Assessment of Biodegradability 1981

Methods for the Examination of Waters and Associated Materials

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Assessment of Biodegradability 1981

Methods for the Examination of Waters and Associated Materials

This booklet contains methods based on the following techniques; although many were originally designed for measuring the biodegradability of surfactants, they are just as effective for other substances:

A Respirometry: basic techniques
B Respiration rate: oxygen electrode method
C Oxygen uptake: manometric respirometer
D CO_2 production (Sturm's method)
E Die-away: Modified OECD (MOST)
F Predictive die-away: modified OECD detergent method (STCSD)
G Die-away: Bunch Chambers (co-metabolism)
H Semi-continuous activated sludge (SCAS)
I Simulation-activated sludge (Husmann/Porous Pot)
J Simulation-biological filter (rotating tube)
K Isolation of bacteria

For full titles see the Tables of Contents below.

These methods sometimes mention specific makes of equipment. In some instances this was the make of equipment used by the developer of the test, in other cases, the method used is slightly dependent on instrument type and variations are given accordingly. No specific endorsement is intended.

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Warning to users

The analytical procedures given in this booklet should only be carried out by competent trained persons, with adequate supervision when necessary. Local safety regulations must be observed. Laboratory procedures should be carried out only in properly equipped laboratories. Field operations should be conducted with due regard to possible local hazards, and portable safety equipment should be carried. Care should be taken against creating hazards. Lone working, whether in the laboratory or field, should be discouraged. Reagents of adequate purity must be used, along with properly maintained apparatus and equipment of correct specification. Specifications for reagents, apparatus and equipment are given in manufacturers' catalogues and various published standards. If contamination is suspected, reagent purity should be checked before use.

There are numerous handbooks on first aid and laboratory safety. Among such publications are: 'Code of Practice for Chemical Laboratories' and 'Hazards in the Chemical Laboratory' issued by the Royal Society of Chemistry, London; 'Safety in Biological Laboratories' (Editors Hartree and Booth), Biochemical Society Special Publication No 5, The Biochemical Society, London, which includes biological hazards, Public Health Laboratory Service Monograph Series No 6 'The Prevention of Laboratory Acquired Infection', HMSO, London.

Where the Committee have considered that a special unusual hazard exists, attention has been drawn to this in the text so that additional care might be taken beyond that which should be exercised at all times when carrying out analytical procedures. It cannot be too strongly emphasised that prompt first aid, decontamination, or administration of the correct antidote can save life; but that incorrect treatment can make matters worse. It is suggested that both supervisors and operators be familiar with emergency procedures before starting even a slightly hazardous operation, and that doctors consulted after any accident involving chemical contamination, ingestion, or inhalation, be made familiar with the chemical nature of the injury, as some chemical injuries require specialist treatment not normally encountered by most doctors. Similar warning should be given if a biological or radiochemical injury is suspected. Some very unusual parasites, viruses and other micro-organisms are occasionally encountered in samples and when sampling in the field. In the latter case, all equipment including footwear should be disinfected by appropriate methods if contamination is suspected.

The best safeguard is a thorough consideration of hazards and the consequent safety precautions and remedies well in advance. Without intending to give a complete checklist, points that experience has shown are often forgotten include: laboratory tidiness, stray radiation leaks (including ultra violet), use of correct protective clothing and goggles, removal of toxic fumes and wastes, containment in the event of breakage, access to taps, escape routes, and the accessibility of the correct and properly maintained first-aid, fire-fighting, and rescue equipment. If in doubt, it is safer to assume that the hazard may exist and take reasonable precautions, rather than to assume that no hazard exists until proved otherwise.

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About this series

This booklet is part of a series intended to provide recommended methods for the determination of water quality. In addition, the series contains short reviews of the more important analytical techniques of interest to the water and sewage industries. In the past, the Department of the Environment and its predecessors, in collaboration with various learned societies, have issued volumes of methods for the analysis of water and sewage culminating in 'Analysis of Raw, Potable and Waste Waters'. These volumes inevitably took some years to prepare, so that they were often partially out of date before they appeared in print. The present series will be published as individual methods, thus allowing for the replacement or addition of methods as quickly as possible without need of waiting for the next edition. The rate of publication will also be related to the urgency of requirement for that particular method, tentative methods being issued when necessary. The aim is to provide as complete and up to date a collection of methods and reviews as is practicable, which will, as far as possible, take into account the analytical facilities available in different parts of the Kingdom, and the quality criteria of interest to those responsible for the various aspects of the water cycle. Because both needs and equipment vary widely, where necessary, a selection of methods may be recommended for a single determinand. It will be the responsibility of the users — the senior analytical chemist, biologist, bacteriologist etc, to decide which of these methods to use for the determination in hand. Whilst the attention of the user is drawn to any special known hazards which may occur with the use of any particular method, responsibility for proper supervision and the provision of safe working conditions must remain with the user.

The preparation of this series and its continuous revision is the responsibility of the Standing Committee of Analysts (to review Standard Methods for Quality Control of the Water Cycle). The Standing Committee of Analysts is one of the joint technical committees of the Department of the Environment and the National Water Council. It has nine Working Groups, each responsible for one section or aspect of water cycle quality analysis. They are as follows:

1.0 General principles of sampling and accuracy of results
*2.0 Instrumentation and on-line analysis
3.0 Empirical and physical methods
4.0 Metals and metalloids
5.0 General nonmetallic substances
6.0 Organic impurities
7.0 Biological methods
*8.0 Sludge and other solids analysis
9.0 Radiochemical methods

The actual methods etc are produced by smaller panels of experts in the appropriate field, under the overall supervision of the appropriate working group and the main committee. The names of those associated with this method are listed inside the back cover.

Publication of new or revised methods will be notified to the technical press, whilst a list of Methods in Print is given in the current HMSO Sectional Publication List No 5, and the current status of publication and revision will be given in the biennial reports of the Standing Committee of Analysts.

Whilst an effort is made to prevent errors from occurring in the published text, a few errors have been found in booklets in this series. Correction notes for booklets in this series are given in the Reports of The Standing Committee of Analysts, published by the Department of the Environment but sold by the National Water Council, 1 Queen Anne's Gate, London SW1H 9BT. Should an error be found affecting the operation on a method, the true sense not being obvious, or an error in the printed text be discovered prior to sale, a separate correction note will be issued for inclusion in the booklet.

* These two Working Groups have now been wound up. Their tasks have been redistributed among the other Working Groups.

T A DICK
Chairman

L R PITTWELL
Secretary

28 October 1982

Introduction to Methods

1 The impact of a compound on the aquatic environment, and thus whether it can be safely discharged to that environment, depends on a number of factors. These include the amount to be discharged, the rate and extent of degradation by biological and physico-chemical processes, its resulting concentration relative to the receiving water, the uses to which that water may subsequently be put, and the toxicity of the compound to aquatic plants, micro-organisms and animals, particularly man. Physico-chemical degradation processes are thought to play only a subsidiary role in the aquatic environment, compared to their roles in the air and in soil, except that adsorption onto solids and assimilation into micro and macro-organisms (bioaccumulation) may play a part. These latter processes, together with biodegradation, are termed "bio-elimination" and may transfer the problem to another environment.

2 The tests in this booklet describe how the aerobic biodegradability of a compound in the aquatic environment may be assessed. Some methods assess true biodegradability, while the property determined by others is bioelimination.

3 Most of the methods described in the literature, eg those involving the use of pure cultures and those in which the ratio of substrate to micro-organism concentration is abnormally high, bear little relation to practical conditions. In others the conditions are chosen to simulate as closely as possible those existing in the environment to which the particular substance is to be discharged.

4 The factors which govern microbial activity are also those which affect the assessment of biodegradability. Discussion of the conditions appropriate to the assessment of biodegradability in the aquatic environment is given by Gilbert and Watson[1], Painter [2] and by others [3,4].

Factors affecting biodegradation

5 Important factors affecting biodegradation of a substance, apart from its structure are:–

(a) the nature, activity and concentration of the microbial population in the inoculum;
(b) the concentration of the test substance – it may be inhibitory;
(c) the presence of essential mineral and trace organic nutrients;
(d) the presence of other organic substrates – cometabolism or inhibition;
(e) the temperature, pH value, and the concentration of dissolved oxygen;
(f) the presence of other compounds which may be inhibitory.

Microbial Population

6 In chemical analysis the reagents used can be described accurately, are reproducible and can be standardized.

In tests requiring micro-organisms as 'reagents' there can be wide differences of response and properties between individuals of the same species. Even greater differences may arise between populations of mixed species. Biodegradability tests determine whether there are micro-organisms capable of degrading the test substance under the conditions of that test. It is therefore necessary to use a wide population of numerous species as an inoculum; in practice this is achieved by using a soil extract, sewage, sewage effluent, river water or activated sludge. Such inocula are more likely to contain one or several species capable of either degrading the test substance or of becoming adapted to do so.

Where ultimate biodegradability or biodegradation values under specific conditions are required, the development of acclimatized cultures by a suitable enrichment technique from a wide spectrum inoculum may be appropriate.

The microflora obtained from each source of inoculum are not constant with time either in numbers or types of micro-organisms present, and since species which degrade a substance do not necessarily do so at equal rates, the rate of degradation may differ from experiment to experiment.

Thus, tests involving mixed cultures of micro-organisms are subject to much greater variation and lack of reproducibility than purely chemical tests.

In the batch die-away type of test, whatever determinand is followed, eg specific analysis, dissolved organic carbon (DOC), chemical oxygen demand (COD), oxygen uptake or carbon dioxide production, the concentration of the inoculum is as important, for quantitative purposes, as its source. This subject is dealt with in paras 14–17 of this introduction.

Concentration of Test Substance

7 The selection of the concentration of the test substance should be made from a consideration of the expected level of production and the consequent estimated discharge levels, which will depend on whether the substance reaches the environment via point or diffuse discharges. It is advisable first to apply a respiration rate test [19] to assess the toxicity of the substance before making the choice and to carry out the biodegradability test at more than one non-inhibitory concentration. Inhibition is often a cause of failure, especially in die-away tests using very low concentrations of inocula (Method E, BOD_5, BOD_{20}).

Nutrients

8 All micro-organisms require a range of elements (eg N, P, S, K, Ca, Zn) for growth, so that the media used must contain adequate concentrations of appropriate salts of these elements. Some species also require trace concentrations of organic substances such as the B group of vitamins and some grow faster in their presence, so that it may be helpful to add such compounds.

Other Carbon Sources

9 Some organic compounds are not capable of supplying carbon and energy to micro-organisms but can be partially degraded to other organic compounds, or even completely to carbon dioxide and water, by organisms which simultaneously grow at the expense of other substrates in the medium. Such a co-metabolic effect may be revealed by adding normal substrates, eg yeast extract, as in the Bunch-Chambers test (Method G); analogue metabolism may be revealed by adding substances similar in structure to the test compound.

Physical Factors

10 Tests must be conducted at temperatures, pH values and concentrations of dissolved oxygen conducive to good growth. Usually values for temperature are chosen between 18° and 25°C, though in special cases it may be desirable to operate at the lower temperatures often found in the aquatic environment eg 8–10°C. Values for pH giving best growth are usually between 6.5 and 8.5 and the buffering capacity of the medium must be sufficient to maintain this range, while the concentration of dissolved oxygen should be greater than 2 mg/l. Anaerobic degradation is dealt with in another booklet in this series [20].

Assessment Using Non-Specific Analytical Methods

11 Until a few years ago most interest was centred on environmentally acceptable and functional (primary) biodegradability, which often run parallel with one another, but more recently interest has shifted to ultimate biodegradability (complete mineralization). The reasons for this change in emphasis include greater re-use of water, coupled with a fear that intermediate metabolic products might have, as yet undiscovered, harmful effects on the environment and on man.

12 The removal of the substance itself is used as the criterion of primary biodegradability. This is determined by chemical analysis specific for the substance or for the group of which it is a member (eg methylene blue activity for anionic

surfactants). To assess ultimate biodegradability the removal of organic matter must be determined, eg by DOC or COD, or indirectly by oxygen uptake or carbon dioxide production, making allowance for organic carbon in bacteria formed from the substance. At present determinations of DOC and COD are less well-developed and often do not give values as precise as those obtained by specific analyses at the concentrations (about 3 mg DOC/1) conventionally used [5]. This has led to the use of higher initial concentrations of substrate which can give rise to inhibition of bacterial growth if low concentrations of inocula are used. In tests employing additional organic substrates (Method G) the application of DOC and COD is much more difficult. In the continuous methods (I and J) the difficulties can be at least partially overcome by operating control vessels treating only the basic synthetic sewage and deducing by difference the organic carbon in effluent arising from the test substance. Fischer's[6] mixing method (coupled units) is yet a further modification.

Available Methods

13 The methods available can be divided broadly into two classes – direct, in which the disappearance of the test substance or DOC is determined and which can be applied only to soluble, non-volatile compounds*; and indirect, in which the oxygen taken up or the carbon dioxide produced during oxidation of the test substance is determined and which can be applied to both soluble and insoluble compounds. In some experimental procedures it is possible to follow the degradation both directly and indirectly.

Among the methods are:

Indirect

(a) Ratio of the 5-day biochemical oxygen demand [21] to COD [22], total organic carbon (TOC) or theoretical oxygen demand (TOD)[23];
(b) Respiration rate (0_2-electrode cell, Method B);
(c) Oxygen uptake in manometric respirometer (Method C; the Japanese official Ministry of International Trade and Industry test, MITI[8]†);
(d) Carbon dioxide production (Sturm method, Method D) and use of C^{14}.

Direct

(e) Die-away (or screening) test (Standing Technical Committee on Synthetic Detergents, STCSD[9]), Method F; Modified OECD test, Method E; L'Association Francaise de Normalization (AFNOR)[10]†; Zahn-Wellens test[11]†;
(f) Die-away test with added organic matter (Bunch-Chambers, Method G);
(g) Longer-term continuous or semi-continuous simulation tests (Husmann units, Porous Pots, Method I; Rotating Tube, Method J; Semi-continuous activated sludge, (SCAS) Method H).

Concentration of Inoculum in Die-Away Tests

14 It was mentioned earlier (para 6) that the concentration of inoculum (ie the number of bacteria added) was an important factor in determining the degree of removal of the test substance by the end of the permitted incubation period in a batch, die-away test. Given that the inoculum contains micro-organisms capable of degrading the test substance and that the substance is not inhibitory at the concentration used, eventually a high degree of removal will, of course, be reached by any size of inoculum; the smaller the inoculum, the longer the time taken.

15 The die-away type of test, in which the decrease in concentration of the substance is followed, arose directly out of the conventional BOD bottle test[12] [21], which employs a seed of 5 ml sewage effluent/l of medium, but of necessity restricts the substrate concentration to 2–5 mg/l.

The method was subsequently developed for anionic surfactants by Eden et al[13], who found that inocula of activated sludge proved more reliable than sewage effluent and that a range of suspended solids concentration in the final test medium of

* Volatile compounds may be assessed by the recent method of Blok[7].

† Not described here.

0.3–30 mg/l had little effect on the degree of removal ultimately reached but that the time required for 90% removal of a given product ranged from 20 to 4 days. Concentrations higher than about 100 mg suspended solids/l gave errors due to adsorption/desorption. The inoculum concentration in the STCSD test, Method F, was thus adopted as 30 mg/l (Table 1). In the derived OECD version of this test[5], the inoculum source and concentration were made flexible, since control tests are required with two standard substances, one hard and one soft. The levels of biodegradation of both standards must fall within acceptable limits (90–95% and <35%, respectively) if the test is to be valid; the size of the inoculum must be increased or decreased to suit these requirements. The usefulness of these tests is that the duration is not excessive (usually up to 14 d) and that, from experience with a number of compounds, passing the test is a reliable indication that the substance will be biodegraded in sewage treatment processes. A drawback is that those substances which respond poorly for one reason or another may prove to be adequately biodegradable in practice.

16 In three other tests approved by the OECD Chemical Group[14] and in the respirometric method, Method C, similar concentrations of inocula are used (Table 1). The French standard AFNOR test[10,14], in which DOC removal is followed, employs $5 \pm 3 \times 10^5$ cells/ml (= about 30 mg suspended solids/l) the cells having been harvested from sewage effluent by ultra-filtration and then resuspended in a much smaller volume. The Japanese MITI test [8,14], which determines accumulative oxygen uptake in an enclosed respirometer, also uses a seed of 30 mg activated sludge suspended solids/l and the revised form of the Sturm test[14,15] Method D, in which carbon dioxide production is determined, uses $10^4 - 2 \times 10^5$ cells/ml derived from the settled supernatant from homogenized activated sludge. However, in the other two tests recommended by the OECD Group much lower concentrations of inoculum are used. The modified OECD test (MOST)[14], Method E, in which the concentration of DOC is followed, employs a fixed inoculum, for purposes of standardisation, of 0.5 ml sewage effluent/l, equivalent to only about 10^2 cells/ml (Table 1). In the Closed Bottle test, which monitors dissolved oxygen concentration, the inoculum concentration is even lower at about 25/ml. In spite of this wide range (25–10^6/ml) of concentration of bacteria in the five OECD tests (indicated in Table 1), all five have the same status in that they are called tests of ready biodegradability. By inference this also applies to the manometric respirometer (Method C) and STCSD (Method F) tests.

17 These lower concentrations of micro-organisms result in much longer times for achieving given degrees of removal or no degradation at all in the time permitted, which has recently been increased from 14 to 19 d to 28 d. Differences in die-away curves, calculated using Monod bacterial kinetics, produced by different sizes of inoculum and their implications for biodegradability assessment are discussed in a recent paper[16]:

Acceptance Removal Values

18 Because of the relatively high variation and lack of reproducibility of results of die-away tests and because of the different reasons for carrying out such tests, results should be interpreted with caution. The OECD Chemicals Group[14] has put forward tentative acceptable removal values for ready biodegradability in the various types of test and these are given in Table 2

These values are recommended in the light of the joint experience of the group. In particular, the values for oxygen and for carbon dioxide are lower than the others, since a proportion of the substrate carbon is converted to new bacterial cells. The level for DOC is lower than for the substance itself, since in the unbalanced environment of a batch die-away test the normal sequence of microbiological reactions is interrupted and more drawn out than in the balanced community existing in the 'equilibrium' conditions in sewage treatment plants.

Somewhat arbitrarily, it was decided that the values in Table 2 should be attained over a period of only 10d within the 28d of the test. Initiation of biodegradability is deemed to take place when 10% removal (or oxygen uptake or carbon dioxide production) has occurred. This arrangement allows up to 18d for "acclimatization" to occur; it is considered that removals taking longer than 10d indicate substances which may be difficult to treat in practice (but see Painter and King,[16]).

Table 1 Inocula and Bacterial Concentrations used in some screening tests.

Heavy type indicates methods approved by OECD Chemicals Group[14]

Method	Inoculum (vol or weight/l medium)	Concentration* of bacteria (organisms/ml)
OECD (MOST) (E)	0.5 ml effluent	$0.5–2.5 \times 10^2$
Closed bottle (Fischer)	1 drop effluent	0.25×10^2
BOD (USA)	1–2 ml effluent	$0.1–1 \times 10^3$
BOD (UK) (Ref 21)	5 ml effluent	$0.5–2.5 \times 10^3$
Sturm† (CO_2) (D)	1% supernatant of homogenized sludge	$10^4 – 2 \times 10^5$
AFNOR (DOC)	[bacteria from effluent 'are counted']	$5 \pm 3 \times 10^5$
MITI (O_2 + DOC)	30 mg MLSS‡	$2–10 \times 10^5$
Respirometer (O_2) (C)	30 mg MLSS	$2–10 \times 10^5$
STCSD (DOC) (F)	30 mg MLSS	$2–10 \times 10^5$
Zahn-Wellens§ (DOC)	1000 mg MLSS	$0.6–3 \times 10^7$

* Calculated from data in Tomlinson, Loveless and Sear (1962), *J. Hyg. (Camb.)* **60**, 365: $1–5 \times 10^5$ organisms/ml – 'total' viable count in sewage effluent; and from Pike and Carrington (1972), *Wat. Pollut. Control* **71**, 583; $0.6–3 \times 10^7$ organisms/mg activated sludge.

† Reference 15
‡ Adapted to glucose. MLSS = mixed liquor suspended solids.
§ Not strictly a screening test.

Table 2 Proposed Pass Levels for Ready Biodegradability Tests

	Percentage of theoretical value, or percentage removal	Method
Oxygen uptake	>60	Ref 21, B, C
CO_2 production	>60	D
Specific substance	>80	E, F, G, H, I, (C,D)
DOC COD	>70	E, F, (C, D)

For the SCAS test, which assesses inherent, rather than ready, biodegradability, the Group has recommended the acceptance level to be 70% DOC removal as evidence of ultimate biodegradation, while more than 20% removal (as specific substance) is evidence of inherent biodegradation, and a search for metabolic intermediates would be worthwhile.

Strategy of Testing

19 Where the biodegradability under a specific set of environmental conditions requires evaluation, the choice of the appropriate test method may be self-evident.

In general, however, most test methods although providing similar conditions to specific natural environments should not normally be regarded as simulation tests, but

rather as tests where the conditions offer a greater or lesser chance of degradation at the concentration tested. Commonly those tests with short time periods and low inocula concentrations offer relatively unfavourable conditions, whereas those with a longer test period, a high inoculum level and enrichment procedures offer highly favourable conditions.

20 In some cases it is sufficient to determine the ratio of BOD_5 to COD. A value of, say, 0.5 would indicate a readily biodegradable substance while values of BOD_{20}: COD of 0.5 would indicate less readily degradable compounds, and further tests would be required.

21 If the substance does not exert a BOD_5 or BOD_{20}, the subsequent strategy adopted is determined by the analyst's purpose, but it is advisable first to assess the toxicity of the substance to bacteria so that a non-inhibitory concentration can be used in biodegradability assessment. A modified BOD test and respiration rate test (in this series, Method B) are suitable for this purpose.

It is also important that the solubility of the substance be determined if not already known. (See paragraph 24).

A suggested stategy is shown in Fig. 1.

