1 ml of the test compound stock solution (6.5) to be tested.	(a) This volume is used when test compounds are added in 100 ml aliquot volumes. (If different aliquots are added, for example in the case of a relatively dilute dispersion, the volume of water should be adjusted accordingly). The final volume of test mixture should be 5 litres. (b) Alternatively, 50 ± 1 ml of activated sludge inoculum (6.14.2) may be added. As described, 50 ml of activated sludge inoculum yields about 30 mg l^{-1} solids per litre of test mixture whereas 7.5 ml of activated sludge inoculum yields about 4 mg l^{-1}. Corresponding volumes of effluent inoculum range between 10 - 100 ml per litre of test mixture.
8.1.1.2	To a second 5-litre bottle add, in sequence with appropriate mixing, the same constituents as described in section 8.1.1.1 but replacing the test compound stock solution (6.5) with the same volume of reference compound stock solution (6.6).	
8.1.1.3	To each bottle, add sufficient water (6.1) to make 5 litres of test mixture. Mix well.	
8.1.2	*Insoluble and volatile liquids*	
8.1.2.1	Prepare the test mixture as described in sections 8.1.1.1, 8.1.1.2 and 8.1.1.3 but omit the test compound stock solution (note c) in the first bottle.	(c) The appropriate amount of test compound should be added later directly into the test vessels, see sections 6.5.2 and 8.4.2.
8.1.3	*Insoluble solids and viscous liquids*	
8.1.3.1	Prepare the test mixture as described in sections 8.1.1.1 and 8.1.1.2 but omit the test compound stock solution in the first bottle. Add the appropriate amount of test compound by the most suitable method (note d).	(d) The procedures used for adding insoluble compounds directly to test vessels are outlined in section 6.5.3.
8.1.3.2	To each bottle, add sufficient water (6.1) to make 5 litres of test mixture. Mix well.	

8.2 Blank control

8.2.1 Prepare the test mixture as described in section 8.1.1.1 but omit the test compound stock solution.

8.2.2 To the bottle, add sufficient water (6.1) to make 5 litres of test mixture.

8.3 Inhibition control

8.3.1 If required, prepare an extra 5 litres of test mixture as described in 8.1.1.1 but containing 50 ± 1 ml of the test compound stock solution (6.5) and 50 ± 1 ml of the reference compound stock solution (6.6). See notes e and f.

8.3.2 To the bottle, add sufficient water (6.1) to make 5 litres of test mixture.

8.4 Test mixture

8.4.1 Mix thoroughly the contents of each of the 5-litre bottles. Whilst mixing, pipette (note g) for example 100 ± 1 ml (V_L litres) of the test mixture from 8.1.1.3 or 8.1.2.1 or 8.1.3.2 and 8.2.2 and, if required, 8.3.2 into separate clean dry test vessels (7.2) (note h).

8.4.2 If liquid test compounds are to be tested, use a syringe (7.4) to add the appropriate volume (note i) of liquid required to give, for example 1 mg of carbon in each test vessel containing test mixture from 8.1.2.1.

(e) The same amount of organic carbon should be added to each test vessel.

(f) Some test compounds will need to be added directly to the test vessels, see notes c and d.

(g) Fill the pipette to the 100 ml mark as quickly as possible while mixing the test mixture in the bottle. Do not allow the meniscus of the test mixture to go above the mark. An alternative volume may be used, but the volume ratio of liquid (test mixture) to air in the test vessel should be the same for all vessels (about 2:1) and the volume of air should contain sufficient oxygen for complete biodegradation to occur.

(h) The greater the number of test vessels the greater the number of determinations that can be carried out and the greater the number of replicate analyses that can be undertaken. Also the greater the number of replicate analyses the greater the confidence in the results and statistical treatment.

(i) The volume of liquid to be added to each test vessel containing test mixture from 8.1.2.1 is calculated from the specific gravity of the liquid and the percentage of carbon in the test compound.

8.4.3 If insoluble solids or viscous liquids are to be tested, add the appropriate amount of test compound directly to the appropriate test vessels containing test mixture from section 8.1.3.2, note d.

8.4.4 If, for soluble test compounds only, the dissolved organic carbon (DOC) is to be determined, a known volume of test mixture from a number of test vessels (note j) containing test compound from 8.1.1.3 and blank control samples (8.2.2) should be removed, then filtered (7.6) and the DOC content determined in the liquid phase (note k) using a suitable carbon analyser (7.1). After the analysis has been completed, the test vessels should be discarded.

8.4.5 Seal the test vessels with, for example butyl rubber septa and aluminium caps. Place the test vessels on an orbital shaker maintained throughout the incubation period at ± 2 °C between 20-25 °C, in the dark. Switch on the shaker (at about 150 - 200 revolutions per minute) and commence the incubation.

8.4.6 Continue incubation (with shaking) normally for a total period of 28 days. At suitable intervals (note l) during incubation, remove a number of test vessels (containing test compound and reference compound including blank control and inhibition control samples) from the orbital shaker. The test mixture in these vessels should be analysed for inorganic carbon or carbon dioxide, as appropriate (see sections 8.5.1, 8.5.2 or 8.5.3) by removing suitable volumes of the liquid phase or gaseous phase as necessary. When the analyses are complete the test vessels should be discarded.

8.4.7 From the results of analysis of the test mixtures containing test compound and blank control samples and for each time interval determine the percent biodegradation (% D_t). Plot a curve of the mean biodegradation at time t versus time (see section 10).

8.4.8 If the curve shows a plateau representing about > 50 % biodegradation within 28 days, the test may be terminated. If

(j) The number of test vessels sampled containing test compound, and blank control sample indicate the number of replicate determinations.

(k) The DOC (OC_i) determined in the filtered test mixture indicates the level of DOC at the start of the test.

(l) The intervals may be daily, weekly or other suitable period of time. At each interval, the number of test vessels (containing test compound and reference compound, including blank control and if required, inhibition control samples), and hence the number of replicate analyses to be carried out is dictated by the time interval and the degree of confidence required. Regular intervals enable the biodegradation process to be followed more easily, and frequent intervals facilitate the biodegradation assessment.

(m) It should be reported that the duration of the test has been extended. Test compounds, requiring more than

biodegradation occurs, but from the curve a plateau is not observed within 28 days, then incubation should be continued and the test extended for a further period of up to 10 days (note m).

28 days incubation, are not classified as readily biodegradable.

8.4.9 If, for soluble test compounds only, the DOC is to be determined, a known volume of test mixture from a number of test vessels (note h) containing test compound from 8.1.1.3 and blank control samples (8.2.2) should be removed on day 28 (or the last day of testing) and filtered (7.6) and the DOC content determined in the liquid phase (note n) using a suitable organic carbon analyser (7.1). After analysis has been completed, the test vessels should be discarded.

(n) The DOC (OC_f) determined in the filtered test mixture indicates the level of DOC at the end of the test.

8.5 Determination of inorganic carbon or carbon dioxide

This section applies to all test compounds, reference compounds, blank control and optional inhibition control samples. The inorganic carbon or carbon dioxide can be determined in three ways (see sections 8.5.1, 8.5.2 and 8.5.3). Since the procedures may generate slightly different results due to the fact that different conditions are used and different determinations undertaken, only one procedure should be used within a single test study.

8.5.1 *Acidification*[6]

8.5.1.1 Calibrate the carbon analyser on the day each analysis is carried out (notes o and p).

(o) To prepare a calibration graph and confirm equal concentrations of inorganic carbon in gaseous and liquid phases, prepare test vessels containing standard solutions of between 0.5 - 10 mg of inorganic carbon per litre of solution using anhydrous sodium carbonate in carbon dioxide-free water (6.12)[9]. Ensure that the volume ratio (normally 1:2) of the head-space volume to the liquid volume is the same as for the test compounds in the test vessels. Acidify the test vessels by injecting through the septum 1 ml of phosphoric acid solution (6.7), shake for 1 hour, withdraw suitable volumes of samples from the head-space gas and liquid phase as appropriate, and analyse for

inorganic carbon or carbon dioxide as necessary. There should be no significant difference between the gaseous concentration (C_H) and liquid phase concentration (C_L).

(p) As a check, routinely, inject for example 1.00 ± 0.05 ml of 1 % carbon dioxide in nitrogen mixture (6.11) into the carbon analyser. This is equivalent to 5 mg of carbon per litre of test mixture with a head-space to liquid volume ratio of about 1:2.