22 Attention needs to be drawn to a number of points. For a soluble compound (>10 mg/l) a number of tests are available and to some extent it is a matter of the analyst's preference as to what choice is made. An indication can be quickly obtained from respiration rate measurements (Method B) but this should be followed up by another method. The usual choice is a die-away test in which DOC removal is determined, either by the MOST procedure (Method E) if the substance is to be discharged via rivers or the STCSD procedure (Method F) if the compound is to reach the environment via a sewage treatment works. The indirect methods – CO_2 evolution (Method D) and manometric determination of oxygen uptake (Method C) – can also be used but are less popular; for insoluble compounds, however, only indirect methods are applicable.

A negative result in these tests for ready biodegradability cannot be taken as evidence that the compound is not biodegradable; further tests should be made, perhaps with a lower concentration of test substance, since it may be toxic at the previous concentration. Alternatively, a higher concentration of inoculum may be used, as indicated in paras 14–16, or an acclimatized seed could be tried.

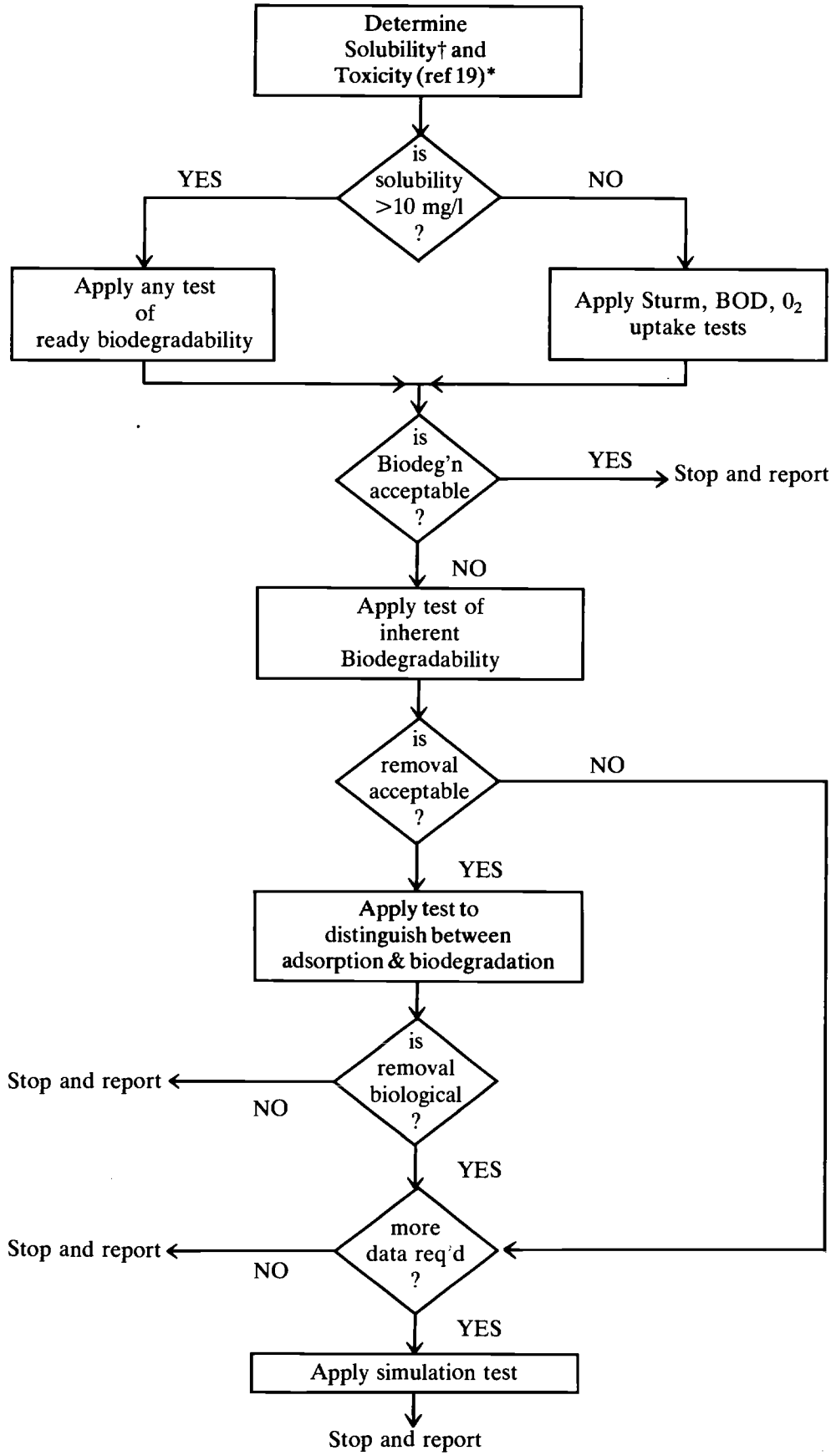
Another variant is to apply the Bunch-Chambers method (Method G) to test the need for co-metabolism. If these fail, the SCAS test (Method H) for inherent biodegradability should be applied. For compounds found to be inherently, but not readily biodegradable, a simulation test (Methods I and J) should be applied, if the compound is to be disposed of via the sewage route. Such tests allow a wide range of micro-organisms to develop and a good chance of acclimatization, as well as co-metabolism.

It cannot be concluded, however, from results of such methods (Methods I and J) in which a high ratio of biomass to test substance is used, that the substance has been biodegraded, since some or all of it may have been adsorbed onto the sludge flocs or biological film, but it may be concluded that the substance has been bioeleminated. To be sure that degradation has occurred, the sludge or biological film must be analysed for the test substance.

The OECD Confirmatory test (Method I) allows a total period of 9 weeks but it has been found that some substances which are not removed in this time can be removed only after longer periods of acclimatization. Thus, in cases of economically important compounds it might be worthwhile lengthening the period of the test.

Finally, it must be emphasized that biodegradability is only one factor involved in deciding whether a compound should be allowed to enter the aquatic environment. Other factors are toxicity to micro- and macro-organisms, and bioaccumulation, as well as the tonnage to be produced and the method of disposal. Thus, these considerations, beyond the scope of this booklet, may come into play at various decision points in Fig. 1 and may affect the course of the investigation.

Figure 1 Strategy for Biodegradability Testing



* An appropriate concentration should be chosen for testing taking into account the solubility and toxicity.
† See para 24.

Legislation

23 National[17] and international (EEC) legislation[18], passed or impending, recommends the application of certain tests for assessing biodegradability; these are indicated in Table 1, together with the SCAS (Method H) and simulation tests (Methods I and J). The OECD Report[14] foresees that other methods, especially for insoluble and volatile compounds, may be developed over the next few years, but only after fruitful research into the kinetics of removal, acclimatization of inocula and DOC analysis as applied to simulation tests.

Solubility Test

24 Weigh out of known weight, eg 100 mg of the substance and add it slowly to 1 litre of distilled water at room or other desired temperature and shake. If all dissolves, add more substance up to 1 g. Shake overnight, then allow to settle and, if necessary, filter. Then determine the concentration of the substance in the filtrate.

For difficult to determine organic substances a Dissolved Organic Carbon analysis[23] is suggested.

Glossary of Terms

1 **Acclimatization**

The processes, including selection and adaptation, by which a mixed population of microorganisms develops the ability to degrade a substance, or develops a tolerance to it.

2 **Activated sludge**

A flocculated mixture of microorganisms and inert organic and inorganic material produced by the aeration of sewage and/or waste water.

3 **Adsorption**

The adherence of a substance to a surface (organic or inorganic) by physico-chemical processes.

4 **Analogue metabolism** (– a special case of co-metabolism)

The process by which a normally non-biodegradable compound is biodegraded only in the presence of a structurally similar compound, which can induce the necessary enzymes.

5 **Bioaccumulation**

The ability of a microorganism to assimilate and retain a chemical unaltered by normal physiological processes.

6 **Biodegradability**

The ability of a compound to undergo microbial attack.

7 **Biodegradation**

The breakdown of a compound by micro-organisms. This may be:

(a) *Primary* – the alteration of the chemical structure of a substance resulting in loss of specific property of that substance;
(b) *environmentally acceptable* – biodegradation to such an extent as to remove undesirable properties of the compound. This will frequently correspond to primary biodegradation but may vary depending on the circumstances under which the products are discharged into the environment;
(c) *ultimate* – the complete breakdown of a compound to either fully oxidised or reduced, simple molecules (eg CO_2, CH_4, H_2O, NO^-_3, NH^+_4) and the formation of new cells.

8 **Bioelimination**

The removal of a compound from the liquid phase in the presence of living micro-organisms by physico-chemical as well as biological processes.

9 **BOD** (Biochemical oxygen demand)

The amount of oxygen consumed by micro-organisms when metabolizing a compound.

10 **COD** (Chemical oxygen demand)

The amount of oxygen consumed during oxidation of a compound with hot acid dichromate; it provides a measure of the oxidizable matter present.

11 **Co-metabolism**

The process by which a normally non-biodegradable compound is biodegraded only in the presence of an additional carbon source. (See also analogue metabolism.)

12 **Complete mineralization**

The complete breakdown of a compound to carbon dioxide, water and oxides of other elements present, including biodegradation of new cells formed.

13 **Degradation**

A process involving simplification of the structure of a compound.

14 **Die-away test**

A term applied to batch biodegradability tests in which the decrease in the initial concentration of a compound is observed with time.

15 **DOC** (Dissolved organic carbon)

The amount of carbon present in organic compounds in aqueous solution.

16 **Inherently Biodegradable**

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

17 **Inhibition**

The effects of a compound, or its metabolites, on micro-organisms which may be manifested as a reduction in: oxygen uptake, substrate degradation, gas evolution or growth.

18 **Inoculation**

The process of adding micro-organisms to a test medium.

19 **Inoculum**

Micro-organisms added to a test medium.

20 **Loading factors** (F/M)

(a) *Sludge loading.* This term relates to the operation of activated sludge plants and is defined as the weight of BOD applied to the plant per day per unit weight of sludge under aeration. It is usually expressed as kg BOD/kg MLSS day.

(b) *Initial sludge loading.* This term relates to the operation of batch processes and is defined as the weight of BOD added at the start of the test per unit weight of micro-organisms. It is usually expressed as g BOD/g MLSS.

(c) *F/M*. This is a somewhat confusing term which may represent either of the above situations.

21 **Mixed liquor suspended solids** (MLSS)

The concentration, expressed as mg dry solids/l, of activated sludge in the aeration chamber of a treatment plant.

22 **Nitrification**

The sequential oxidation of ammonium salts to nitrite and nitrate by micro-organisms.

23 **Readily biodegradable**

An arbitrary classification of chemicals which have passed certain specified screening tests for ultimate biodegradability; these tests are so stringent that such compounds will rapidly and completely biodegrade in a wide variety of aerobic environments.

24 **Respiration rate**

The weight of oxygen taken up in unit time by unit volume of sample (mg/l h). The *specific respiration rate* of a sample is the weight of oxygen taken up in unit time by unit weight of activated sludge, usually expressed as mg/g h.

25 **Screening (indicative) tests**

Relatively simple, batch tests which may be used for preliminary assessment of biodegradability, treatability or toxicity of a test compound.

26 **Simulation tests**

Tests designed to predict the rate of biodegradation of a compound under relevant environmental conditions.

27 **TOC** (Total organic carbon)

The total amount of organic carbon in an aqueous solution/suspension.

28 **TOD** (Theoretical oxygen demand)

The total amount of oxygen required to oxidize a compound completely to mineral products, calculated from its formula.

29 **Toxicity**

The extent to which a test compound adversely affects micro-organisms.

30 **Treatability**

The amenability of compounds to removal during biological treatment without adversely affecting the normal operation of the treatment processes. Adverse effects are usually: reduced removal of chemical oxygen demand and/or dissolved organic carbon, inhibition of nitrification and/or anaerobic sludge digestion, etc.

Sample Collection and Preservation

Only methods I and J have specific sections on these topics. In general, samples should be as representative as possible, considering the nature of the sample and the information required. Preservatives are not recommended and must not be used at all if by their use the biodegrading organisms may be inhibited or killed. If samples are unstable due to biological activity, storage at 4 ± 1°C is suggested, but tests should be started as speedily as possible. This should be taken into account when planning any sampling programme. (See also reference 24).

A Respirometry: Basic Techniques

Introduction

The degree to which oxygen is taken up by a chemical or waste water in the presence of suitable micro-organisms is a measure of the biodegradation of the test material. Respiration measurements can be made in two distinct ways. In the first, the sample absorbs oxygen from a confined atmosphere; either the atmosphere is recharged with oxygen to maintain a constant volume *(Method AA – electrolytic respirometer)* or the change in volume is measured by means of a calibrated scale *(Method AB – Hach respirometer)*. In the second method the decrease is observed of the concentration of dissolved oxygen in the sample, which is confined so that no further replenishment of the dissolved oxygen can take place *(Method AC – oxygen electrode respirometer)*. The application of the manometric respirometers is described in method C and the electrode respirometers in method B.

It should be noted that none of these methods distinguishes between chemical and biological oxidation. If such distinctions are required, it will be necessary to devise suitable experiments. With the exception of a few chemicals and some trade wastes, the vast majority of oxygen demand is biological in nature and oxygen uptake rates are thus referred to as rates of respiration. Oxygen uptake due to nitrification and carbonaceous oxidation may be distinguished by means of a specific inhibitor (see method B).

Method AA Electrolytic Respirometer

AA.1 Performance Characteristics of the Method

AA.1.1	Property determined	Oxygen uptake (respiration) expressed as weight, and % of theoretical.
AA.1.2	Types of sample	Sludge alone or mixed with waste waters or solutions or suspensions of chemicals.
AA.1.3	Basis of method	Replenishment, by controlled electrolysis, of oxygen consumed by a confined, inoculated sample and air.
AA.1.4	Range of application	0–250 mg/l h; 0–100%.
AA.1.5	Standard deviation	Not available Example of duplicate values obtained on one occasion: All contain 30 mg sludge solids/l and 100 mg/l of substrate, where appropriate. Vol of reaction mixture = 550 ml

Substrate	mg O_2 taken up in 28d
None	23, 24 12, 18
sodium benzoate	107,108
diethylene glycol	62, 96
pentaerythritol	86, 96
t-butanol	25, 33
2-chloro-aniline	11, 16

AA.1.6	Limit of detection	1 mg (current chosen produces 1 mg O_2 in 1 min).
AA.1.7	Interferences	See Section AA.3
AA.1.8	Time required	Up to 28 days Operator time: 10 h per run up to 6 units.

AA.2 Principle

A measured volume of test material is stirred in a closed flask and the consumption of oxygen is determined by measuring the quantity of oxygen required to maintain constant volume in the respirometer flask. A magnetic stirrer is used to mix the contents of the flask and form a vortex which permits rapid transfer of oxygen to the sample; carbon dioxide is formed, but mostly remains in solution as carbonate or bicarbonate. Gas-phase carbon dioxide is absorbed in potassium hydroxide solution, and the fall in pressure due to this and the uptake of oxygen as a result of microbial

respiration causes the interruption of an AC circuit in a manometer – electrolytic cell device attached to the respirometer. This in turn switches a preselected stabilised DC current through the electrolytic cell releasing oxygen at its anode which replenishes oxygen consumed in the respirometer.

AA.3 Interferences

AA.3.1 Toxic materials may cause inhibition and care should be taken to ensure that they do not enter the system except by design. Examples of materials which may be present adventitiously are chlorine from tap water and chromate from glass cleaning operations. No special precautions, other than rinsing with distilled water, are needed to ensure removal of these substances.

AA.3.2 If silicone grease is used on the ground glass joints, care should be taken to avoid its entering the main body of the flask as it will cause interference by reducing the rate of oxygen transfer.

AA.4 Hazards

Hazards associated with handling sewages and industrial wastes may be encountered, and appropriate precautions should be taken.

AA.5 Reagents

AA.5.1 **Water** Distilled or deionized water is normally used.

AA.5.2 **Activated sludge**

The source of the activated sludge used will depend on the aim of the experiment. Normally, activated sludge is taken from a plant treating predominantly domestic sewage and should be in the endogenous phase. Details of sludge preparation are given in Method C.

AA.5.3 **Allyl thiourea solution**

Inhibition of nitrification in activated sludges which have not previously been exposed to the inhibitor can be achieved by adding allyl thiourea solution (5 ml of 2.5 g/litre to each litre of test sample) so as to generate a concentration of inhibitor of 12.5 mg/litre.

AA.5.4 **8% sodium hydroxide solution W/V.**

AA.5.5 **20% potassium hydroxide solution W/V.**

AA.5.6 **Nutrient-phosphate buffer**

The following solutions, A–D, are made up using de-ionized or distilled water, and analytical grade reagents.

Solution A:

21.75 g dipotassium hydrogen phosphate
8.5 g potassium dihydrogen phosphate
33.4 g dibasic sodium phosphate dihydrate
5.0 g ammonium chloride
– made up to 1 l; pH value is 7.2.

Solution B

22.5 g magnesium sulphate heptahydrate – made up to 1 l.

Solution C:

27.5 g calcium chloride – made up to 1 l.

Solution D:

0.25 g ferric chloride hexahydrate – made up to 1 l.
The final buffer contains 3 ml each of solutions A, B, C and D per litre in deionized or distilled water; it contains 4 mg N/l and phosphate at 0.9375 mM.

AA.6 Apparatus

The apparatus is that developed by the former Water Pollution Research Laboratory in 1968 and originally intended for the determination of rapid respiration rates.

The equipment is in two parts, (i) a constant temperature water bath (180 cm × 30 cm × 17 cm) in which are immersed the six reaction flasks and, (ii) a central console (56 cm × 30 cm × 75 cm).

AA.6.1 **Reaction flask** (See also the note on p22)

A flat-flanged reaction flask of 500 ml nominal volume (actual volume about 720 ml), and its electrolytic cell are shown in Fig. 2. The flask has a 5-neck demountable lid. The central neck is closed by a suitable screwthread cone adapter containing a glass rod supporting a glass dish to contain the CO_2–absorbent.
The other necks contain:
(i) the manometer-electrolytic cell
(ii) a stopcock
(iii) a stopper, and
(iv) optionally a dissolved oxygen (DO) probe, which is otherwise replaced by a similar stopper to (iii).

The stoppers are extended to reduce the volume of the gas phase. PTFE sleeves and gaskets should be used to give an air-tight seal at the ground glass joints. This avoids the use of silicone grease which can cause interference (see Section AA.3).

AA.6.2 **Electrolytic – Manometer Cell**

The electrolytic cell is shown in detail in Fig. 2. Three platinum electrodes are incorporated in the cell, one acts as a level detector whilst the other two comprise the anode and cathode of the electrolytic cell. The cell electrolyte is 8% sodium hydroxide.

AA.6.3 **Stirrers**

The contents of the reaction flasks are stirred by PTFE coated slave magnets ("followers"). The stirrer drive motors are placed beneath the water bath and must be capable of effective magnetic coupling not only through the flask wall, but through the water bath as well. Not all shapes of slave are suitable: an egg-shaped spinbar and a 32 mm "circulus" have been used successfully in the round-bottomed flasks.

Very rapid stirring (at rates of the order of 1000 rev/min) is needed to achieve the necessary rates of oxygen transfer with the more actively respiring samples. Two types of magnetic stirring motor are available, one electrical and one driven by compressed air. Experience has shown that the air-driven motors are more reliable than the electric motors, and that either should be fitted flush with the bottom of the bath. The bottom of the water bath is 'dished', both to locate the reaction flasks centrally over the motors, and if necessary to minimise the distance between the master and slave magnets.

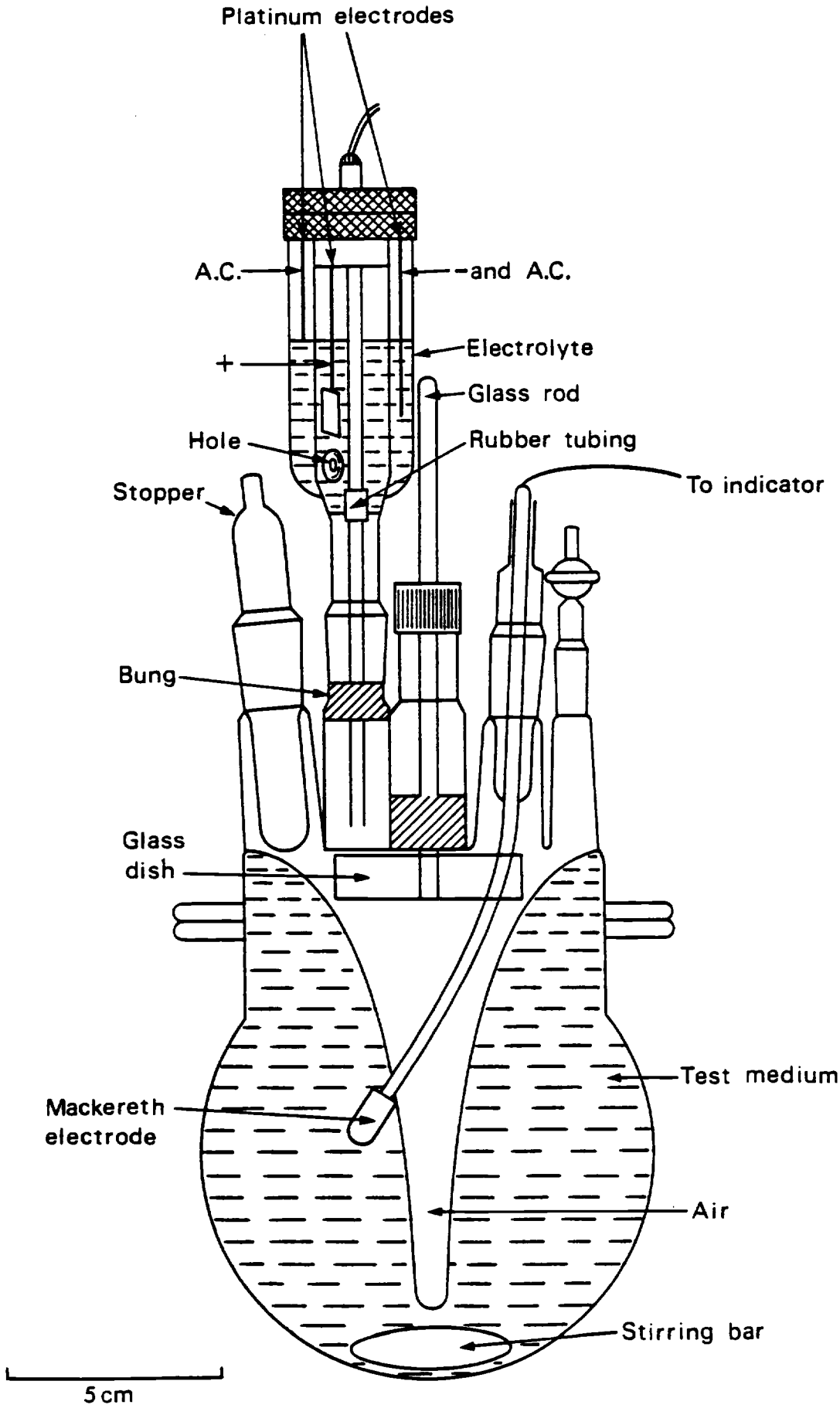
AA.6.4 **Control Console**

The control console contains all the necessary power supplies, circuitry, controls, and printing counters. As each channel is independent, a single timer cam could be used to operate all channels simultaneously but in order to reduce power requirements individual cams, arranged in sequence, are provided.