8.5.1.2 At the appropriate time interval, remove from the shaker a number of test vessels (note h) containing test compound, reference compound and blank control sample for analysis. Add, by injection through the septum, approximately 1 ml of phosphoric acid solution (6.7) to each test vessel and replace on the shaker. Shake for 1 hour to equilibrate. Remove the test vessels from the shaker, withdraw 1.00 ± 0.05 ml of the head-space gas from each vessel and inject into the carbon analyser. Also analyse the liquid phase. Read off the amount of inorganic carbon from the appropriate calibration curve and convert to a concentration (C_H). See note o.

8.5.2 *Two-phase analysis*[7]

8.5.2.1 Calibrate the carbon analyser on the day each analysis is carried out (note q).

(q) A calibration for gas samples should be prepared by injecting suitable volumes of, for example 0.2 % (v/v) mixture of carbon dioxide in nitrogen (6.11) into the carbon analyser. Similarly, for liquid samples, by injecting standard solutions of sodium hydrogen carbonate in carbon dioxide-free water (6.13) in a range up to 20 mg of carbon per litre.

8.5.2.2 A number of test vessels (note h) containing test compound, reference compound and blank control sample should be removed from the shaker. Using a gas syringe (note r) withdraw a volume of the head-space gas from each test vessel. Inject the volume of gas into the carbon analyser and determine the inorganic carbon. Convert the amount of inorganic carbon determined to a concentration value (C_H).

(r) The volume of gas required for an initial concentration of 10 mg of carbon per litre of test mixture varies between 0.5 - 1.0 ml, and depends on the extent of biodegradation. The same volume of sample should be used as used for the calibrations.

8.5.2.3 Similarly, analyse the liquid phase (note s) by removing a volume of liquid with a syringe (7.4). Inject the volume into the carbon analyser and determine the inorganic carbon content. Convert the amount of inorganic carbon to a concentration value (C_L).

(s) The volume of liquid required will typically be between 50 - 200 μl, depending upon the initial concentration of the test compound and the extent of biodegradation.

8.5.3 *Conversion of carbon dioxide to Carbonate*[10]

8.5.3.1 Calibrate the carbon analyser on the day each analysis is carried out (note t).

(t) Inject standard solutions of sodium hydrogen carbonate in carbon dioxide-free water (6.13) in the range 0.5 to 20 mg of carbon per litre as dissolved inorganic carbon.

8.5.3.2 A number of test vessels (note h) containing test compound, reference compound and blank control sample should be removed from the shaker. Inject 1 ml of sodium hydroxide solution (6.10) through the septum of each vessel and return the vessel to the shaker. Shake for 1 hour to equilibrate. Remove the test vessels from the shaker and allow the contents to settle. Using a syringe (7.4) withdraw a suitable volume (note u) of the supernatant liquid from the test vessel for analysis. Inject the liquid into the carbon analyser (note v) and determine the inorganic carbon content. Convert the amount determined to a concentration value (C_L).

(u) The volume of liquid will normally be between 50 - 200 μl and will depend on the concentration of test compound and the extent of biodegradation.

(v) Care should be taken to ensure the carbon analyser is not damaged by the alkaline solution.

---

9 Calculations

9.1 Assuming 100 % conversion of organic carbon in the test compound to inorganic carbon in each test vessel, the theoretical inorganic carbon (ThIC) in excess of that produced in the blank control sample will be same as the amount of organic carbon (OC) added as test compound. Hence

$$ThIC = OC$$

The amount of inorganic carbon (IC) in the test vessel is given by:

IC = (mass of carbon in the liquid phase + mass of carbon in the gaseous phase)

Thus:

$$IC = (V_L \times C_L) + (V_H \times C_H) \qquad (1)$$

Where:

V_L is the volume of the test mixture in test vessel (litres);

C_L is the concentration of inorganic carbon in the liquid phase (mg of carbon per litre of test mixture);
V_H is the volume of head-space gas in the test vessel (litres). This is the difference between the volume of the test vessel (V_{TV}) and the volume of liquid in the test vessel (V_L).
C_H is the concentration of inorganic carbon in the head-space gas (mg carbon per litre).

The calculation of IC for the three procedures used (see sections 8.5.1, 8.5.2 or 8.5.3) can be obtained in the following manner.

For 8.5.1 After acidification to a pH value of less than 3, and equilibration of the concentrations of inorganic carbon in the liquid and gaseous phases, the concentration of inorganic carbon in the gas phase can be assumed to be the same as that in the liquid phase. Hence $C_L = C_H$ and equation 1 can be used with the value of C_H determined.

For 8.5.2 For this two phase approach, equation 1 should be used with the calculated values of C_L and C_H.

For 8.5.3 Following conversion of carbon dioxide to carbonate, the negligible amount of inorganic carbon in the gaseous phase can be ignored, i.e. $C_H = 0$. Substituting this in equation 1 enables IC to be determined.

For each interval where test vessels are removed and the contents analysed, the percent biodegradation (% D_t) can be calculated using:

$$\% D_t = [(IC_t - IC_{bt}) \times 100] / OC \qquad (2)$$

where:

IC_t = mean inorganic carbon (in mg) in the test vessels at time t;
IC_{bt} = mean inorganic carbon (in mg) in blank control vessels at time t;
OC = organic carbon (in mg) in the test compound added to the test vessel at the start of the test.

If, for soluble test compounds only, DOC is determined (see sections 8.4.4 and 8.4.9) the percent biodegradation, % D_{oc}, can be calculated using:

$$\% D_{oc} = 100 [1 - (OC_f - OC_{Bf}) / (OC_i - OC_{Bi})] \qquad (3)$$

where

OC_i is the mean concentration of organic carbon in the test vessels at the start of the test (mg l^{-1});
OC_f is the mean concentration of organic carbon in the test vessels at the end of the test (mg l^{-1});
OC_{Bi} is the mean concentration of organic carbon in the blank control vessels at the start of the test (mg l^{-1});
OC_{Bf} is the mean concentration of organic carbon in the blank control vessels at the end of the test (mg l^{-1}).

9.2 A statistical treatment of the results can be carried out where replicate analyses are undertaken. At each interval, the percent biodegradation, % D_t, for each individual test vessel can be calculated. From these values, the mean percent biodegradation (mD_t) at

time t, and the corresponding standard deviation should be calculated. Finally, the 95 % confidence limits, CI, should be calculated using:

$$CI = (t \times s) / \sqrt{n}$$

where

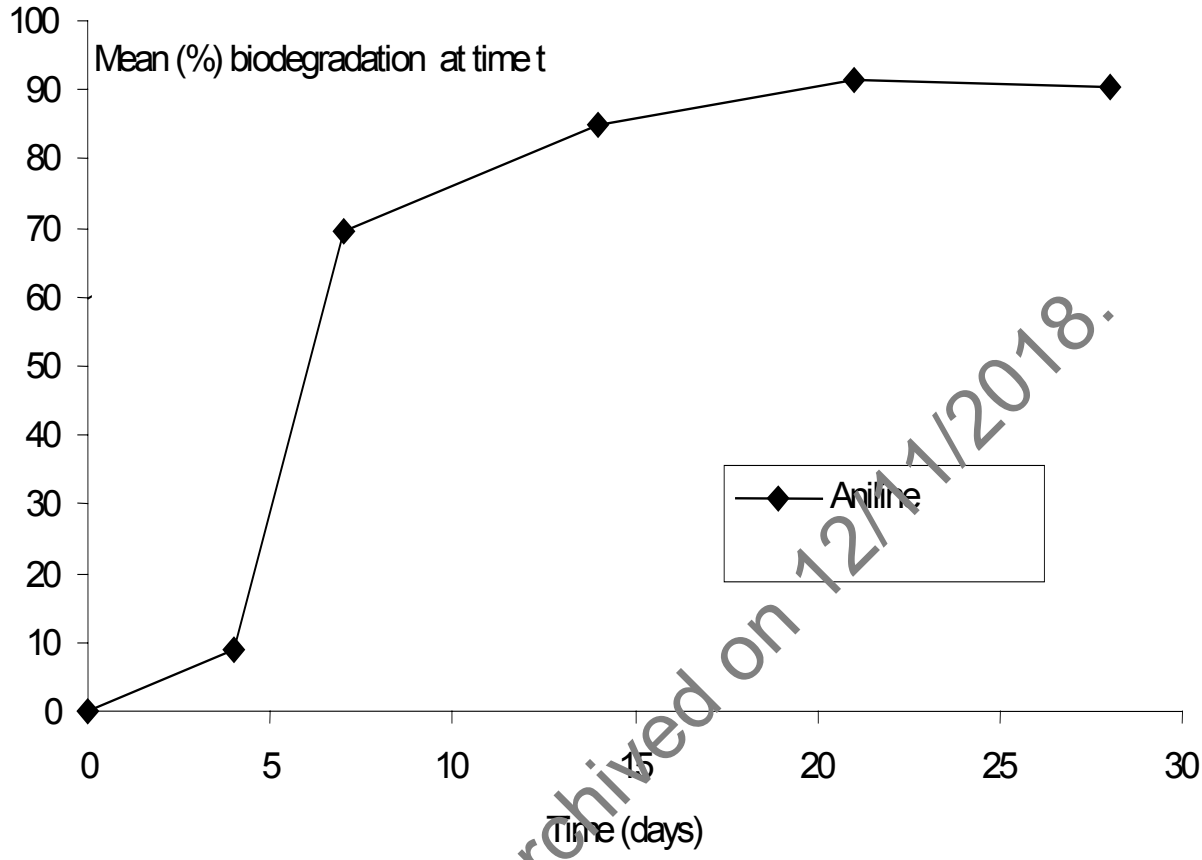
s is the standard deviation;
t is the two-sided student t-test value at n-1 degrees of freedom at the stated level of confidence;
n is the number of individual values used to obtain the standard deviation, (i.e. the number of replicate analyses)