The solid state logic and printing counter drive circuits are assembled on three plug-in printed circuit boards for each channel, the total of eighteen cards being contained in a card frame at the top of the unit. Commercially available items have been used throughout and for ease of assembly no chassis is employed, all components being mounted on the base and panels.

Details of design and construction of the control console are available from the Water Research Centre on request, and can be obtained commercially from pHox Instruments Ltd, Shefford, Herts.

FIG.2 AUTOMATIC ELECTROLYTIC RESPIROMETER



AA.6.5 Basis of Operation

The consumption of oxygen by the sample causes a reduction in the volume of the gas phase and the level of electrolyte falls in the outer annulus of the cell. Ultimately the level falls below that of the side contact and a small AC current flowing between the contact and the cathode ceases. A standard cam timer (synchronous motor driven) allows a logic circuit to interrogate the condition of the AC 'probe' circuit once every minute and on the next operation after the AC current has ceased, the logic circuit causes the electrolyte current to be switched on at a pre-set value, and the time, in minutes from the start of the experiment, to be printed out by an electro-mechanical printer. If the oxygen supplied by electrolysis is sufficient to re-make the AC circuit, the electrolytic current ceases at the end of 1 min, otherwise electrolysis continues for further consecutive periods of 1 min until the AC circuit is remade, the printer operating at the beginning of each minute of electrolysis.

The results are obtained as a series of printed times, each corresponding to a known increment of oxygen.

AA.7 Procedure

Step	Procedure	Notes
AA.7.1	**Preparation of Apparatus**	
	Assemble the six reaction flasks and place them in the constant temperature water bath. Add 8% sodium hydroxide solution to the electrolysis cells to just below the level of the sensing probes. Switch the power on and adjust the individual current for each channel (note a). Allow 1 to 2 mg oxygen to be supplied to each vessel by suitable adjustment of the level of the electrolyte. Leave the flasks for a period, e.g. overnight and, if the flasks are air-tight no further evolution of oxygen should take place. Although one stopper on each flask will have to be moved again, this procedure considerably reduces the possibility of overlooking leaks.	(a) The current normally used is 402 mA which corresponds to the evolution of 2 mg of oxygen in 1 min or 201 mA, 1 mg of oxygen in 1 min. (See also note on next page for a variant flask design).
AA.7.2	**Preparation of Activated Sludge**	
	Collect the mixed liquor on the day of the test. (See method C.) Determine the suspended solids (or volatile suspended solids) of the thickened activated sludge (see Ref 27 and 33). Place the activated sludge in the water bath and aerate until use (note b).	(b) The inoculum should be a small proportion of the total volume and therefore high suspended solids are required (3000 mg/l or above).
AA.7.3	**Characterization of Test Materials**	
	Determine the COD, and if not known, the DOC of the test material by standard methods (Refs 22 and 23).	
AA.7.4	**Preparation of Flasks**	
	Calculate the required volume of nutrient solutions, buffer, test material and distilled water and add these to the flasks. (See Table 1, Method C, for an example.) The final volume of liquid in the flasks should be 550–620 ml, leaving a gas	(c) Increasing the volume of liquid reduces errors but also reduces the rate of solution of oxygen. Work with the minimum gas space for measuring respiration rates at the lower end of the range and *vice versa*.

Step	Procedure	Notes
AA.7.4 (contd)	Preparation of Flasks (contd)	
	volume of not more than 170 ml and not less than 100 ml (note c).	
	Start the magnetic stirrers and adjust so that a smooth rapid stirring in the flask is obtained. Switch on the electrolysis cells and allow to run for 15 minutes. During this period add a volume of inoculum to give the required concentration of MLSS. Add 2 ml of 20% potassium hydroxide solution (AA 5.6) to the glass dish using a syringe.	
AA.7.5	Measurement of Oxygen Uptake	
	Insert the stoppers and close the stopcocks. Finally, press the 'reset' button on the printers and allow the apparatus to run.	
	Make good any evaporation losses from the electrolytic cell by the addition of distilled water, and replenish the water bath with tap water when necessary. Usually no further attention is required other than occasional checks to see that adequate stirring is maintained.	
AA.7.6	From the print-out of times of oxygen production (2 mg if 402 mA for 1 min and *pro rata*), plot a respiration curve. This can be done by plotting, against time, the weight of oxygen taken up or the percentage of theoretical oxygen uptake, after correction for the oxygen utilized by a control containing the inoculum but no test substance.	

Note Modified Reaction Flask (AA.6.1)

It is possible to use a two neck flask. One neck carries the cell unit (as in Fig. 2). The other neck carries a hollow stopper which is fitted with a vent tap at the top and a hook at the bottom from which hangs a small glass dish. The Mackereth electrode, which is only needed if the oxygen content of the liquid is to be monitored, may be omitted.

AA.8 Sources of error

AA.8.1 Barometric pressure

An increase in barometric pressure reduces the volume of the gas phase and also causes more air to dissolve, causing the electrolytic cell to supply oxygen to restore the original volume of gas. Similarly, a decrease in pressure retards the operation of the cell. A correction may be made graphically, using the results of a control experiment, or by calculation. A correction of 1 mg oxygen is necessary for a change in pressure of 5 mm Hg with 620 ml liquid and 110 ml of gas, while for 550 ml liquid and 180 ml gas the correction is 1 mg per 3 mm Hg. It is imperative to make these corrections or to allow for them in a control experiment using the same gas/liquid ratio as in the test.

AA.8.2 Oxygen deficit

Errors due to the development of an oxygen deficit in the liquid phase become evident only when the respiration rate is high. The rates found in biodegradability tests will be too low for this to occur.

AA.8.3 **Time scale**

Because the sample should reach equilibrium in the reaction flask before readings are started an uncertainty may arise in the time scale. This would be small for biodegradability assessments but can be almost certainly eliminated by equilibrating the buffered samples with air at the temperature of the experiment.

Method AB Manometric Method (Hach)

AB.1 Performance Characteristics of the Method

AB.1.1	Property determined	Oxygen uptake (respiration) rate.
AB.1.2	Type of sample	Sludge alone or mixed with waste waters or solutions/supsensions of chemicals.
AB.1.3	Basis of method	Oxygen consumption of a confined inoculated sample plus air is measured by means of a calibrated closed-end manometer.
AB.1.5	Standard deviation	Not available Examples of duplicates of oxygen uptake (mg) after 28 days:

Control	23	31.5	
Sodium benzoate	160	167	(a)
Sodium stearate	172	220	(b)
Sulphanilic acid	30	40	100(c)
Diethylene glycol	107	145	(d)

AB.1.6	Limit of detection	Depends on sample volume and scale chosen, e.g.: 416 ml, scale 0–35, Limit 0.5 mg/l 240 ml, scale 0–350, Limit 5 mg/l.
AB.1.7	Interferences	See Section AB.3
AB.1.8	Time required	Operator time: 10 h per run up to 6 units

Notes: (a) a typical soluble easily biodegradable compound – reproducible results
(b) a typical insoluble compound
(c) and (d) examples of erratic compounds, (c) in triplicate

AB.2 Principle

A measured volume of test material is stirred in a partially filled bottle, which is connected to a closed-end mercury manometer. Oxygen consumption is measured by observing the change in level of the mercury column in the manometer. Carbon dioxide evolved into the bottle atmosphere is absorbed in a small amount of alkali held in a small cup within the bottle cap. Temperature control is normally achieved by placing the equipment in an incubator.

AB.3 Interferences

AB.3.1 Toxic materials may cause inhibition and care should be taken to ensure that they do not enter the system except by design. Examples of materials which may be present adventitiously are chlorine from tap water and chromate from glass cleaning operations. No special precautions, other than rinsing with distilled water, are needed to ensure removal of these substances.

AB.3.2 If silicone grease is used on the rims of the bottles, care should be taken to avoid its entering the main body of the flask as it will cause interference by reducing the rate of oxygen transfer.

AB.4 Hazards

Normal hazards associated with handling sewages and industrial wastes may be encountered, and appropriate precautions should be taken.

AB.5 Reagents

AB.5.1 **Water**

Distilled or deionized water is normally used.

AB.5.2 **Activated sludge**

The source of the activated sludge used will depend on the aim of the experiment. Normally, activated sludge is taken from a plant treating predominantly domestic sewage and should be in the endogenous phase. Details of sludge preparation are given in method C.

AB.5.3 **Allyl thiourea solution**

Inhibition of nitrification in activated sludges which have not previously been exposed to the inhibitor can be achieved by adding allyl thiourea solution (5 ml, 2.5 g/litre) to each litre of test sample so as to generate a concentration of inhibitor of 12.5 mg/litre.

AB.5.4 **20% potassium hydroxide solution W/V.**

AB.5.5 **Dilution water**

AB.5.5.1 *Solution A:*

Potassium dihydrogen phosphate, 8.5 g; di-potassium hydrogen phosphate, 21.75 g; disodium hydrogen phosphate dihydrate, 33.4 g; ammonium chloride, 5.0 g; dissolved in distilled water and made up to 1000 ml. the pH value should be 7.2.

AB.5.5.2 *Solution B:*

Magnesium sulphate heptahydrate, 22.5 g dissolved in distilled water and made up to 1000 ml.

AB.5.5.3 *Solution C:*

Calcium chloride dihydrate, 36.3 g dissolved in distilled water and made up to 1000 ml.

AB.5.5.4 *Solution D:*

Ferric chloride hexahydrate, 0.25 g dissolved in distilled water and made up to 1000 ml.

The dilution water is made up to contain 3 ml of each of solutions A–D per litre of distilled water.

AB.6 Apparatus

AB.6.1 The apparatus used for these studies is a constant volume respirometer, measuring oxygen uptake manometrically. Each individual unit (Fig. 3) consists of a dark glass bottle connected to a closed-end mercury manometer. Carbon dioxide produced during respiration is absorbed by potassium hydroxide placed in the seal cup and the oxygen taken up is measured as a decrease in pressure by reading directly the BOD (mg/l) on a scale*. The contents of each bottle are individually stirred by a PTFE covered stirrer-bar driven by magnets attached to a motor.

A commercially available form of the apparatus is manufactured by the Hach Chemical Co., Ames, Iowa, U.S., whose agents in the UK are Camlab Ltd, Cambridge.

AB.6.2 **Basis of Operation**

The appropriate volume of sample material is introduced into the bottle, buffer, dilution water and inoculum are added as required, and connection made to the manometer. Thermal equilibrium is allowed to become established, then the manometer is sealed, and readings are commenced. The equipment is normally placed in an incubator to ensure that measurements are made at a defined temperature.

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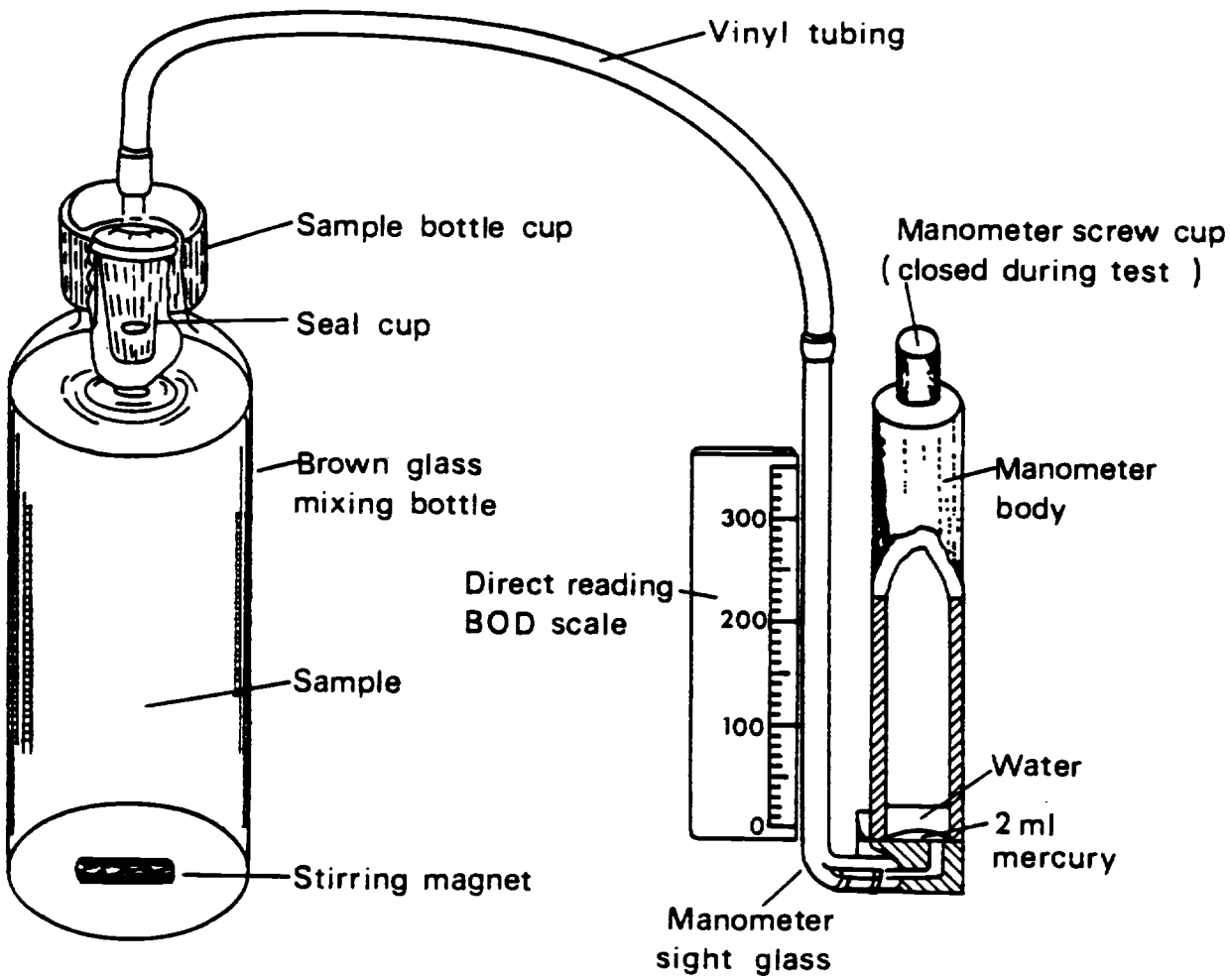
* Mathematically calibrated by the method of Caldwell and Langelier, given in Section AB9.

FIG.3 HATCH-TYPE MANOMETRIC APPARATUS SHOWING ONE CELL



AB.6.3 Seed Concentration

The concentration of seed micro-organisms is normally 30 mg/l. The MLSS of the activated sludge is determined on the day of use and volume required per litre of dilution water is calculated as follows:

$$V = \frac{X \times 1000}{y}$$

where V = Volume of seed ml per litre of dilution water
X = Desired seed concentration (normally 30 mg/l)
y = MLSS of activated sludge (mg/l)

AB.6.4 Volume of Test Medium

The volume of the test medium and hence the amount of test compound that must be dispensed to each bottle depends on the anticipated BOD of the test compound. The theoretical oxygen demand can usually be calculated for pure compounds or formulated products of known composition or the chemical oxygen demand (COD) may be measured if no information is available. Consideration of the structure of the compound may yield information on the likely BOD. Table 3 indicates the appropriate volume – scale relationship.

AB.7 Procedure

Step	Procedure	Notes
	Effluents and water soluble compounds	
AB.7.1	For each sample to be tested calculate the total volume of dilution water required (note a) and half fill a measuring cylinder of appropriate volume with aerated distilled water.	(a) The volume required depends on the anticipated BOD (see Section AB.6.4.) The greater the oxygen uptake expected, the larger the volume of air required to satisfy the demand and hence the smaller the volume of solution to be tested in each bottle.
AB.7.2	Add to this measuring cylinder 3 ml per litre (final volume) of each of the solutions 5.5.1–5.5.4 followed by the appropriate volume of activated sludge seed as calculated above (AB.6.3) (note b)	(b) The activated sludge can be varied from the 30 mg l^{-1} level stated. Also the seed can be obtained from other sources (e.g. laboratory units acclimatized to the test compound) depending on the objectives of the experiment.
AB.7.3	Finally add the appropriate volume of sample or test compound solution (note c) and make the total volume in the measuring cylinder up to the required level with distilled water.	(c) Calculated from a knowledge of the stock solution concentration, the desired test concentration and the volume of solution being prepared.
AB.7.4	Mix thoroughly and dispense the required volume into each of three Hach bottles (note d).	(d) Normally three replicate bottles are used to ensure reproducibility. If equipment availability is limited, fewer bottles can be used.
AB.7.5	Set up the blank Hach bottles (seed and dilution water only) and if necessary, standards (e.g. aniline, 100 mg/l, in seeded dilution water).	
AB.7.6	Place a magnetic follower in each bottle and then apply a thin smear of grease around the rim of the bottles (note e).	(e) Ensure that the grease does not come into contact with the test solution where it could interfere.
AB.7.7	Place a seal cup to which has been added 2 drops of 20% w/v potassium hydroxide (note f) in the mouth of each bottle and then appropriately position the bottles on the Hach stirring apparatus.	(f) The potassium hydroxide absorbs any carbon dioxide gas produced.

Step	Procedure	Notes
AB.7.8	Apply a further smear of grease to the top of the seal cup and then switch on the stirrer motor checking that each bottle is correctly agitated.	
AB.7.9	Open the manometer caps, check that each manometer contains 4–5 drops of water and then lightly grease each manometer cap seal before loosely replacing on the manometer body. (note g).	(g) The closed-end manometers compensate for changes in atmospheric pressure.
AB.7.10	Screw down the bottle caps loosely and then leave the whole apparatus to reach thermal equilibrium (30–60 minutes) (note h).	(h) This time can be shortened by bringing all solutions to the experimental temperature before filling the bottles.
AB.7.11	After this time period, tighten both bottle and manometer caps, loosen the manometer scale screws and set the zero mark on the scale level with the top of the mercury column. Tighten scale screws.	
AB.7.12	Record time and date. After a further 30 minutes check the reading on the manometer. If either a negative or a positive reading has resulted in any of the manometers briefly loosen both manometer and bottle caps then reclose. Repeat AB7.11.	
AB.7.13	Continue to record manometer values daily until the end of the experiment, and plot the respiration curve. This can be done by plotting, against time, the concentration of oxygen take up or the percentage of theoretical oxygen uptake, after correction for the oxygen utilised by a control containing the inoculum but no test substance.	
	Viscous or solid samples (not water soluble)	
AB.7.14	Follow steps AB7.1 to AB7.2.	
AB.7.15	Make up the volume of dilution water to the appropriate level with distilled water (note i).	(i) In most cases the volume of compound to be added is an insignificant proportion of the seeded dilution water and need not be taken into account.
AB.7.16	Place viscous samples directly into the Hach bottles then add the required volume of dilution water.	
AB.7.17	For solid samples, place the required volume of dilution water into a clean wash bottle and then wash the sample into the appropriate Hach bottle.	
AB.7.18	Follow steps 7.5 to 7.13.	

AB.8 Sources of error

Barometic pressure

Unlike most manometric respirometers, this version is not subject to errors due to changes in barometric pressure because of the closed-end manometer.

Table 3 Volume – scale relationships and conversion factors for Hach BOD bottles

Sample volume (ml)	Selected[1] scale	Conversion[2] factor	Expected BOD[3] (mg/l)
416	0–35	1.0	0–35
416	0–350	0.1	0–35
352	0–70	1.0	0–70
352	0–350	0.2	0–70
305	0–350	0.3	0–100
293	0–350	0.33	0–150
240	0–350	0.5	0–175
190	0–350	0.75	0–250
157	0–350	1.0	0–350
123	0–350	1.4	0–500
123	0–700	0.7	0–500
93	0–350	2.0	0–700
93	0–700	1.0	0–700

Notes (1) The Model 2173 apparatus only has a scale from 0–350. The Model 2173 A scale can be varied as follows: 0–35; 0–70; 0–350; 0–700.
(2) The averaged results must be multiplied by this correction factor before blank correction.
(3) Where there is an alternative, it is advantageous to take the largest volume possible with the smallest scale range. This makes scale reading easier and minimises errors in multiplication.

AB9 Calibration of the manometer scale on the Hach respirometer

For a scale reading change of x cm during the experiment the wt (mg) of oxygen absorbed by the volume of liquid, v, is calculated as follows:–

BOD (wt in mg of O_2 adsorbed by volume v) =

$$\frac{32\ T_N.x}{22.4.T_I.P_N}\left[V_1 + P_I\frac{\pi d^2}{4} - \frac{\pi d^2 x}{4}\right]$$

where

T_N = standard temperature (°K)
T_I = temperature of experiment (°K)
P_N = standard pressure (cm of mercury)
P_I = atmospheric pressure (cm of mercury)
d = diameter of sight glass tubing
V_1 = volume of air in vessel A plus the length (L) of flexible tubing, at the start of the experiment

$$V_1 = V - v + \frac{\pi D^2 L}{4}$$

where

V = volume of glass vessel A
v = volume of liquid in glass vessel
D = diameter of flexible tubing
L = length of flexible tubing

AB9.1 Calibration procedure

The manometer sight glass tube is of constant diameter (d = 0.15 cm) and a calibration scale is provided by the manufacturer (20 divisions = 5.1 cm). The length of the 'Tygon' tubing is constant (25 cm), as is its diameter (0.65 cm). The volume of the glass bottle is constant (500 ml) and the volume of the liquid sample (v) is known. From these data the BOD corresponding to a scale division can be calculated at the start of the experiment for a given temperature and pressure.

Normally the temperature is kept constant throughout the experiment and the apparatus and test solutions are brought up to this temperature at the start. Theoretically corrections should be made to the calibration for the atmospheric

pressure prevailing at t = O. In practice this is not done and a change in pressure ($\triangle P$) from the assumed norm (76 cm Hg) will cause an error in BOD reading of about

$$\frac{\triangle P}{(P_I + V_1 . \frac{4}{\pi d^2}} \times 100\%$$

For tubing of 0.15 cm diameter this will always be negligible.

Once the apparatus is in operation correction for changes in barometric pressure do not need to be made to the daily readings.

Method AC
Oxygen Electrode Respirometer

AC.1 Performance Characteristics of the Method

AC.1.1	Parameter determined	Oxygen uptake (respiration) rate.
AC.1.2	Types of sample	Sludge alone or mixed with waste water or solutions/suspensions of chemicals.
AC.1.3	Basis of method	Decrease in dissolved oxygen concentration with time of a well aerated, confined inoculated sample, excluding gaseous phase, is measured using an oxygen electrode.
AC.1.4	Range of application	0–4 mg O_2/l min; 0–240 mg/g h for sludge containing 1000 mg MLSS/1.
AC.1.5	Standard deviation	See table below.
AC.1.6	Limit of detection	0.05 mg O_2/l min.
AC.1.7	Interferences	See Section AC.3.
AC.1.8	Time required	Operator time: 15 min for one determination.

Sample	Respiration rate mg/g h		
	mean	SD	n
1. Sludge alone (5250 mg MLSS/1)	8.0	0.22	4
2. (1) washed and centrifuged four times	16.5	2.4	4
3. (2) plus sewage	44.0	2.8	4

AC.2 Principle

Suitable aerated samples are introduced into a closed vessel containing an electrode which detects the decrease in dissolved oxygen concentration which is recorded as a function of time. The slope of the resulting line is the respiration rate in mg O_2/litre per unit time. By measuring the mixed liquor suspended solids (MLSS), or volatile solids (MLVSS) of the samples under test, it is possible to express respiration as specific oxygen uptake rate, mg O_2/g ML(V)SS hour.

AC.3 Interferences

AC.3.1 Toxic materials may cause inhibition and care should be taken to ensure that they do not enter the system except by design. Examples of materials which may be present adventitiously are chlorine from tap water and chromate from glass cleaning operations. No special precautions, other than rinsing with distilled water, are needed to ensure removal of these substances.

AC.3.2 Some substances may affect the calibration of the electrode; this may be checked, if suspected, by appropriate experiments in the absence of bacteria.

AC.4 Hazards

Normal hazards associated with handling sewages and industrial wastes may be encountered, and appropriate precautions should be taken.

AC.5 Reagents

AC.5.1 **Water**

Distilled or deionized water is normally used.

AC.5.2 **Activated sludge**

The source of the activated sludge used will depend on the aim of the experiment. Normally, activated sludge is taken from a plant treating predominantly domestic sewage and should be in the endogenous phase. Details of sludge preparation are given in method C, Section 5.2.

AC.5.3 **Allyl thiourea solution**

Inhibition of nitrification in activated sludges which have not previously been exposed to the inhibitor can be achieved by adding allyl thiourea solution (5 ml, 2.5 g/litre) to each litre of test sample so as to give a concentration of inhibitor of 12.5 mg/litre.

AC.5.4 **Electrode zero calibration solution**

Prepare and use a solution of the composition recommended by the electrode manufacturer.

AC.6 Apparatus

AC.6.1 Equipment is required to confine the sample so that it is not in contact with air, to stir the sample, and to measure the decrease in concentration of dissolved oxygen within it. This can be assembled from general purpose laboratory equipment or, alternatively, proprietary equipment can be purchased and suitably adapted.

One type of proprietary device, which is readily available, is the Rank respirometer, and this can be obtained in cell capacities ranging from 7 ml to 50 ml. A respirometer using this equipment is shown schematically in Figure 4.

Suitable systems can also be constructed from standard laboratory glassware, such as conical flasks or BOD type bottles, incorporating DO probes, such as those available from Yellow Springs Instruments (YSI), Electronic Instruments Ltd (EIL), or International Biophysics Corporation (IBC). Typical arrangements are shown in Figures 5 and 6.

The Rank electrode requires a suitable operating circuit, as shown in Figure 4, and a chart recorder with a sensitivity in the range 1–20 mV full scale deflection (fsd).

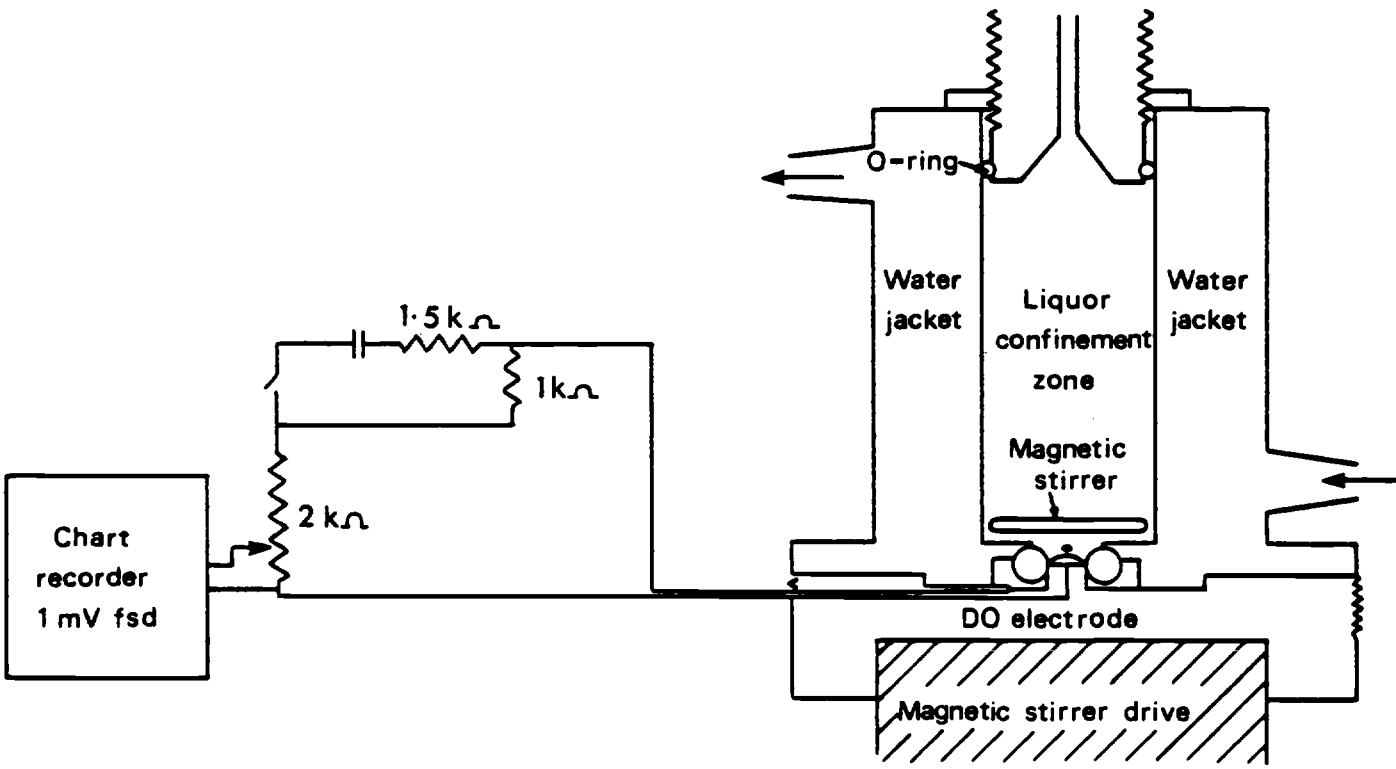
The YSI and IBC probes require their associated DO meters, but the EIL probe can be connected to any suitable pH or mV meter. The YSI and IBC instruments should be connected to a suitable chart recorder, preferably having 100–500 mV fsd sensitivity range, using the circuit shown in Fig. 5. The EIL probe should be used with a meter which has an output signal compatible with the sensitivity range of the available chart recorder.

For consistent results, it is important to maintain the test vessel and contents at a constant, known temperature since slight temperature variations may lead to significant changes in concentration of dissolved oxygen, and to a lesser extent in respiration rate.

AC.6.2 **Basis of Operation**

The cell or bottle is filled with the sample under test, the DO probe and/or stopper is inserted, and the rate of decrease of dissolved oxygen concentration is measured, ideally by recording on a suitable chart recorder.

FIG.4 RANK TYPE RESPIROMETER



1cm. Cell only is to scale

FIG.5 TYPICAL ARRANGEMENT USING YSI BOD-TYPE DO METER

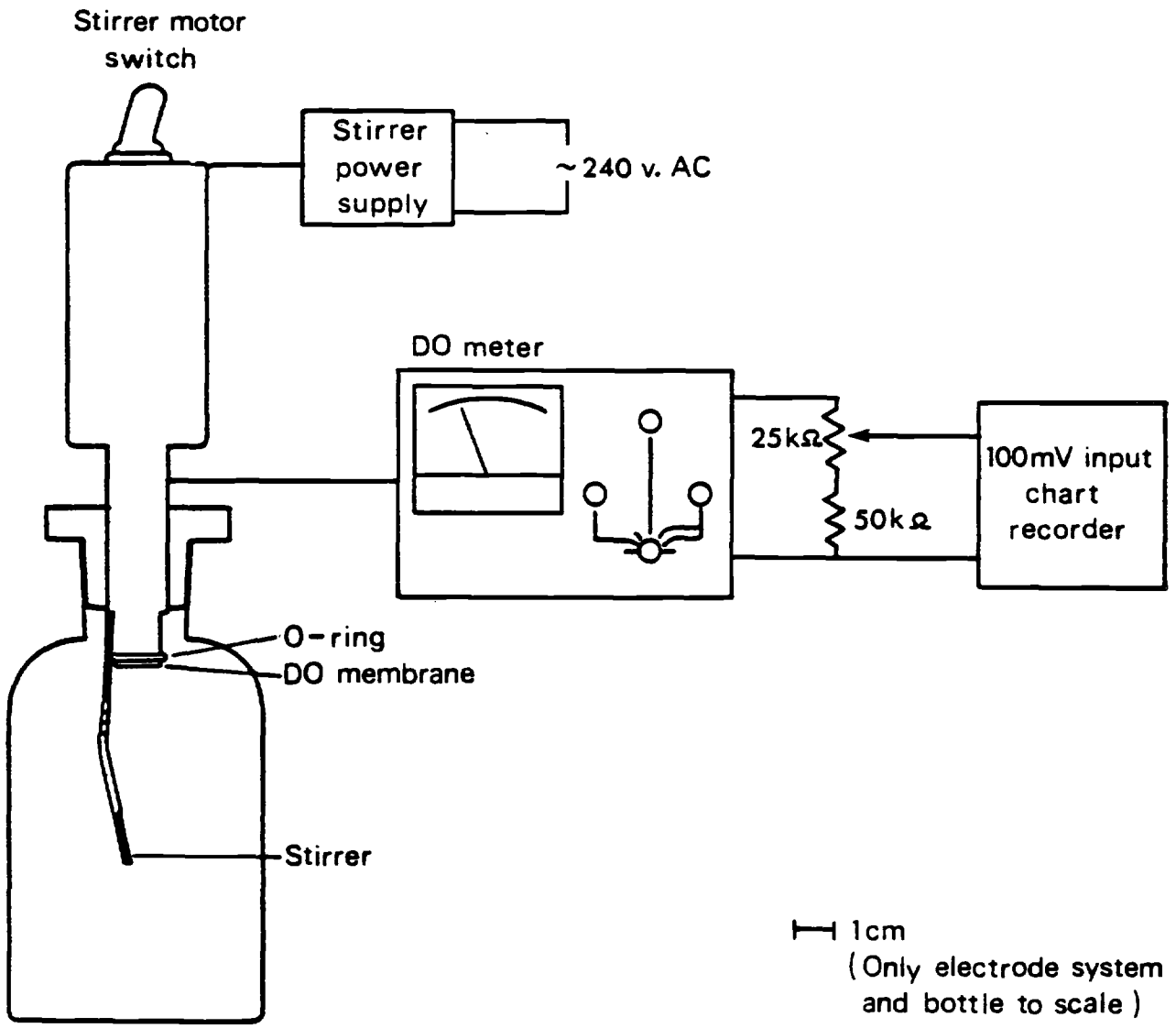
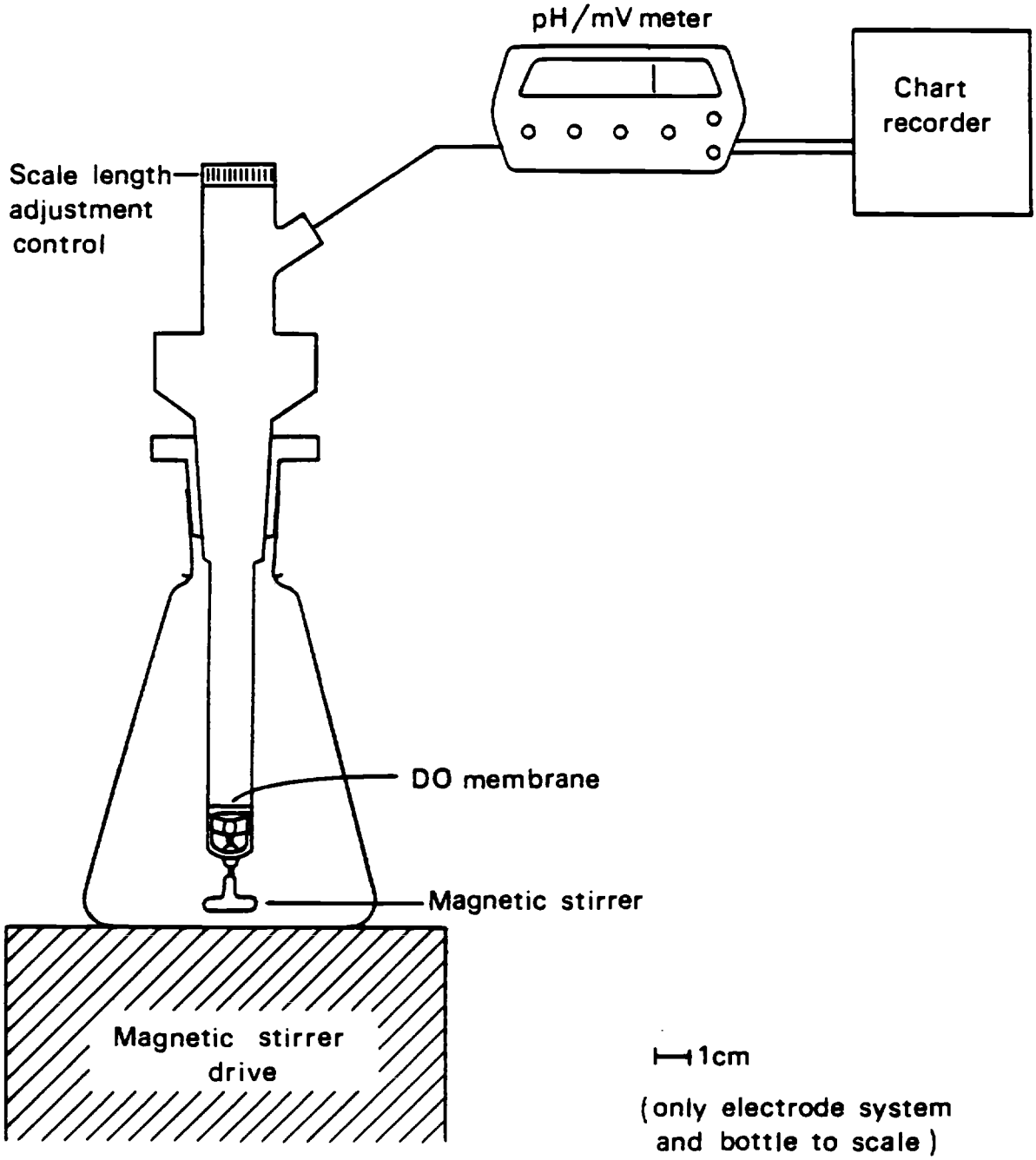


FIG.6 TYPICAL ARRANGEMENT USING EIL TYPE DO ELECTRODE



Step	Procedure	Notes
	Set up the oxygen electrode according to the manufacturer's instructions	
AC.7.1	Calibration of respirometer: zero calibration	
AC.7.1.1	*Rank type*	
	Fill the cell with the zero DO calibration solution (AC5.4), insert the magnet and stopper and start the stirrer motor. Adjust the recorder zero offset control so that the recorder pen is set at scale zero.	
AC.7.1.2	*YSI and IBC types*	
	Fill the bottle or flask with the zero DO calibration solution, insert the probe, and start the stirrer motor.	
	Move the function switch to DO 10 mg/l range. The meter needle should fall to 0.2 mg/l. If it does not, the electrode requires servicing. (Note a.)	(a) If the needle does not indicate zero, do not adjust the zero control to make it indicate zero. Refer to the manufacturer's handbook on electrode servicing.
	Adjust the recorder zero offset control so that the recorder pen is set at zero.	
AC.7.1.3	*EIL type*	
	Fill the bottle or flask with the zero DO calibration solution, insert the probe, and start the magnetic stirrer.	
	Select the pH range and adjust the buffer control until the meter needle indicates zero (note b).	(b) If there is insufficient adjustment on the buffer control to permit this, as is likely to be the case with pH/mV meters other than EIL models 7010/7020, 7030 or 7050, the mV range should be used instead.
	Adjust the recorder zero offset control so that the recorder pen is set at scale zero.	
AC.7.2	Air-saturated water calibration	
	Prepare a sample of clean air-saturated water at the required temperature.	
	Determine the dissolved oxygen concentration in the sample by the method described in this series (note c).	(c) See reference 25.
	(a) *Rank type*	
	Thoroughly rinse the cell and then fill with the air-saturated water, insert the magnet and stopper and start the stirrer motor (note d).	(d) Sensor response varies with stirring speed below a minimum critical value. This value should be determined by increasing the stirrer speed until no increase in meter reading occurs and the reading is steady.
	Adjust the circuit potentiometer 'fine' control until the recorder pen indicates the correct value as determined for DO concentration	
	(b) *YSI and IBC types*	
	Rinse the bottle or flask well and then fill it with clean air-saturated water. Insert the DO probe and start the stirrer. With the function switch on the appropriate DO concentration range, adjust the meter reading using the DO calibration control until the meter needle indicates the correct DO concentration (note e).	(e) The stirrer speed is not adjustable but the standard speed is sufficient to give an optimum response.

Step	Procedure	Notes
	YSI	
	Adjust the circuit potentiometer until the chart recorder indicates the correct DO concentration also.	
	IBC	
	The recorder should give the same reading as the DO meter, if not, the electrode requires servicing.	
	EIL type	
	Rinse the bottle or flask thoroughly and then fill it with clean air-saturated water. Insert the DO probe and start the stirrer motor (note f).	(f) If stirrer speed is too slow, a bumpy recorder trace will be obtained during a respirometry run. Ideally, the trace should be smooth and straight.
	Turn the scale length control on top of the probe fully anti-clockwise and allow the meter reading to stabilise for about five minutes. When a steady reading is obtained, slowly turn the scale length control clockwise until the meter reading corresponds to the dissolved oxygen concentration present in the sample, (note g). The normal scale calibration range is 0–14 mg/l. The chart recorder should now also read the correct value without further adjustment.	(g) If it does not, the recorder input does not match the output signal from pH/mV meter. In this case the recorder sensitivity range should be changed.
	The pH/mV meter should now read the correct value for DO concentration (note h).	(h) This will be the case with EIL meters such as models 7030, 7050. Meters with separate slope/ offset controls will require further adjustment. In this case, the slope control should be adjusted until the meter needle indicates the correct value of DO concentration in the air-saturated water.
	The chart recorder should also read the correct value for DO concentration.	
AC.7.3	**Preparation of Test Samples**	
	Bring the aerated sludge and test solutions to the required temperature and then mix them in the necessary proportions to yield the test samples.	
AC.7.4	**Measurement of respiration rate**	
	Transfer sufficient sample completely to fill the cell, bottle or flask (note i).	(i) The mixed samples should contain at least 5 mg/l of dissolved oxygen and if necessary should briefly be vigorously aerated prior to transfer to the respirometer.
	(a) *Rank type*	
	Insert the stirrer magnet and the stopper (note j).	(j) In all cases a small quantity of sample will be displaced from the respirometer vessel so that air is effectively excluded from the test sample.
	(b) *YSI and IBC*	
	Insert the DO probe.	
	(c) *EIL type*	
	Insert the stirrer magnet and the DO probe.	
	Switch on the stirrer, and the recorder chart drive. Allow the test to proceed until a sufficient chart trace has been obtained to allow the gradient of the line to be determined accurately (note k).	(k) For most purposes, a suitable chart drive speed is 10 mm/min. An adequate recorder trace is normally obtained within 10 minutes. See also note h.