10 Expression of results

Following the calculations a graph may be plotted of the mean percent biodegradation at time t versus time t (see figure 1). If, from the graph, a plateau phase is indicated, calculate a mean percent biodegradation in this region and report this value as the “degree of biodegradation” for the test compound. If a plateau phase is not observed, the highest mean percent biodegradation value calculated is reported as the “degree of biodegradation” for the test compound. At the same time, corresponding graphs should be plotted for the reference compound (to demonstrate that the micro-organisms used in the test are capable of biodegrading compounds) and if appropriate the inhibition control sample. The amount of inorganic carbon formed in the blank control vessels should also be recorded.

Information on the toxicity of the test compound may be useful in the interpretation of data from test vessels exhibiting a low degree of biodegradation. If, for inhibition control samples, the mean percent biodegradation is below, say 25 %, and insufficient biodegradation of the test compound is observed in test vessels containing only the test compound, then it may be assumed that the test compound is inhibitory at the concentration used. In these cases, it may be necessary to repeat the test with a lower concentration of test compound. Using lower concentrations may however reduce the precision of the method.

Figure 1 Example of plot of mean percent biodegradation at time t versus time t



11 Checking the validity of results

The activity of the inoculum is demonstrated by the degree of removal of the reference compound. If the carbon dioxide produced as a result of biodegradation of the reference compound is not greater than, say 60 % of the theoretical amount within 14 days of the start of the biodegradation test, the test may be regarded as being invalid and should be terminated. The test should then be repeated with an inoculum from another source.

The value for $\%D_{oc}$ should be at least as much as $\%D_t$ (at the end of the test). The value for $\%D_{oc}$ can be, and often is, as high as 100%, and may be significantly higher than $\%D_t$. This is due to the fact that some of the organic carbon is converted to new bacterial cells rather than to carbon dioxide. (This is not always the case as illustrated by the data in shown in table 1).

In addition, the inorganic carbon produced within 28 days using the inoculum blank control should not exceed 1.3 mg of carbon per litre of test mixture for a 10 % (v/v) effluent inoculum. Also, the inorganic carbon produced within 28 days using the inoculum blank control should not exceed 2.5 mg of carbon per litre of test mixture for an activated sludge inoculum containing 4 mg of dry matter per litre of test mixture.

12 References

1. Standing Committee of Analysts. Assessment of Biodegradability 1981. *Methods for the Examination of Waters and Associated Materials*. in this series, HMSO, ISBN 0117516619

2. Organisation for Economic Co-operation and Development. Revised Guidelines for Ready Biodegradability Paris, 1993.

3. The recovery of carbon dioxide in the Sturm test for ready biodegradability. D Weytjens, I van Ginneken and H A Painter, *Chemosphere*, 1994, **28**(4), pp801-812.

4. General method for determining anaerobic biodegradation potential, D P Shelton and J M Tiedje, *Applied and Environmental Microbiology*, 1984, **47**, pp850-857.

5. Standing Committee of Analysts, The Assessment of Biodegradability in Anaerobic Digesting Sludge 1988, *Methods for the Examination of Waters and Associated Materials*. in this series, ISBN 0117521914.