Step	Procedure	Notes
	Caution should be exercised when the uptake exceeds 2 mg/l min, since the response time of the electrode may mask changes in the actual DO, which could underestimate the actual respiration rate.	
	Stop the recorder chart drive. Thoroughly rinse the cell, bottle or flask (note l).	(l) Otherwise cross contamination may occur and cause erroneous results.
AC.7.5	**Determination of MLSS**	
	Determine the MLSS of the relevant samples by the method described in this series (notes m and n).	(m)See reference 27. (n) Care should be taken to ensure the specimens withdrawn for solids determination are drawn from well-shaken liquors..
AC.7.6	**Calculation of results**	
	Measure the gradient of the recorder respiration trace and calculate the result as mg O_2/l.h. Divide this result by the MLSS concentration (g/l) to obtain the specific respiration rate as mg O_2/g h (note o)	(o) The recorder trace may not be linear initially and if a determination continues until the DO concentration falls below 1 mg/l and becomes rate limiting, the slope again deviates from linearity. The central portion of the trace should be used for the calculation of respiration rate.

B Assessing the Biodegradability of Chemicals and Industrial Waste Waters using Respiration Rate Measurements

Introduction

The two respiration methods presented here must be considered only as preliminary tests giving provisional indications rather than final conclusions, although positive oxygen uptake is usually evidence of biodegradation. (Exceptions to this are certain uncouplers of phosphorylation which can stimulate respiration of endogenously respiring sludge but which themselves are not readily biodegraded.) Lack of oxygen uptake, however, cannot be taken as evidence of non-biodegradability and a re-assessment should be made by other methods in this booklet (e.g. methods D, E, F or H).

Inevitably, with some substances and industrial waste waters, overlapping effects can be obtained, that is, at low concentrations they can be oxidized while at higher concentrations they are toxic.

In this booklet, biodegradability is assessed using sludge in the basal, or endogenous, state. While toxic substances may reduce the endogenous respiration rate, sludges are much more sensitive in an actively respiring state. Methods for assessing toxicity and inhibition, using mixtures of sludge and sewage, are given in another booklet (see reference 19).

The two methods which are given in Section B9 are:

Method I *Assessment of Biodegradability for Soluble and partially soluble chemicals.*

Method II *Percentage theoretical Oxygen Uptake.*

B1 Performance characteristics

B1.1	Property determined	Biodegradability of a chemical or a waste water, and percentage of theoretical oxygen uptake.
B1.2	Type of sample	Soluble substances, insoluble substances (see B5.4); mixtures; or industrial waste water.
B1.3	Basis of method	Determination of the effect of test substance on the respiration rate of activated sludge.
B1.4	Range of application	Varying degrees of stimulation of respiratory activity of the activated sludge micro-organisms.
B1.5	Standard deviation	Examples: n = 3 High rate sludge alone 16.0 ± 1.9 mg O_2/g h + aspirin 22.8 ± 2.6 mg O_2/g h Low rate sludge alone 2.32 ± 0.09 mg O_2/g h + caffeine 8.15 ± 0.59 mg O_2/g h
B1.6	Interferences	Substances which may be auto-oxidised. Substances which affect the calibration of the oxygen electrode (see Section B 3).
B1.7	Time required for determination	Operator time method I: 15 min (one determination at one concentration) method II: 2–6 h (or more).

B2 Principle

The effect is determined of the addition of test substance or industrial waste on the respiration rate of activated sludge; the rates are measured using an oxygen-electrode.

Increase in respiration rate of sludges, previously brought to a near endogenous state, usually indicate that the test material is biodegradable. The percentage of theoretical oxygen uptake can be calculated in some cases by measuring the respiration rate of a number of sub-samples of aerated mixtures of sludge and test material at timed intervals until the basal rate is reached.

B3 Interferences

B3.1 Some chemicals may be auto-oxidized and take up oxygen in the absence of micro-organisms, thus appearing to be biodegradable. The presence of this effect can be detected by placing an aerated solution of the test substance in a closed bottle, fitting an oxygen electrode and observing whether oxygen is taken up.

B3.2 Inhibitory substances may be present in the activated sludge collected from a sewage works receiving industrial discharges. If the object of the test is to investigate the effect of a chemical upon the activated sludge of a particular sewage works, then the sludge should not be pretreated. If more general studies are to be made, the sludge may be washed before use. Soluble substances can be removed by centrifugation and washing of the sludge as described in Section B7. Adsorbed substances are more difficult to remove and culturing the sludge with synthetic sewage for several days may be necessary.

Activated sludge may be diluted with sewage effluent, to the suspended solids concentration required for testing, but if the effluent is known, or thought, to contain inhibitory substances tap water may be used instead.

B4 Hazards

B4.1 **Hygiene**

Sewages and derived activated sludges may contain potentially pathogenic organisms, therefore appropriate precautions should be taken when handling these materials.

Vessels containing aerated activated sludge should be loosely covered to avoid splashing and fine spray.

B4.2 **Chemicals**

Test chemicals, or components of the waste waters tested, may be toxic and should be regarded as potentially dangerous; precautions should be taken to avoid contact with skin and clothing, and to avoid breathing dust or vapour.

B5 Reagents

B5.1 **Activated sludge**

Activated sludge is taken from the outlet of the aeration tank of a plant treating domestic sewage or mixed industrial and domestic sewage (see Section B7). It should be kept aerated before use and have a concentration of suspended solids of at least 3 g/l, but for respiration rate measurements it is used at approximately 1.5 g/l.

B5.2 **Solution or suspension of test substance**

Stock solutions of test chemicals are prepared in distilled water, e.g. 1 g/l or 10 g/l. Saturated solutions of less soluble compounds should be prepared.

Further, increases in respiration rate have been observed with suspensions (colloidal and coarser) of such compounds as starch, stearate, cellulose[26], so that this method may also be used to test suspensions of insoluble compounds.

B5.3 **Industrial waste water**

Fresh, representative samples of the industrial waste waters to be tested should be collected from the sites and, if necessary, stored at 1–4°C to avoid changes.

B5.4 **Standard test substances**

Stock solutions of appropriate standards are also prepared; e.g. a 10 g/l solution of glucose, which should be made freshly before use, (glucose is useful for checking the activity of the sludge).

B6 Apparatus

B6.1 **Oxygen electrode**

Suitable oxygen electrodes are made by e.g. Electronic Instruments Ltd (EIL), Yellow Springs Instruments (YSI), International Biophysics Corporation (IBC), etc., and a respirometer-cell by Rank Bros. (see method AC).

B6.2 **Recorder**

A suitable potentiometric recorder for continuous recording of the dissolved oxygen is required.

B6.3 Usual laboratory glassware; including BOD bottles if the oxygen electrode does not include a sample cell.

Note: BOD bottle or sample cell hereafter called the 'electrode vessel'.

B6.4 **Circulatory water bath**

Samples are kept immersed in the water bath before use to avoid temperature variations. It is also used for maintaining the respiring mixture at the correct temperature either directly, or by passage of the water through the jacket of the Rank cell.

B6.5 **Compressed air supply**

Compressed air is passed through a cotton-wool strainer and wash bottle of distilled water, and used for aerating activated sludge samples.

B7 Collection and Preparation of the Activated Sludge

Activated sludge, preferably with a concentration of suspended solids of >3 g/l, is taken from the outlet of the aeration tank of a sewage works or from a unit in the laboratory maintained for the purpose. If necessary the activated sludge may be sieved to remove coarse extraneous material. The concentration of activated sludge should be adjusted to about 3 g suspended solids/l with tap water.

If the activated sludge is obtained from a sewage works receiving industrial discharges, it may be necessary to centrifuge the sludge (after the preliminary settling of coarse solids) e.g. at 1100 g for 10 min, and to wash it with tap water (or an isotonic solution) see Section B3.2, to remove any interfering substances that may be present in the industrial waste water. The washed sludge is recentrifuged and then resuspended in synthetic sewage to give a suspended solids concentration of approximately 3.0 g/l and aerated overnight before use.

The sludge (washed or unwashed) is kept aerated for a few hours at a rate to maintain the solids in suspension and the concentration of dissolved oxygen above 2 mg/l. It is ready for use when the sewage or synthetic sewage has been metabolised and the sludge is in the endogenous (basal) state. If it cannot be used within 24 h, the sludge should be fed daily, e.g. with OECD synthetic sewage, but should not be used until the basal respiration rate has been reached.

The concentration of suspended solids is determined just before use and, if necessary, is diluted with tap water or a mixed salts solution to the required solids concentration.

Care should be taken to see that the sludge is well mixed and aerated when test portions are removed for respiration rate determinations so that representative samples are obtained.

B8 Preparation and Calibration of the oxygen electrode

The oxygen electrode is calibrated according to the manufacturer's instructions (see method AC and Ref 25).

Step	Procedure	Notes
B9.1	Determine the concentration of suspended solids on an aliquot of the activated sludge to be used (note a)	(a) see references 27 and 33.
	Method I	
B9.2	Assessment of Biodegradability	
B9.2.1	*Endogenous or basal respiration rate R_B*	
	Mix a sample of aerated activated sludge (note b) with a concentration of suspended solids of about 3 g/l with an equal volume of aerated tap water (note c). Fill the electrode vessel (bottle or cell) with the well-aerated mixture (note d) close (by fitting the electrode probe and/or stopper) ensuring that no air bubbles are trapped.	(b) Prepared as described in Section 7 and diluted, with tap water or sewage effluent if necessary. If the effect of pH value is being investigated, the sludge should be adjusted beforehand to the required value. (c) Aeration tends to cool the sludge and water so they should be placed in a water bath some time before use. (d) If the mixture is not well aerated the concentration of dissolved oxygen will soon reach zero.
	Stir at the pre-set speed (note e) and record the decrease in concentration of dissolved oxygen at a suitable chart speed, e.g. 600 mm/h, for about 5 min, or until a suitable length of trace has been obtained so that the gradient may be measured.	(e) The speed of stirring affects the measurements of dissolved oxygen, therefore the optimum speed should be determined beforehand and kept unchanged during the test.
	For adequately-soluble chemicals, proceed to B9.2.3	
	For less-soluble chemicals and for industrial wastes, proceed to B9.2.2 and then B9.2.4.	
B9.2.2	Empty out the electrode vessel, and repeat the procedure on another portion of mixture in B9.2.1 (note f).	(f) The procedure is repeated to obtain the required number of replicates, usually 3.
B9.2.3	*Respiration rate with soluble chemicals, R_S* When the basal rate has been measured (note g) and with the same sludge mixture add (note h) the required amount of solution of test substance (note i), allow to mix, re-close the electrode vessel and continue to record the fall in the concentration of dissolved oxygen after addition of the test substance for a suitable length of time.	(g) As described in B9.2.1 (h) A syringe or pipette may be used to introduce the test substance into the mixture in the electrode vessel. Alternatively, the sludge and test substance may be mixed separately and a portion of this placed in the electrode vessel.
	When a satisfactory trace has been obtained (note j) empty the electrode vessel and rinse with distilled water. Repeat the whole procedure (B9.2.1 and B9.2.3) as required to obtain replicate values.	(i) A range of concentrations may have to be tried; 10–100 mg/l is normally suitable. If more than 10% of the volume is added, a control with the same volume of tap water should be tested to allow for dilution of the sludge. (j) Any interference in the trace caused by the addition should be ignored.
B9.2.4	*Respiration rate with industrial wastes and less-soluble chemicals R_S*	
	Dilute the required volume (note k) of industrial waste, or sparingly-soluble test substance, to a standard volume (e.g. 250 ml) with tap water, and aerate.	(k) A range of volumes may have to be tried. For industrial wastes a ratio of 1:1 (v/v) (waste: sludge) is normally the maximum needed.

Step	Procedure	Notes
B9.2.4 contd	Add to an equal volume of aerated activated sludge with about 3 g suspended solids/l. Mix thoroughly and aerate.	
	Fill the electrode vessel with well aerated mixture and record the respiration rate (note 1) as described in B9.2.1.	(l) If the test substance or industrial waste is readily biodegraded, the maximum respiration rate will be achieved immediately after mixing and will fall rapidly with time, especially if the concentration of limiting substate(s) is not well above the saturation constant.
B9.2.5	When a satisfactory trace has been obtained, empty the electrode vessel and rinse with distilled water.	
	Repeat B9.2.4 as required (note m) to obtain replicates.	(m)The whole procedure B9.2.4 must be repeated because the respiration rate of the original mixture may have fallen (note 1).
B9.3	**Method II** **Determination of Percentage Theoretical Oxygen Uptake**	
B9.3.1	Place 1 l of aerated activated sludge (note n) into a 3 l beaker and continue aerating.	(n) Prepare the activated sludge as described in Section B7; it should contain about 3 g suspended solids/l.
	Determine the basal respiration rate on a sample of this sludge (note o) and return it to the beaker. Add 1 l of a solution of test substance so that a final concentration of 100 mg/l is obtained or 1 l of industrial waste or of diluted industrial waste (note p)	(o) As described in B9.2.1. (p) 100 mg/l is convenient but if the substance is toxic a lower concentration may have to be used.
	Mix thoroughly, note the time and continue aerating. Immediately, transfer a sample to the electrode vessel and determine the respiration rate, (B9.2.1) noting the time after mixing.	
	Replace this sample in the beaker and continue aerating. Determine the respiration rate at timed intervals (note q) until the rate has returned to the basal level previously determined.	(q) Determine the repiration rate at, say, 15 min intervals at first. The time interval may be increased when there is little change in the respiration rate.

B10 Expression of Results

B10.1 *Method of calculation*

The respiration rate is calculated from the slope of the straight line portion of the respiration curve traced on the chart recorder and is expressed as mg oxygen/l min or mg oxygen/l h. (Only exceptionally will respiration curves have no linear portion, e.g. at initial concentrations of test substance at or below its "saturation" constant, especially in the presence of very active sludges.)

The specific respiration rate is taken as the mean of replicate (usually 3) values calculated from the following formula:–

$$R = \frac{\text{Respiration rate (mg/l h)}}{\text{concentration of suspended solids (g/l)}}$$

$$= \text{mg oxygen/g solids h.}$$

B10.2 **Method I**

If there is a statistically significant increase in the specific respiration rate, R_S, in the presence of the test substance or industrial waste compared with the basal rate R_B, the test material is biodegradable. This can be checked by a comparison of means using a 't' test.

The relationship between the concentration of a biodegradable substance and the increase in respiration rate above the endogenous rate is normally hyperbolic, obeying the enzyme kinetics of Michaelis-Menten. This is characterised by the existence for each substance of a concentration ("saturation" constant), usually below 20 mg/l, at which the respiration rate increase is one-half of the maximum rate obtained when excess of the substance is present. The relationship is more complex for substances which show the dual properties of biodegradability and toxicity.

B10.3 **Method II**
Determination of Percentage of Theoretical Oxygen Uptake

Respiration rates obtained under 9.3 are plotted against time and curves of the general shape shown in Fig. 7 are obtained. The area under the curve from the time of addition of the substance or industrial waste to the sludge until the rate reaches the endogenous value is equivalent to the oxygen taken up in oxidising the added organic matter plus that due to endogenous metabolism. The latter amount is allowed for by determining the area of the shaded portion only. Let the oxygen taken up in oxidizing the added organic matter be equivalent to Q mg oxygen/g solids. Thus the total oxygen taken up

$$= Q \times MLSS \times V \text{ g}$$

where MLSS = concentration of suspended solids (g/l)
and V = volume of activated sludge

For a pure substance, added at initial concentration C and having a theoretical oxygen requirement of T g/g, the total theoretical requirement
$= T \times C \times V$ g so that proportion of theoretical oxygen uptake exerted

$$= \frac{Q \times MLSS \times V}{T \times C \times V} = 100\%$$

$$= \frac{Q \times MLSS}{TC} \times 100\%$$

Note: The theoretical oxygen uptake of a substance may be calculated, assuming that the substance is completely oxidised to CO_2, water etc. e.g. for glucose:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$$

$$\therefore \quad T = \frac{\text{No. of oxygen atoms} \times \text{MW of oxygen}}{\text{MW of substance}}$$

for glucose

$$= \frac{12 \times 16}{180} = 1.07 \text{ g oxygen/g glucose}$$

For a mixture or industrial waste

In this case the theoretical oxygen requirement cannot be calculated and the COD value of the waste, as finally diluted in the sludge-waste mixture, should be substituted, so that

$$\% \text{ COD taken up} = \frac{Q \times MLSS \times V}{C' \times V} \times 100\% = \frac{Q \times MLSS}{C'} \times 100\%$$

Where C' = COD of waste water, or mixture, after allowing for dilution with sludge.

NB Since the test substance may be converted to new cells as well as oxidized to CO_2, a low percentage of theoretical oxygen uptake does not necessarily mean that the test substance has not been fully biodegraded. (See Introduction to the methods.)

B11 Sources of Error

B11.1 To avoid cross contamination, the electrode vessel must be carefully rinsed out after each test substance or sodium sulphite has been used.

B11.2 The concentration of suspended solids affects the respiration rates obtained with biodegradable substances since the ratio of food: micro-organisms, or inhibitor: micro-organisms is a critical factor. Therefore a particular solids concentration (1500 mg/l) is chosen and this is used for all measurements in order to achieve comparability.

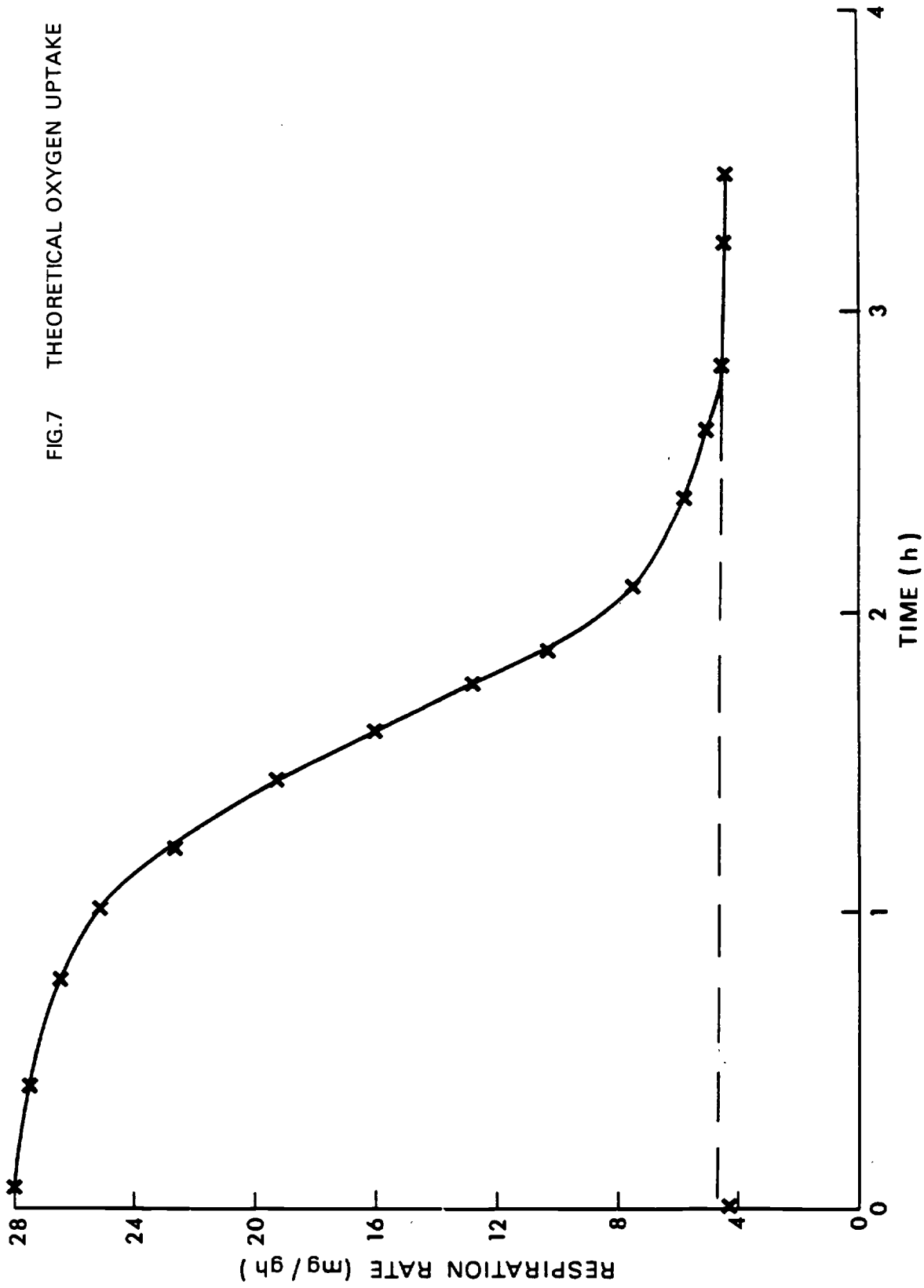


FIG.7 THEORETICAL OXYGEN UPTAKE

B11.3 The percentage of theoretical oxygen uptake is not always a true reflection of the biodegradability of a substance, since it is possible, though rare, to achieve no biodegradation while observing oxygen uptake. The percentage of theoretical uptake is usually significantly below 100% because some of the carbon and hydrogen is not fully oxidized but is converted into new cellular material.

B12 Checking the Accuracy and Validity of Results

The accuracy of the biodegradability method can be checked by carrying out a number of replicates for each concentration of test substance (e.g. 3), and taking the mean respiration rate calculated from these. Outliers are disregarded. The validity of the results can be checked using a standard substance e.g. glucose. If its respiration rate falls within experimental error of the expected result (a net rate of 20–30 mg/g h for 100 mg/l glucose), the sludge is showing normal activity, and the respiration rates for the test substances are considered to be valid.

C Assessment of Biodegradability by means of a Manometric Respirometer

Introduction

This method offers some advantages over others; it requires little attention and no chemical analyses, although DOC can be determined, additionally, at the beginning and end of incubation. At least qualitative results can be obtained with insoluble compounds and the test may be used to assess toxicity to bacteria. Among disadvantages are the rather high concentration of test substance needed and the possible interference from nitrification. As with other tests, abiotic degradation may be assessed and should be corrected for by the use of suitable tests under sterile conditions.

This method is very similar to the standard Japanese method[28] for ready biodegradability.

C1 Performance Characteristics

C1.1 Property determined	Biodegradability by means of oxygen uptake
C1.2 Types of sample	Organic compounds which are non-volatile and soluble to at least 100 mg/l; may be less reproducible for insoluble chemicals.
C1.3 Basis of method	Comparison of the increased oxygen uptake obtained (over a period of not more than 28 days) by adding a known amount of test substance to a mineral medium, inoculated with activated sludge, with the theoretical (or chemical) oxygen demand.
C1.4 Limit of detection	For electrolytic respirometer – oxygen uptake – 1 mg oxygen (see Method AA), for Hach manometer – 1–5 mg oxygen, depending on sample volume (see Method AB).
C1.5 Precision	Varies, but usually RSD of O_2 uptake ± 10% (see Method A).
C1.6 Interferences	Any substance inhibitory to bacterial growth; substances interfering in the rate of oxygen transfer. Nitrification (see Section C3).
C1.7 Time required for test	Overall – up to 28 days. Operator time 20 h per run up to 6 units. (Includes preparation of solutions and cleaning apparatus.)

C2 Principle

A measured volume of inoculated medium containing a known amount of test substance is stirred in a closed flask and the consumption of oxygen is determined either by measuring the quantity of oxygen required to maintain constant gas volume in the respirometer flask, or from the change in volume or pressure in the apparatus. Evolved CO_2 is absorbed in a solution of potassium hydroxide.

The amount of oxygen taken up by the substance (corrected for blank) is expressed as a percentage of the COD or theoretical oxygen demand calculated from the formula of the compound.

C3 Interferences

C3.1 Toxic materials may cause inhibition and care should be taken to ensure that they do not enter the system except by design. Examples of materials which may be present adventitiously are chlorine from tap water and chromate from glass cleaning operations. No special precautions, other than rinsing with distilled water, are needed to ensure removal of these substances.

C3.2 If silicone grease is used on ground glass joints care should be taken to avoid its entering the main body of the flask as it will cause interference by reducing the rate of oxygen transfer.

C3.3 Oxidation of ammonium salts (nitrification) present in the medium and formed from nitrogen-containing organic compounds can cause abnormally high oxygen uptakes, especially towards the end of incubation when it may be detected as an increased slope in the respiration curve after a plateau has been reached.

C4 Hazards

C4.1 **Hygiene**

Activated sludges derived from sewage may contain potentially pathogenic organisms, therefore appropriate precautions should be taken when handling them to avoid the risk of infection.

C4.2 **Chemicals**

Precautions should be taken if the test substance is thought to be toxic, or if its properties are unknown.

C5 Reagents

C5.1 **Water**

Distilled or deionised water is used.

C5.2 **Activated sludge**

Activated sludge is collected from a treatment works or a laboratory unit receiving municipal sewage which contains no single major industrial waste water. The sample is taken preferably from the end of the aeration tank or return sludge line so that little external substrate remains and the microorganisms are in the 'endogenous' phase: this will give the largest possible difference between the oxygen uptake of the sludge alone and in the presence of the test substance. If the sludge is actively respiring on external substrate, it can be brought to the 'endogenous' phase either by aerating for a few hours before use or by centrifuging, washing with medium (C5.5), re-centrifuging and resuspending in the medium. This latter treatment should be applied if it is suspected that the sludge contains inhibitory matter.

The 'endogenous' respiration rate of activated sludge decreases slowly with time, but taken over a period of several days the rates are usually in the range 2–6 mg O_2/g solids h. This can readily be checked in a few minutes from measurements using an oxygen electrode respirometer (Method B) and by determination of the suspended solids concentration by a standard method. Once obtained in the 'endogenous' phase, the sample of sludge should be kept aerated and used within 24 hours. (If an acclimatized inoculum is required, sludge should be taken from a laboratory-scale plant treating sewage containing the test substance or from a semi-continuous activated sludge (SCAS) vessel (Method H) receiving the substance for a period, usually up to 3 months.)

A concentration of 30 mg suspended solids/l, giving an oxygen uptake of about 2 mg/d per litre of medium over the first ten days or so, has been found suitable for concentrations of test substance in the 50–150 mg/l range.

C5.3 **8% Sodium hydroxide solution (w/v).**

C5.4 **20% Potassium hydroxide solution (w/v).**

C5.5 **Medium**

Chemicals should be of analytical grade.

A suitable medium may be prepared from the following solutions:–

(a) 21.75 g dipotassium hydrogen phosphate
8.5 g potassium dihydrogen phosphate
33.4 g dibasic sodium phosphate dihydrate
5.0 g ammonium chloride
dissolved in 1 l distilled water (C5.1).

(b) 22.5 g magnesium sulphate heptahydrate dissolved in 1 l distilled water (C5.1).

(c) 36.4 g calcium chloride dihydrate dissolved in 1 l distilled water (C5.1).

(d) 0.25 g ferric chloride hexahydrate dissolved in 1 l distilled water (C5.1).

The final medium (C7.5) contains 3 ml each of solutions A, B, C and D per litre of deionized or distilled water (C5.1) and contains 4 mg N/l and phosphate at 0.9375 m M.

Sufficient trace elements and vitamins are added with the activated sludge inoculum.

C5.6 **Stock solution of test substance** – For soluble substances preferably 5 g/l in distilled water (C5.1) but not lower than 0.1 g/l.

For sparingly soluble and insoluble substances weigh out the appropriate amount of test substance and suspend it directly in the nutrient solution.

C5.7 **Stock solution of glucose or sodium acetate** 5 g/l in distilled water (C5.1).

C6 Apparatus

C6.1 **Suitable respirometers** are the Hach, electrolytic (see Method A), the Sapromat[29] and that of the Japanese Ministry of International Trade and Industry (MITI)[28].

C6.2 **Water bath or constant temperature incubator**

C7 Test Procedure

Step	Procedure	Notes
7.1	Preparation of Apparatus Assemble the reaction vessels according to the manufacturer's instructions (see method A) and place them in the constant-temperature water-bath, or in a constant-temperature room.	
C7.2	Preparation of Activated Sludge Collect a sample of activated sludge and prepare it, as described in Section C5.2, to be in the 'endogenous' state and of the appropriate concentration. Place the sludge in the water-bath and aerate until required (note a).	(a) The inoculum should be a small proportion of the total volume; the concentration of suspended solids should be in the range 2–4 g/l.
C7.3	Characterisation of Test Substances Determine the COD and, if not known, the DOC of the substance to be tested.	
C7.4	Prior Assessment of Toxicity of Test Substance If this step is required, determine the respiration rate of the sludge in an oxygen electrode respirometer and add the test substance at the required concentration during the measurement (note b). The degree of inhibition is indicated by the reduction in the respiration rate.	(b) See method B.

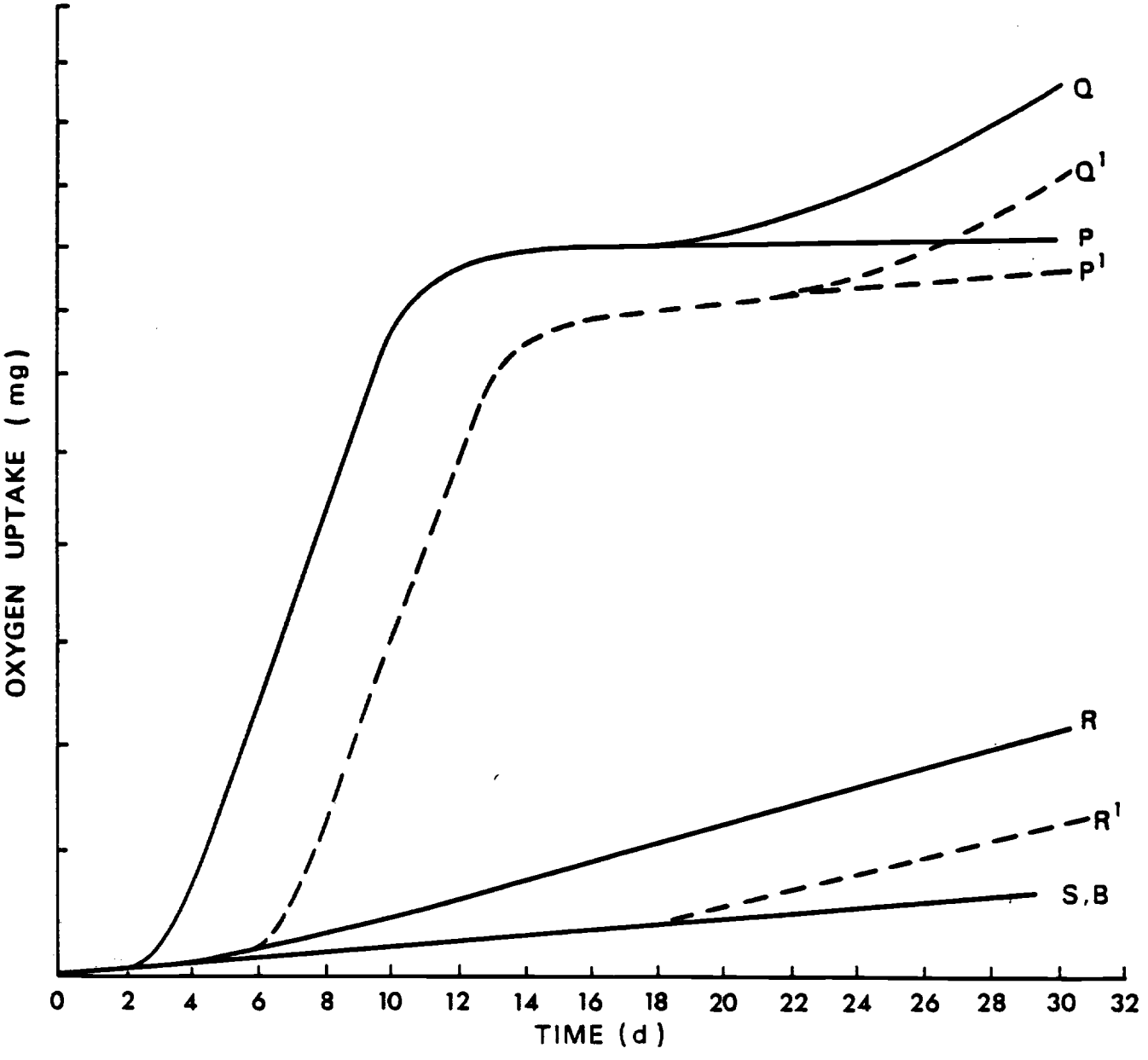
Step	Procedure	Notes
C7.5	Preparation of vessels	
C7.5.1	Calculate the amounts of distilled water (C5.1) required for each vessel (see Table 4 for examples) and add these amounts. Add appropriate amounts of each nutrient solution A, B, C and D (C5.5) to each vessel. Add sufficient volume of stock solution of test substance (C5.6) to one pair of vessels (3,4; Table 4) to give the required test concentration e.g. 100 mg/l (this should be shown to be non-inhibitory (C7.4)). Similarly, to check the activity of the inoculum, add sufficient volume of a stock solution of a standard substance (C.5.7) (glucose or acetate) to a second pair of vessels (5,6; Table 4) to give the required test concentration e.g. 100 mg/l.	
	A third pair of vessels (1,2; Table 4) are controls (note c).	(c) If further flasks are available, other concentrations of test substance may be assessed and/or its toxicity may be determined (Table 4).
	If the physico-chemical uptake of oxygen is required, include a sterile vessel and water containing test substance (but no inoculum) which has been sterilised by membrane filtration.	
C7.5.2	*Assessment of Toxicity of Test Substance*	
	If the toxicity of test substance is to be determined, add to a pair of vessels (7, 8; Table 4) stock solutions of both test substance and standard to give 100 mg/l of each.	
C7.5.3	*Measurement of Oxygen Uptake*	
	Inoculate the vessels (except the physico-chemical control) with the required volume of activated sludge (note d) and start the measurement of oxygen uptake.	(d) A final concentration of 30 mg suspended solids/l is required.
C7.6	Continuation of Test	
	Usually no further attention is required other than to take the necessary readings and make occasional checks to see that adequate stirring is maintained. Stop the experiment, when desired, usually after no more than 28 d (note e).	(e) If biodegradation has commenced but not reached a plateau, the test period may be extended.

C8 Expression and Interpretation of Results

Calculate the oxygen uptake values from the readings obtained as described in method A.

Plot the results as oxygen uptake against time (Fig. 8). Essentially four types of curve can be obtained. Curve P (Fig. 8) asymptotes to an uptake value indicating a biodegradable substance, while curve Q has a plateau, with a further rise, indicating a biodegradable substance with the occurrence in the latter of cryptic growth, or, more likely, nitrification, which can be investigated by appropriate analysis. Curve R is a slowly rising curve indicating a slowly degradable substance and curve S is more or less identical with the control (no test substance, curve B) indicating a non-biodegradable compound. Curves P, Q and R could be preceded by lag phases (Curve P', Q' and R') indicating that acclimatization of the bacterial population had occurred.

FIG.8 TYPICAL OXYGEN UPTAKE CURVES



C9 Calculation of Results

For curves of the type P, R and S:
read off the oxygen uptake after 28 d = BOD
read off the oxygen uptake of blank (curve B) after 28 d = B
For curves of the type Q:
read off the oxygen uptake at the plateau = BOD
read off the oxygen uptake of blank at this time = B

The percentage oxygen uptake of theoretical oxygen demand (TOD) or of COD, is calculated as

$$\frac{\text{BOD}-\text{B}}{\text{TOD or COD}} \times 100$$

for both best substance and standard.

If the percentage of theoretical oxygen uptake for the standard is not ± 10% of the expected value (e.g. 60% for glucose) inspection of the curves may reveal that either the inoculum blank was too high or that the inoculum was inactive. In either case, the assessment should be repeated.

C10 Toxicity

If the percentage oxygen uptake of the standard in the presence of the test substance is significantly lower than the normal value, the test substance can be said to be toxic to the sludge bacteria. In this case the experiment should be repeated using a lower concentraion of test substance.

Table 4 Typical scheme for preparation of reaction mixtures
Total volume of liquid 1000 ml†

Flask	Additions						
	Distilled water (C5.1) (ml)	Nutrient solutions (C5.5–A,B,C and D) (ml)	Stock solution of substance (C5.6)*		Stock solution of standard (C5.7)		Inoculum‡ (C5.2)
			Volume (ml)	Final concn. (mg/l)	Volume (ml)	Final concn. (mg/l)	
1, 2 Control	978		0	0	0	0	10
3, 4 Test 1	958		20	100	0	0	10
5, 6 Standard	958	3 of	0	0	20	100	10
7, 8 Toxicity 1	938	each	20	100	20	100	10
9, 10 Test 2	958		10	50	0	0	10
11, 12 Toxicity 2	948		10	50	20	100	10

* If the test substance is not sufficiently soluble (i.e. about 5 g/l), a larger volume (up to 500 ml) of a weaker solution (down to about 0.1 g/l) can be used, reducing the volume of water added correspondingly.

† or pro rata according to the size of the respirometer vessel.
‡ assuming concentration to be 3 g suspended solids/l.

D Screening Test for the Determination of Biodegradability by measurement of CO_2 Evolution (Sturm)

Introduction

This procedure is based on the CO_2 evolution method of Sturm[30.15] and a modified version has been described by the OECD[31].

Since it does not employ analysis of the test compound, it is useful for assessing the biodegradability of insoluble as well as soluble organic chemicals, though the amount of biodegradation achieved for insoluble materials will depend on the effectiveness of the agitation to keep the material in suspension and therefore available for bacterial attack. The production of CO_2 from an organic compound necessarily means that the compound has been broken down, so that comparison of the calculated theoretical CO_2 production with that observed gives a measure of the extent of biodegradation. However, the theoretical value (i.e. 100% biodegradation) will never be reached even with readily biodegradable substances, since only a proportion of the test substance is used in respiration for energy, while some breakdown products are incorporated as new cells, which, in the duration of the test (28 d) will not be broken down.

D1 Performance Characteristics

D1.1	Property determined	The biodegradability of an organic compound, expressed as CO_2 produced as a percentage of the theoretical production.
D1.2	Type of sample	Organic compounds which are soluble or insoluble, non-volatile and not inhibitory to micro-organisms at the concentration used (10–20 mg/l).
D1.3	Basis of method	Measurement of the amount of CO_2 produced when the test compound is incubated with micro-organisms under aerobic conditions.
D1.4	Precision	Glucose at 20 mg/l n = 6 86.8 ± 4.1% of theoretical Other examples: (duplicates)

	% theoretical CO_2 evolution	
	10 mg C/l	20 mg C/l
Aniline	48, 108	68, 51
Glucose	34, 88	— —
4-nitrophenol	92,118	81, 78
diethylene glycol	9, 5	24, 37
tetra-propylene benzene sulphonate	4, 3	3, 21

D1.5	Interferences	Inhibitory or volatile substances (see Section 3)
D1.6	Time required for test	28 days Operator time: approx 50 h for 10 individual tests.

D2 Principle

The test compound is dissolved, or suspended, in an inorganic medium at concentrations of 10 and 20 mg/l. The medium is inoculated with a relatively large number ($10^5 - 10^6$/ml) of micro-organisms from a mixed population and aerated at constant temperature. By measuring the amount of carbon dioxide produced and comparing the observed values with the theoretical yields calculated from a knowledge of the carbon content of the test compound, a measure of the ultimate biodegradability can be made.

D3 Interferences

Substances or their breakdown products having an inhibitory or toxic effect on the biological system will give falsely low results.

Gaseous or volatile substances may interfere in the estimation of carbon dioxide in effluent gas.

D4 Hazards

D4.1 Hygiene

Where an inoculum originating from an effluent of sewage treatment is used, precautions should be taken to avoid the risk of infection from potentially pathogenic micro-organisms present in the unknown population.

D4.2 Chemicals

Test chemicals may be toxic and should be regarded as potentially hazardous; precautions should be taken to avoid contact with the skin and clothing and to avoid breathing dust or vapours.

Barium hydroxide is extremely poisonous (S1) and must be handled with great care.

D5 Reagents

D5.1 Deionized or distilled water free from toxic substances particularly metals such as copper.

D5.2 Test medium

Reagents should be of analytical grade.

The following stock solutions (a) to (e) are required.

(a) *Calcium chloride dihydrate* 36.4 g dissolved in 1 l water (D5.1).
(b) *Magnesium sulphate heptahydrate* 22.5 g dissolved in 1 l water (D5.1).
(c) *Ferric chloride hexahydrate* 0.25 g dissolved in 1 l water (D5.1).
(d) *Phosphate buffer:–*

potassium dihydrogen orthophosphate	8.5 g
dipotassium hydrogen orthophosphate	21.75 g
disodium hydrogen orthophosphate dihydrate	33.4 g
ammonium chloride	1.7 g

dissolved in 1 l of water (D5.1)

(e) *Ammonium sulphate* 40 g dissolved in 1 l water (D5.1).

Stock solutions (a) to (e) should be stored in the dark, preferably in a refrigerator, and discarded at the first sign of sediment, turbidity or biological growth.

The final test medium should contain per litre of water (D5.1)

1 ml solution (a)
1 ml solution (b)
4 ml solution (c)
2 ml solution (d)
1 ml solution (e)

The pH of the medium should be 7.2 ± 0.2

D5.3 Stock solutions of test substances

For soluble substances prepare a 1 g/l solution of the test compound in distilled water (D5.1). If the pH of this solution is outside the range 3–10, it should be adjusted with HCl or NaOH to pH 7 ± 1.

For sparingly soluble substances 4 litres of a 10 or 20 mg/l solution of the test compound are prepared and an appropriate volume of each of the nutrient solutions (a) to (e) added to the mixture (see D5.2).

For insoluble substances, weigh out the required amount and add this to each test bottle as appropriate.

D5.4 **Reference compound**

Prepare a 1 g/l solution of the reference compound (e.g. dextrose) in distilled water (D5.1).

D5.5 **Standard Barium Hydroxide Solution** (0.0125M)

Dissolve 4.0 g of barium hydroxide octahydrate in 1 litre of water in a stoppered flask. Filter through paper and seal the clear filtrate, preferably with a soda lime guard tube, to prevent adsorption of CO_2 from the air. The solution is standardised against 0.05M hydrochloric acid using phenolphthalein as an indicator.

D5.6 **Standard Hydrochloric Acid** (0.05M)

Dilute 4.5 ml of concentrated hydrochloric acid to 1 litre with distilled water. Standardise using sodium carbonate (AR).

D5.7 **Sodium hydroxide** (10M)

Prepare sufficient sodium hydroxide (10M) for use in the CO_2 absorbers if compressed air is to be used.

D5.8 **Inoculum**

Activated sludge freshly collected from a plant treating predominantly domestic sewage is aerated for 2–4 h to ensure the removal of soluble substrate. A portion of this (e.g. 500 ml) is homogenised for 2 min in a blender, settled for 30 min and the supernatant decanted. If the supernatant contains high levels of suspended solids after settling for 30 min, allow further time for settling before decanting. Sufficient supernatant is required for a 1% inoculum for each test flask.

D6 Apparatus

D6.1 **CO_2 scrubbing apparatus (see figure 9)**

To remove CO_2 from compressed air the following is required for up to 12 units.

4 × 1 l plastic bottles containing approximately 700 ml 10M sodium hydroxide
1 × 1 l flask containing approximately 700 ml of 0.05M barium hydroxide
1 × 1 l flask as a liquid trap.

These bottles are connected in series to the air supply using plastic tubing. A suitable manifold is required to supply CO_2-free air to each test bottle. The gas flow to each culture vessel is controlled by a capillary tube (20 cm long, 0.25 mm bore) inserted between the manifold and the culture vessel.

D6.2 **CO_2-free gas (alternative to D6.1)**

To avoid removing CO_2 from compressed air, bottled nitrogen and oxygen gases may be used mixed in the ratio 4:1. The flow of each gas should be controlled by needle valves and suitable flow meters.