6. Headspace determination of evolved carbon dioxide in a biodegradability screening test, J Struijs and J Stoltenkamp, *Ecotoxicology and Environmental Safety*, 1990, **19**(2), pp204-211.

7. The application of dissolved inorganic carbon measurements to the study of aerobic biodegradability, R R Birch and R J Fletcher, *Chemosphere*, 1991, **23**(4), pp507-524.

8. Biodegradation tests for poorly soluble compounds, A de Morsier, J Blok, P Gerike, L Reynolds, H Wellens, W J Boetinck, *Chemoshere*, 1987, **16**(4), pp833-847.

9. The ISO headspace carbon dioxide biodegradability test. *Chemosphere*, N S Battersby, 1997, **34**(8), pp1813-1822

10. An inherent biodegradability test for oil products: description and results of an international ring test, N S Battersby, D Ciccognani, M R Evans, D King, H A Painter, D R Peterson and M Starkey, *Chemosphere*, 1999, **38**, pp3219-3235.

Table 1 Performance data

(all test compounds at initial concentration of 10 mg of carbon per litre of test mixture)

Within batch

Compound	Number of days	Mean per cent biodegradation[#]	RSD (%)	Number of replicates
Soluble chemicals				
monoethanol	22	93	1.7	10
1, 6-hexanediol	22	85	1.9	10
2,4,6-trichlorophenol	22	92	3.0	7
pentaerythritol	28	84	4.6	6
Volatile chemicals (1-2 μl used)				
ethanol	15	97	2.5	4
acetone	15	91	2.4	4
ethyl acetate	15	94	5.1	4
Sparingly soluble compounds				
sodium stearate*	28	89	1.3	6
benzyl acetate**	28	101	1.2	4
diethyl phthalate**	28	104	2.4	5

[#] based on carbon dioxide produced as a % of theoretical.
* added as a solution; precipitates in the medium.
** water-insoluble liquid.
RSD relative standard deviation

Between batch for benzyl alcohol

Mean per cent biodegradation	Number of replicates	95 % Confidence limits
96.3		92.9-99.8
97.8	5	94.8-100.8
93.8	5	92.5-95.0
92.1	5	88.7-95.6
97.0	5	95.9-98.2
Overall mean 95.4	5	
RSD 2.49 %		

Data provide by Unilever Research Laboratory, Port Sunlight, UK.

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users would like to receive advanced notice of forthcoming publications please contact the Secretary on the Agency's web-page.

Standing Committee of Analysts
Environment Agency (National Laboratory Service)
56 Town Green Street
Rothley
Leicestershire, LE7 7NW
http://www.environment-agency.gov.uk/nls

Standing Committee of Analysts
Members assisting with this method

N Battersby
D Bealing
R Birch (deceased)
C Burford
A Colley
M Daniel
M T Douglas
R Drake
M Evans
D Fearnside
R Fletcher
P Gale
D Gore
J W Handley
S Henderson
R Jenkins
C Mead
D Morris
R Milne
H A Painter
N Rehman
G Roberts
J R Snape

CONTACTS:

ENVIRONMENT AGENCY HEAD OFFICE

Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol BS32 4UD

www.environment-agency.gov.uk
www.environment-agency.wales.gov.uk

ENVIRONMENT AGENCY REGIONAL OFFICES

ANGLIAN
Kingfisher House
Goldhay Way
Orton Goldhay
Peterborough PE2 5ZR

MIDLANDS
Sapphire East
550 Streetsbrook Road
Solihull B91 1QT

NORTH EAST
Rivers House
21 Park Square South
Leeds LS1 2QG

NORTH WEST
PO Box 12
Richard Fairclough House
Knutsford Road
Warrington WA4 1HG

SOUTHERN
Guildbourne House
Chatsworth Road
Worthing
West Sussex BN11 1LD

SOUTH WEST
Manley House
Kestrel Way
Exeter EX2 7LQ

THAMES
Kings Meadow House
Kings Meadow Road
Reading RG1 8DQ

WALES
Cambria House
29 Newport Road
Cardiff CF24 0TP

ENVIRONMENT AGENCY
GENERAL ENQUIRY LINE
08708 506 506

ENVIRONMENT AGENCY
FLOODLINE
0845 988 1188

ENVIRONMENT AGENCY
EMERGENCY HOTLINE
0800 80 70 60

ENVIRONMENT AGENCY