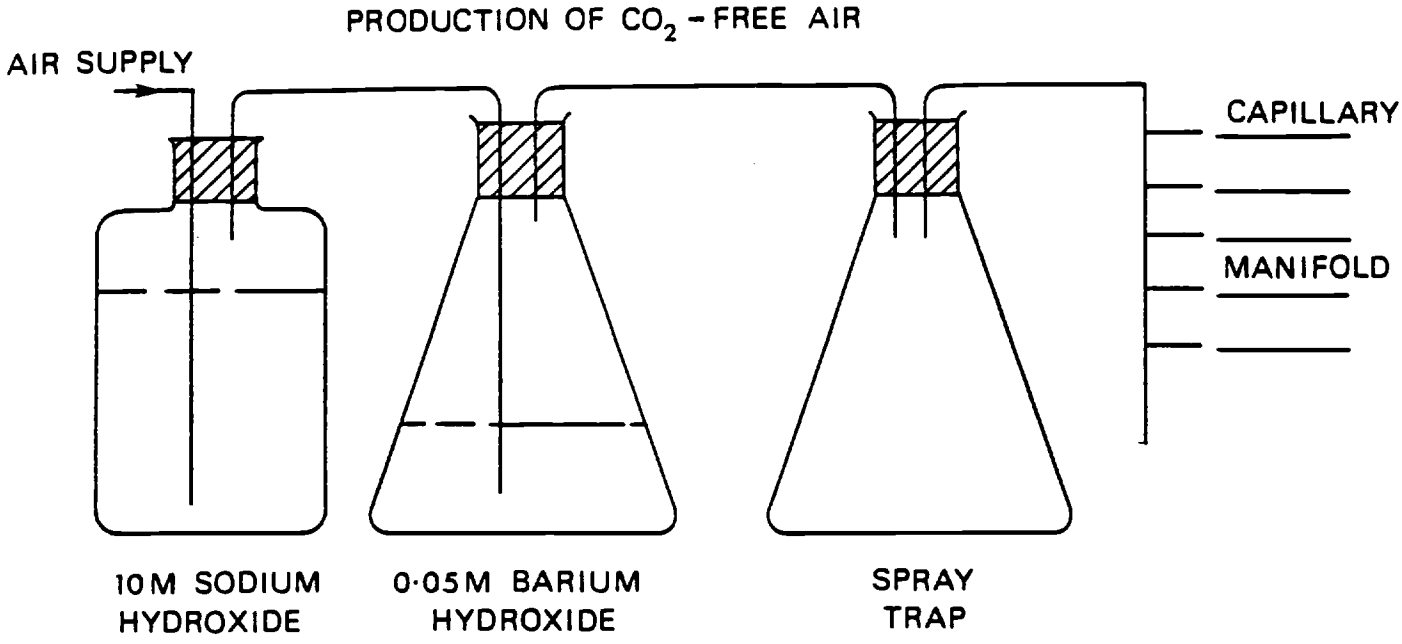
D6.3 **CO_2 production and absorption apparatus**

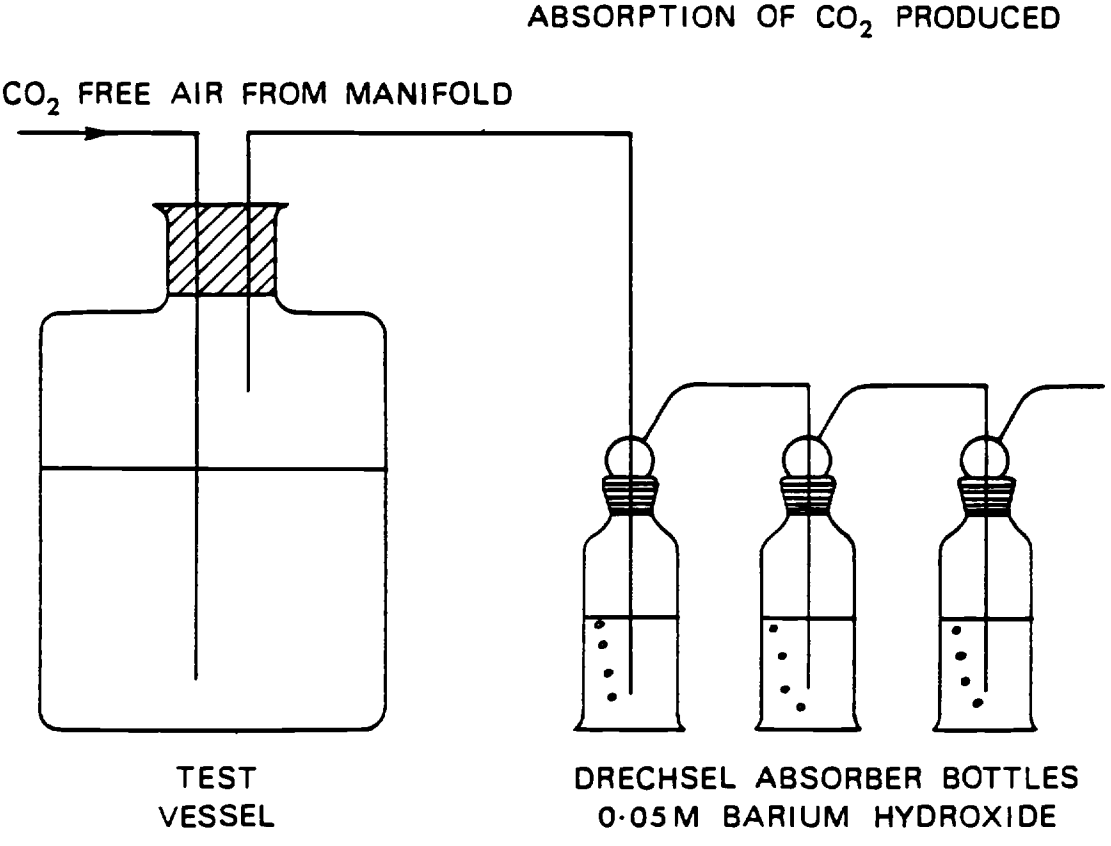
5 l bottles (two for each test material and controls).
100 ml absorber bottles (3 for each test bottle, plus adequate spares for changing absorber solutions).

The absorbers are connected in series to the outlet of each test bottle with plastic tubing.

D6.4 **Suitable blender**

FIG.9


PRODUCTION OF CO_2 - FREE AIR
AIR SUPPLY
CAPILLARY
MANIFOLD
10M SODIUM HYDROXIDE
0·05M BARIUM HYDROXIDE
SPRAY TRAP


ABSORPTION OF CO_2 PRODUCED
CO_2 FREE AIR FROM MANIFOLD
TEST VESSEL
DRECHSEL ABSORBER BOTTLES
0·05M BARIUM HYDROXIDE

Step	Procedure	Notes
D7.1	To a series of 5 l bottles add 3750 ± 25 ml of water (D5.1) and add the following:– 1. 4 ml of calcium chloride solution (a) (D5.2). 2. 4 ml of magnesium sulphate solution (b) (D5.2). 3. 16 ml of ferric chloride solution (c) (D5.2). 4. 8 ml of phosphate buffer (d) (D5.2). 5. 4 ml of ammonium sulphate solution (e) (D5.2). 6. 40 ml of inoculum (D5.8).	
D7.2	Assemble the apparatus as shown in Figure 9, adding 100 ml of 0.0125M barium hydroxide to each absorber bottle.	
D7.3	Aerate the test culture at 50–100 ml/min with CO_2–free gas for a period of 24 h (note a) at 24 ± 3°C in the dark.	(a) This purges the solution and the equipment of carbon dioxide. It is not necessary to titrate the barium hydroxide at this stage.
D7.4	Test substances Add 80 ml of the stock solutions of test substances (D5.3) to individual bottles respectively to give a concentration of 20 mg/l. To another set similarly add 40 ml of stock solutions of test substances to give 10 mg/l. Add water (D5.1) to make all volumes up to 4 l (note b). *Reference substance* Add 80 ml of the stock solution of reference compound (D5.4) to each of two bottles and add water (D5.1) to make the volume up to 4 l. *Controls* Add water (D5.1) to a further two bottles to make the volume up to 4 l.	(b) For sparingly soluble and insoluble compounds see section D5.3.
D7.5	Recharge the proximal absorber with 100 ml of fresh barium hydroxide and restart the air flow.	(c) Titrations should be made on the first absorber solution, as needed, that is, before any $BaCO_3$ is evident in the second trap, approximately every other day for the first 10 days, then every fifth day until day 25. (d) It is essential to release the pressure in this way to prevent barium hydroxide being forced into the culture vessel.
D7.6	After the required time interval (note c) stop the air supply to the apparatus and remove the bungs in the absorbers starting with the trap farthest from the culture vessel (note d).	
D7.7	Remove the absorber bottle nearest to the culture vessel, pipette 20 ml of the solution into a conical flask (note e) and titrate with standard hydrochloric acid using phenolphthalein indicator.	(e) Barium hydroxide is extremely poisonous and a pipette bulb should always be used.
D7.8	Repeat the titration with a second aliquot of barium hydroxide solution.	

Step	Procedure	Notes
D7.9	Pipette 100 ml of fresh barium hydroxide into a clean dry absorber (note f), move the two remaining bottles one position closer to the culture vessel and complete the absorber series by placing a recharged absorber in the vacant position. The air flow is then re-started.	(f) The residual barium hydroxide is discarded and any barium carbonate deposits dissolved by washing with dilute hydrochloric acid. The absorber is thoroughly rinsed with distilled water to remove acid and dried.
D7.10	Repeat steps D7.6–D7.9 as necessary until day 27 (note g). On the 27th day of the test add 1 ml of concentrated hydrochloric acid to each culture vessel (note h).	(g) The test may be finished earlier if a plateau is observed before day 28. (h) Addition of acid drives off any CO_2 remaining in solution.
D7.11	Aerate for a further 24 hours and titrate the barium hydroxide in each of the absorbers.	

D8 Calculation of Results

The theoretical CO_2 production per mg of test compound is calculated as follows:

$$\text{mg } CO_2/\text{mg test compound} = \frac{44 \times P}{12 \times 100}$$

where P = % carbon in compound under test.

Since carbon dioxide is produced by endogenous respiration of the micro-organisms in the seed and by biodegradation of the residual organics present in the inoculum at the start of the test, a correction must be applied. The amount of carbon dioxide produced from the test material is therefore proportional to the difference in the amounts of standard hydrochloric acid required to titrate the excess barium hydroxide in the control and test traps.

Since 1 ml of 0.05M HCl ≡ 1.1 mg CO_2 produced, the amount of CO_2 in each trap (100 ml)

$$= (T_c - T_t) \times 5 \times 1.1 \text{ mg}$$

where T_c = mean titre of 20 ml control (ml 0.05M HCl).
and T_t = mean titre of 20 ml test (or reference) solution (ml 0.05M HCl)

The total CO_2 produced from the test material is obtained by summation of the individual results, including the CO_2 evolved after addition of acid. The extent of biodegradation (= CO_2 produced expressed as % of theoretical CO_2 production) is calculated as follows:

$$\% \text{ Biodegradation} = \frac{\text{total mg } CO_2 \text{ produced} \times 100}{\text{theoretical mg } CO_2/\text{mg compound} \times \text{mg test compound used}}$$

The course of biodegradation may be followed by plotting a graph of percentage biodegradation against time.

D9 Sources of Error

A common reason for low production of CO_2 is that the test substance is inhibitory at the concentrations used (20 and 10 mg/l). If the toxicity at these concentrations is not known it should be determined to see whether this is the reason.

Tests could be carried out at concentrations lower than 10 mg/l but these may be impracticable if the amount of CO_2 produced from test materials is small compared with that from the control.

D10Checking the Validity of Results

The activity of the inoculum is shown by the degree of removal of the reference substance (D5.4). If the CO_2 produced from this within 10 days of the start of biodegradation is not greater than 60% of the theoretical, the test is invalid and should be repeated with an inoculum from other source.

Also the CO_2 produced in 28 d from the inoculum control should not exceed 17 mg per litre of medium.

E Modified OECD Screening Test for the Determination of Biodegradability

Introduction

The modified OECD screening test[32] is designed to pick out substances which are readily biodegradable. It employs a low concentration (10^2/ml) of microorganisms and has a maximum duration of 28 days. Any substance which requires a period greater than 18 days for acclimatisation of the relevant bacteria or the presence of another substrate (co-metabolism) will be found poorly – or non-biodegradable. However, this does not necessarily mean that this substance will not be adequately biodegraded in sewage treatment, but merely that further testing is necessary. Substances passing this test (>70% DOC removed) can be assumed to be extensively biodegraded in sewage treatment and will be rapidly removed from bodies of water, and thus do not need any further work on them.

E1 Performance Characteristics

E1.1	Property determined	Ready, ultimate biodegradability under aerobic conditions, expressed as removal of DOC
E1.2	Type of sample	Organic compounds which are non-volatile, water soluble and not inhibitory at the concentration used in the test (5–40 mg dissolved organic carbon (DOC)/l).
E1.3	Basis of method	'Die-away' test in an open vessel, with the test substance as the sole source of carbon. Removal of test substance is assessed by DOC analysis.
E1.4	Precision	Duplicate values on two occasions:
E1.5	Bias	Tends to give a lower biodegradability than continuous tests, since there are a lower concentration and smaller range of microorganisms present.
E1.6	Interferences	Inhibitors of bacterial growth. (See Section E3)
E1.7	Time required for determination	A maximum of 28 days. Operator time approx. 10 h (for one test substance) including analysis.

	% DOC removed	
	1	2
aniline	84,91	— —
glucose	100,100	100,100
4-nitrophenol	99,99	0,0
diethylene glycol	2,68	31,44
dobane JNQ sulphonate	50,40	— —
tetrapropylene sulphonate	0,0	— —

E2 Principle

The test procedure is based on the modified OECD screening test[32].

The test compound is dissolved in an inorganic medium at a concentration of 10–40 mg C/l, the medium is inoculated with a relatively low concentration (10^2/ml) of microorganisms from a mixed population and aerated at constant temperature for not more than 28 days. Biodegradation is followed by DOC analysis. Control flasks are run in parallel to determine the DOC blank. The procedure is checked by means of a reference substance of known biodegradability.

E3 Interferences

Any chemical substance in the air, or in solution may adversely affect the growth of microorganisms, examples are: organic solvents, toxic metals, strong alkalis, biocides.

The concentration of test substance chosen may be inhibitory to the growth of microorganisms. If this is the case, the test should be repeated using a lower concentration (to a minimum of 5 mg C/l). Intermediates or products of biodegradation of the test substance may also be inhibitory. Substances interfering in the chemical analytical method used may give false results.

E4 Hazards

E4.1 Hygiene

An inoculum originating from effluent of sewage treatment is used, therefore precautions should be taken to avoid the risk of infection from potentially pathogenic microorganisms present in the unknown mixed population.

E4.2 Chemicals

If the test substance is toxic or its properties are unknown, it should be handled with care.

E5 Reagents

E5.1 Deionized or distilled water

The water should be free from toxic substances, particularly metals such as copper. In view of the need to determine DOC in the range 0–40 mg/l, the water should have a low organic carbon content. It is suggested that the water used should contain not more than 10% of the DOC introduced via the test material and that the same batch of water be used for a test series.

E5.2 Nutrient solution

To 900 ml of water (E5.1) add 1 ml of each of the following solutions (a) to (f), in the order given, mixing between additions, and make up to 1000 ml. Where possible, reagents should be of analytical grade.

(a) 36.4 g calcium chloride dihydrate dissolved in 1 l water (E5.1)
(b) 22.5 g magnesium sulphate heptahydrate dissolved in 1 l water (E5.1)
(c) 0.25 g ferric chloride hexahydrate dissolved in 1 l water (E5.1)
(d)

Potassium dihydrogen phosphate	8.5 g
Dipotassium hydrogen phosphate	21.75 g
Disodium hydrogen phosphate dihydrate	33.4 g
Ammonium chloride	20.0 g
Water (E5.1)	1000 ml

(e) *Trace element solution*

Manganous sulphate tetrahydrate	39.9 mg
Boric acid	57.2 mg
Zinc sulphate heptahydrate	42.8 mg
Ammonium molybdate	34.7 mg
Iron chelate ($FeCl_3$,EDTA)	100 mg

Dissolved in 1 l water (E5.1)

Either:

(f) *Vitamin solution*

Biotin	0.2 mg
Nicotinic acid	2.0 mg
Thiamine	1.0 mg
p–Aminobenzoic acid	1.0 mg
Panthothenic acid	1.0 mg
Pyridoxamine	5.0 mg
Cyanocobalamine	2.0 mg
Folic acid	5.0 mg
Water	100 ml

The solution is sterilised by filtration through a membrane (pore size 0.2 μm).

Or, instead of (f) a freshly prepared solution of yeast extract: 15 mg per 100 ml of water (E5.1) may be used.

The pH value should be 7.2 ± 0.2.

The nutrient solution is prepared immediately before use.

Stock solutions (a) to (e) should be stored in the dark, preferably in a refrigerator, and discarded at the first sign of sediment, turbidity, or biological growth.

E5.3 **Stock solution of test substance**

The DOC content of the test substance should be determined. A solution of the test substance containing 1 g C/l is prepared. From this, a second solution containing the chosen test concentration (10–40 mg C/l) is prepared to check the concentration by analysis.

NOTE: When testing sparingly soluble materials, it may not always be possible to prepare a stock solution as described above. In these circumstances a 10–40 mg C/l solution of the test compound is prepared and 1 ml of each nutrient solution (a) to (f) is added to each litre of this solution.

E5.4 **Stock solution of reference**

A solution of 1 g C/l of the reference substance e.g. sodium acetate, sodium benzoate, or freshly distilled aniline is prepared. From this a solution containing the chosen test concentration (10–40 mg C/l) is prepared for checking by analysis.

E5.5 **Inoculation**

Any of the following three alternatives, or composite of them, may be used as inoculum.

E5.5.1 *Inoculum from secondary effluent*

The source of the inoculum is preferably a secondary effluent of good quality collected from a treatment plant receiving a predominantly domestic sewage. The effluent must be kept under aerobic conditions 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 use. The inoculum must be used on the day of collection.

E5.5.2 *Inoculum from soil*

100 g of soil (fertile, not sterile and not recently treated by fertilisers, herbicides etc.) 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 a coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and until use. The inoculum must be used on the day of collection.

E5.5.3 *Inoculum from a surface water*

An inoculum is drawn from a suitably polluted 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.

E5.5.4 *Composite inoculum*

Equal volumes of the 3 inoculum samples are united, mixed well, and the final inoculum drawn from this mixture.

The viability of all of the above inocula is checked by means of the reference substance.

E6 Apparatus

E6.1 **2–1 Erlenmeyer flasks**

The flasks must be carefully cleaned with e.g. alcoholic hydrochloric acid, rinsed and dried before used to avoid contamination with residues from previous tests. The flasks must also be cleaned before their first use, since they may be contaminated.

E6.2 **Shaking machine** accommodating 2–1 Erlenmeyer flasks either with automatic temperature control, or used in a constant temperature room at 20–25°C.

E6.3 **Membrane filtration apparatus**

Membrane filters 0.2 µm pore size.

Membrane filters are suitable if they neither release carbon nor adsorb the test substance in the filtration step.

E6.4 **Suitable carbon analyser**

E7 Procedure

Step	Procedure	Notes
E7.1	The test materials are evaluated simultaneously in duplicate together with duplicate standard and controls.	
E7.2	Add to 2 l of nutrient solution (E5.2) a suitable volume of solution of test substance (E5.3) to give the required DOC concentration (10–40 mg C/l) (note a) and 1 ml of inoculum (0.5 ml/l).	(a) If the test substance is sparingly soluble, dissolve a minimum of 20 mg in 2 l distilled water (E5.1), and add 2 ml of each of the nutrient solutions (a) to (f) in E5.2 (see note in (E5.3), and then add the inocolum.
	For the reference substance add a suitable volume of stock solution (E5.4) to obtain the desired test concentration and inoculate as for test solutions.	
	For the controls (blank) do not add test substance or reference but inoculate in the same way.	(b) e.g. take 50 ml sample, filter through a membrane of 25 mm diameter (0.2 µm pore size), discard the first 20 ml and collect the next 30 ml for analysis in duplicate.
E7.3	Withdraw a suitable volume of test, reference and control solutions, membrane filter (note b) and determine the initial concentration of organic carbon in duplicate by analysis.	
E7.4	Divide each solution (test, standard and control) into two reaction flasks (E6.1). Loosely cover the neck of each flask e.g. with aluminium foil so that the passage of air is not impeded (note c).	(c) Cotton-wool plugs are unsuitable, because the DOC content may be increased by cotton fibres in the test solutions.
E7.5	Place the flasks on a shaker and agitate gently (note d) for 28 days. The temperature should remain in the range of 20–25°C during the test, and the flasks should be shielded from light (note e)	(d) Agitation ensures complete mixing and maintenance of fully aerobic conditions. (e) Photodegradation may occur if the solutions are incubated in strong light, and/or the growth of algae may interfere in the test.

Step	Procedure	Notes
	Sample Collection and Preservation	
E7.6	During the biodegradation test the DOC concentrations are determined in duplicate at the start (day 0) and on the 28th day, and at least three other determinations should be carried out (note g).	(g) Removal of unnecessarily large sample volumes should be avoided – usually 50 ml will suffice. The sampling programme should be modified according to the progress of the test, so that assessment of the biodegradation level 10 d from the start of degradation of any substance is possible (see E9.1). The test may be terminated if the concentration of test substance reaches a constant low level before day 28.
E7.7	If samples cannot be analysed immediately they should be membrane-filtered (note b) and stored at 1°C.	

E8 Calculation of biodegradability

E8.1 The percentage biodegradation of the test (or reference) substance is calculated from the following formula:–

$$\text{At time 't', \% biodegradation} = \frac{(C_o - B_o) - (C_t - B_t)}{(C_o - B_o)} \times 100$$

Where C_o = mean initial concentration of DOC in the test (or reference) samples,
B_o = mean initial DOC concentration in the controls,
C_t = mean concentration of DOC in the test (or reference) samples at time 't',
B_t = the mean DOC concentration of the controls at time 't'.

E8.2 Plot a curve of percentage biodegradation against time.

E9 Sources of error

E9.1 The activity of the proposed inoculum should be determined before starting the test, and re-determined if a different source is used, since inactivity of the inoculum will give invalid results. The activity should be such that at least 70% DOC of the reference substance is removed in 10 days counting from the day that the observed level of biodegradation first reaches 10%.

E9.2 A common reason for low, or nil, removal of DOC is that the concentration of test substance is too high and causes inhibition. If the toxicity is not known this should be determined and, if appropriate, a further biodegradability assessment should be made at a suitably lower concentration.

E9.3 To avoid contamination, residues of test substance from previous tests must be removed by careful cleaning of all glassware used (see E6.1).

E9.4 Any loss by evaporation must be compensated for by addition of distilled (or deionized) water before sampling.

E9.5 It is advisable to filter a solution of test substance at the chosen test concentration to check that no adsorption onto the membrane occurs after the initial portion of filtrate is discarded. If the test substance is adsorbed onto the membranes, the samples should be centrifuged instead of filtered before analysis e.g. at 4000 g for 15 min in a refrigerated centrifuge, keeping the temperature below 26°C. Also a check should be made to ensure that the test substance does not adsorb significantly onto the walls of the flask.

E10 Checking the validity of the results

The activity of the inoculum is shown by the degree of removal of the reference substance (E5.4). If this has not been biodegraded by 70% DOC within 10 days of the start of biodegradation the test is invalid, and should be repeated with an inoculum from another source.

F Static Die-Away Test for the Assessment of Biodegradability based on the method of the UK Standing Technical Committee on Synthetic Detergents[9]

Introduction

Other screening methods in this series described how to assess whether a compound is readily (methods C, D, E) or inherently (methods G, H) biodegradable. Sometimes a prediction is required as to whether a compound will be degraded under normal conditions of sewage treatment without resort having to be made to a simulation test (method I, J).

Compounds which are judged to be readily biodegradable will be removed to an acceptable extent during sewage treatment, and those which are *not* inherently biodegradable (those which fail test method H after many weeks) will not normally be so removed, unless they are adsorbed onto activated sludge or biological filter film. On the other hand, there is doubt about compounds which are inherently, but not readily, biodegradable. The method described here is similar to method E (MOST) but employs a much higher concentration of micro-organisms, similar to that used in the Sturm test (method D). This allows compounds to be degraded which would not pass the MOST method, yet which degrade at a sufficiently high specific rate to be removed in sewage treatment processes under normal conditions, retention time of sludge 3–10 d[16]. As with screening tests for ready biodegradability, failure does not imply that the substance will escape biodegradation in sewage treatment.

The method is based on data collected during many years experimental work on anionic surfactants. The behaviour of the test substance is compared with that of two surfactants: one is removed to a low degree in sewage treatment, the other, though well removed, is not readily degraded in stricter tests with smaller inocula (method E).

If primary biodegradation is to be determined, specific analysis is carried out; for determination of ultimate biodegradation, dissolved organic carbon (DOC) analysis is necessary[23].

F1 Performance characteristics

F1.1	Property determined	Biodegradability (primary or ultimate) under aerobic conditions (expressed as % removal of substance or DOC).
F1.2	Type of sample	Organic compounds which are water soluble, non-volatile and not inhibitory at the concentration used in the test (5–40 mg dissolved organic carbon (DOC)/l).
F1.3	Basis of method	'Die-away' of the test substance in an open vessel, is followed by DOC analysis (ultimate biodegradability) or specific chemical analysis (primary biodegradability).
F1.4	Precision	Duplicate values:– %DOC removal sodium benzoate 96,98 4-nitrophenol 93,95 sulphanilic acid 39,96 3-amino benzoic acid 14,20 o-phenylene diamine 21,22 N-methylaniline 0,0

F1.5	Bias	Tends to give a lower biodegradability than continuous tests, since there is a lower concentration and smaller range of micro-organisms present.
F1.6	Interferences	Inhibitors of bacterial growth (see Section F3).
F1.7	Time required for determination	A maximum of 28 days. Operator time approx. 8 h per batch of 8 compounds; DOC analysis 12 h.

F2 Principle

F2.1 The method is based on the "static" test procedure described by the Standing Technical Committee on Synthetic Detergents[9].

F2.2 The test substance is dissolved in an inorganic medium at a concentration of 10–40 mgC/l, the medium is inoculated with a relatively large concentration (10^5–10^6/ml) of microorganisms from a mixed population (30 mg activated sludge solids/l) and aerated at constant temperature for not more than 28 days. Biodegradation is followed by DOC analysis (ultimate) or by specific analysis (primary). The procedure is checked by means of a reference substance(s) of known biodegradability.

F3 Interferences

Any chemical substance in the compressed air, e.g. organic solvents, or in solution, e.g. toxic metals, which may adversely affect the growth of microorganisms.

The concentration of test substance chosen may inhibit the growth of microorganisms. If this is the case, the test should be repeated using a lower concentration – to a minimum of 5 mg C/l. Substances interfering in the chemical analytical method may give rise to false results.

F4 Hazards

F4.1 **Hygiene**

An inoculum originating from activated sludge is used, therefore precautions should be taken to avoid the risk of infection from potentially pathogenic microorganisms present in the unknown mixed population.

F4.2 **Chemicals**

If the test substance is toxic or its properties are unknown, it should be handled with care.

F5 Reagents

F5.1 **Deionized or distilled water**

The water should be free from toxic substances, particularly metals such as copper. In view of the need to determine DOC in the range 0–40 mg/l, the water should have a low organic carbon content. It is suggested that the water used should contain not more than 10% of the DOC introduced via the test material and that the same batch of water should be used for a test series.

F5.2 **Nutrient Solution**

Where possible, reagents should be of analytical grade.

To 900 ml of water (F5.1) add 1 ml of each of the following solutions (a) to (d), in the order given, mixing between additions, and make up to 1000 ml.

(a) 36.4 g calcium chloride dihydrate dissolved in 1 l water (F5.1).
(b) 22.5 g magnesium sulphate heptahydrate dissolved in 1 l water (F5.1).
(c) 0.25 g ferric chloride hexahydrate dissolved in 1 l water (F5.1).
(d) Potassium dihydrogen phosphate 8.5 g
Di-potassium hydrogen phosphate 21.75 g
Di-sodium hydrogen phosphate dihydrdate 33.4 g
Ammonium chloride 20.0 g
Dissolve in 1 l water (F5.1)

Sufficient trace elements and vitamins B are added via the inoculum.

The pH value should be 7.2 ± 0.2.

This solution is prepared immediately before use.

Stock solutions (a) to (d) should be stored in the dark, preferably in a refrigerator, and discarded at the first sign of sediment, turbidity, or biological growth.

F5.3 **Stock Solution of Test Substances**

A solution containing 1 g C/l of test substance is prepared. From this, a solution containing 10–40 mg C/l is prepared to check the concentration by analysis.

NOTE: When testing sparingly soluble materials, it may not always be possible to prepare a stock solution as described above. In these circumstances 10–40 mg C/l solution of the test compound is prepared and 1 ml of each nutrient solution (a) to (d) added to each litre of this solution.

F5.4 **Standards for Biodegradability**

Stock solutions of soft and hard standards containing 1 g C/l are prepared. From these, solutions containing 10 mg C/l of each are prepared and analysed to check the concentration.

F5.4.1 *Soft standard*

The soft standard should be a substance which is known to be biodegraded by over 80% (primary), or over 70% (DOC), in activated sludge sewage treatment. It should be readily biodegraded in this test, but not in the MOST procedure with an inoculum of 0.5 ml effluent/l.

F5.4.2 *Hard standard*

The hard standard should not be biodegraded by more than 50% (primary) in activated sludge sewage treatment. It should not be removed by more than 20% DOC in this test, or in the MOST procedure.

F5.5 **Inoculation**

Activated sludge from the treatment of largely domestic sewage is used as the source of the inoculum to obtain a mixed population of aerobic heterotrophic microorganisms.

The mixed liquor suspended solids (MLSS) concentration of the activated sludge sample is determined by a standard method[27,33]. Normally the soluble organic carbon introduced with a small volume (10 ml/l) of activated sludge seed is insignificant (<0.8 mg C/l) when diluted in the test medium. However, if any specific inhibitors are present, the activated sludge should be centrifuged e.g. at 1100 g for 10 min, the supernatant discarded, the sludge washed with tap water or an isotonic solution, recentrifuged, and the supernatant again discarded before using the sludge as an inoculum. If the inhibitors is strongly bound to the activated sludge and washing does not remove it, it may be necessary to feed the sludge with synthetic sewage for a number of days before use.

Normally, 30 mg MLSS/l final medium suffices as inoculum.

F6 Apparatus

F6.1 **Erlenmeyer flasks, 2 l**

The flasks must be carefully cleaned with e.g. alcoholic hydrochloric acid, rinsed and dried before use to avoid contamination with residues from previous tests. The flasks must also be cleaned before their first use since they may be contaminated.

F6.2 **Shaking machine** to give adequate aeration and mixing (e.g. 12 cm amplitude, 68 oscillations per minute) to accommodate these flasks, either having an automatic temperature control, or used in a constant temperature room.

F6.3 **Suitable covers for flasks** e.g. loosely applied aluminium foil.

F6.4 **Membrane filtration apparatus** e.g. 30 ml capacity with suitable membranes (0.2 μm pore size) which will neither adsorb nor leach out organic carbon.

F6.5 **Suitable carbon analyser**

F7 Procedure

Step	Procedure	Notes
F7.1	Set up controls, samples and standards in duplicate.	(a) If the test substance is sparingly soluble, dissolve the equivalent of 20 to 80 mg C in 2 l distilled water (F5.1), and add 2 ml of each of the nutrient solutions (a) to (d) in F5.2 (see note in F5.3), and then add the inoculum.
F7.2	Controls Add to 2 l of nutrient solution (F5.2.) 30 mg MLSS/l inoculum.	(b) The soft and the hard standards are tested at a suitable concentation (5–20 mg/l).
F7.3	Test Add to 2 l of nutrient solution (F5.2) 20–80 ml of solution (F5.3) of test substance (note a) and 30 mg MLSS/l inoculum.	(c) e.g. take a 50 ml sample, filter through a membrane of 25 mm diameter (0.2 µm pore size), discard the first 20 ml and collect the next 30 ml for analysis in duplicate.
F7.4	Standards Add to 2 l of nutrient solution (F5.2) 10–40 ml of solution of soft and hard standards respectively (F5.4.1 and (F5.4.2) (note b) and 30 mg MLSS/l inoculum. (The total volume added is the same as in F7.3).	(d) Agitation should ensure complete mixing of the test solution and maintenance of fully aerobic conditions.
F7.5	Withdraw an appropriate volume of each solution (F7.2, F7.3 and F7.4) membrane filter (note c) and determine the concentration of the test substance and/or DOC in duplicate. Divide each of the remaining solutions equally between two flasks (F6.1). Apply a suitable cover to the mouth of each vessel.	(e) If solutions are incubated in the light, growth of algae may interfere in the test, and/or photo-degradation may occur.
F7.6	Place the vessels on the shaker and agitate (note d). The temperature of 20–25°C must be maintained during the test, and the vessels shielded from light (note e).	(f) Removal of unnecessarily large sample volumes should be avoided, usually 50 ml will suffice. The sampling programme should be modified according to the progress of the test, so that assessment of the biodegradation level 10 d from the start of degradation of any substance is possible (see F9.1). The test may be terminated if the concentration of test substance reaches a constant low level before day 28.
F7.7	Sample Collection and Preservation During the test the DOC and/or specific chemical concentrations are determined in duplicate at the start (day 0) and on the 28th day and at least three other determinations should be carried out (note f).	
F7.8	If the samples cannot be analysed immediately, they should be membrane filtered (note c) and stored at 1°C	

F8 Calculation of Biodegradability

F8.1 The percentage biodegradation of the sample and standard is calculated from the following formula:

$$\text{at time 't', \% biodegradation} = \frac{(C_o - B_o) - (C_t - B_t)}{(C_o - B_o)} \times 100$$

where C_o = mean initial concentration of test substance (for primary biodegradation) or DOC (for ultimate biodedegradation)

B_o = mean initial concentration of test substance (should be zero) or DOC in the controls,

C_t = mean concentration of test substance or DOC at time 't'

B_t = mean concentration of test substance or DOC in the controls at time 't'.

F8.2 Plot a curve of percentage biodegradation against time.

F9 Sources of Error

F9.1 The activity of the inoculum should be determined before starting the test (e.g. by respiration rate measurements. See Method B) and redetermined if a different source is used, since low activity of the inoculum will give invalid results. The activity should be such that at least 70% DOC of the soft reference substance is removed in 10 days counting from the day that the observed level of biodegradation first reaches 10%.

F9.2 A common reason for low, or nil, removal of DOC is that the concentration of test substance is too high and causes inhibition.

If the toxicity is not known, it should be determined, and if appropriate a further biodegradability assessment should be made at a suitably lower concentration.

F9.3 To avoid contamination, residues of test substances from previous tests must be removed by careful cleaning of all glassware used (see F6.1).

F9.4 Any loss by evaporation must be compensated for by addition of distilled (deionised) water before sampling.

F9.5 With an inoculum of activated sludge there is the possibility of carryover of soluble carbonaceous material with sludge. This is monitored by simultaneously running a control flask inoculated with the same amount of sludge as the test flasks. The DOC concentration in this control is determined at each sampling time, and subtracted from the appropriate test DOC value. An inoculum of 30 mg MLSS/l was found to produce an insignificant increase (<0.8 mg C/l) in the organic carbon concentration at time O and after incubation for 28 d.

If the sludge contains any material known to be toxic or inhibitory, the sludge should be washed to remove any soluble material which may be carried over (see section F5.5).

F9.6 It is advisable to filter a solution of test substance at the chosen test concentration to check that no adsorption onto the membrane occurs after the initial portion of filtrate has been discarded. If the test substance is absorbed onto the membrane, the samples may be centrifuged instead of filtered, before analysis, e.g. at 4000 g for 15 min in a refrigerated centrifuge, keeping the temperature below 26°C. Also a check should be made to ensure that the test substance does not adsorb significantly onto the walls of the flask.

F10 Checking the Validity of results

The test is considered valid if the soft standard is biodegraded by at least 80%, as assessed by specific chemical analysis (70% DOC), within 14 days of the start of biodegradation (i.e. 10% removed), and the hard standard by less than 50% specific analysis (20% DOC).

If the soft standard is not biodegraded by the stipulated amount, the test should be repeated with an inoculum from another source. If more than 20% DOC of the hard standard is biodegraded, the test should be repeated with a smaller inoculum until the hard standard is biodegraded by no more than 20% DOC while the soft standard is still biodegraded by over 70% DOC.

F11 Interpretation of results

Any substance which is biodegraded to the same, or a greater, extent than the soft standard in terms of DOC will be acceptably removed in activated sludge sewage treatment. Those substances biodegraded no more than the hard standard probably will not be acceptably removed in activated sludge sewage treatment and should be subjected to further tests, perhaps with an acclimatized inoculum.

Intermediate levels of DOC removal are more difficult to interpret. In the case of alkyl(dodecyl)benzene sulphonates 70% removal of DOC has been considered acceptable, since it is assumed that at this level the more recalcitrant part of the molecule (the benzene ring) has been broken down. Similar considerations may be made with other benzenoid compounds, but for other compounds an acceptable level is difficult to set. Therefore, for substances removed to the extent of 20–70% DOC in the screening test, appropriate simulation tests are necessary before confident predictions can be made. (See Strategy Section in Introduction to the Methods, at the beginning of this booklet).

G Modified Die Away Test for the Determination of Primary Biodegradability by Cometabolism (Bunch/Chambers)

Introduction

Some compounds are amenable to biodegradation only if other organic compounds (co-metabolism) or specific compounds (analogue metabolism) are being simultaneously biodegraded. The procedure described here, to be applied only to compounds found not be readily biodegradable, allows an assessment to be made of degradation in the presence of yeast extract, inoculated with sewage, and also permits a moderate degree of acclimatization. Since other organic compounds are present, primary but not ultimate biodegradation is measured.

G1 Performance characteristics

G1.1	Property Determined	Inherent primary biodegradation in the presence of alternative organic substrates, expressed as percentage removal of the specific substance.
G1.2	Type of Sample	Organic compounds which are non-volatile, water-soluble and non-inhibitory at the concentration tested (20 mg/l).
G1.3	Basis of method	Die-away test with additional organic nutrients present and opportunity for acclimatisation and co-metabolism to occur. Removal is followed by specific chemical analysis.
G1.4	Precision	No data available
G1.5	Interferences	Inhibitors of bacterial growth. (See Section G3).
G1.6	Time Required for Determination	A maximum of four weeks. Operator time is approximately 2 hours per compound (4 sub-cultures), excluding specific analysis.

G2 Principle

This method is a modification of that described by Bunch and Chambers[35]. The test compound (up to 20 mg/l) is dissolved in a medium containing yeast extract as an additional source of carbon, the medium is inoculated with sewage (10% v/v) and aerated at constant temperature. After 7 days the extent of degradation is determined by specific chemical analysis and the test solution is used to inoculate another sub-culture. A further two sub-cultures are similarly made, the whole test period covering 28 days. The procedure is checked by means of a reference substance of known biodegradability and preferably of similar chemical structure.

G3 Interferences

Any chemical substance in the air, or in solution, which may adversely affect the growth of micro-organisms, examples are: organic solvents, toxic metals, strong alkalis, biocides.

The concentration of test substance chosen may inhibit the activity of the micro-organisms. If this is the case the test should be repeated using lower concentrations. Intermediates or products of biodegradation of the test substance may also be inhibitory. Substances interfering in any specific chemical analytical method used may give false results.

G4 Hazards

G4.1 **Hygiene**

An inoculum originating from sewage is used, therefore precautions should be taken to avoid the risk of infection from potentially pathogenic micro-organisms present in the unknown population.

G4.2 **Chemicals**

If the test substance is toxic or its properties unknown it should be handled with care.

G5 Reagents

G5.1 **Deionized or distilled water** free from toxic substances, particularly metals such as copper.

G5.2 **Nutrient solution**

To 900 ml of water (G5.1) add 1 ml of each of the following solutions (a) to (d) in the order given, mixing between additions, and make up to 1000 ml.

Where possible reagents of analytical grade should be used.

a) *calcium chloride solution*

Dissolve 36.4 g calcium chloride dihydrate in distilled water and make up to 1 l.

b) *Magnesium sulphate solution*

Dissolve 22.5 g magnesium sulphate heptahydrate in distilled water and make up to 1.1.

c) *Ferric Chloride solution*

Dissolve 0.25 g ferric chloride hexahydrate in distilled water and dilute to 1 l.

d) *Phosphate buffer.*

Dissolve 8.5 g potassium dihydrogen phosphate 21.75 g dipotassium hydrogen phosphate, 33.4 g disodium hydrogen phosphate dihydrate and 1.7 g ammonium chloride in about 500 ml distilled water and make up to 1 l.

The above solutions should be stored in the dark, preferably in a refrigerator, and discarded at the first sign of sediment, turbidity or biological growth.

e) *Yeast extract*

Add 0.055 g of yeast extract to each litre of the nutrient solution. The medium should be used within 3 h of preparation.

G5.3 **Stock solution of test compound**

A solution containing 2 g/l of test compound is prepared. If the test substance is sparingly soluble it may not be possible to prepare a stock solution as described below. In these circumstances make a solution containing a 20 mg/l of test substance and add 1 ml of each of the solutions (a) to (d) above and 55 mg yeast extract per litre.

G5.4 **Stock solution of reference substances**

A solution of 2 g/l of reference substance is prepared. This should be a chemical of known biodegradability, preferably with a similar structure to the test compound.

G5.5 **Inoculum**

Freshly collected settled sewage is used as the inoculum. The sewage should be predominantly domestic waste water.

G6 Apparatus

G6.1 **250 ml Erlenmeyer flasks.**

G6.2 **Reciprocating shaker.**

Step	Procedure	Notes
	TEST SUBSTANCES, REFERENCE SUBSTANCE AND CONTROLS ARE TESTED SIMULTANEOUSLY IN TRIPLICATE.	
G7.1	Dispense 90 ml medium (G5.2) into 250 ml Erlenmeyer flasks.	
G7.2	Shake all flasks until the medium is saturated with oxygen (note a).	(a) The shaker should be set so that it imparts an adequate swirling motion to keep the liquid in the flasks fully aerobic. (At 100 rpm and an amplitude of 5 cm, the solution will be saturated with oxygen in approximately 20 min.)
	Test solutions	
G7.3	To each of three flasks containing 90 ml of medium, add 1 ml of solution of the test compound (G5.3) (note b).	(b) This will result in a final concentration of about 20 mg/l.
	Prepare a similar set of 3 flasks for each test compound to be evaluated	
	Reference solutions	
G7.4	To each of three similar flasks containing 90 ml of medium, add 1 ml of a solution of a known biodegradable reference compound (G5.4) (note c).	(c) The reference compound is used to check the activity of the inoculum, and to validate the test (see section G10)
Controls		
G7.5	Also take three similar flasks containing 90 ml of medium for controls.	(d) The specific analytical procedure for the test substance should be applied to an inoculated control (and to uninoculated control, if necessary) before and after incubation for 7 d to establish whether any interference occurs.
	Inoculation	
G7.6	Add 10 ml of settled sewage to all flasks.	
G7.7	Verify initial concentration of the test compound by removing a sample from each flask for analysis; this will also demonstrate any initial adsorption loss that might be attributed erroneously to biodegradation (note d).	
G7.8	Place the flasks on a reciprocating shaker and incubate in the dark at 20° ± 1°C. (note e).	(e) There is a possibility that the solutions may become anaerobic if the rate of solution of oxygen is inadequate. The flasks should therefore be open to the atmosphere and the dissolved oxygen concentration checked to ensure that the shaker speed has been correctly set.
G7.9	Observe the flasks the day following inoculation. They should be visibly turbid (note f). If they are not turbid (note g), repeat the procedure with the same and three lower concentrations of the test compound.	(f) If the compound is non-toxic and the bacteria used to inoculate the solution were viable, then within 24 hours the solution will become turbid due to biological growth on the yeast extract. (g) The absence of turbidity would indicate that the test compound is toxic, or the inoculum was not viable.

Step	Procedure	Notes
G7.10	After incubation for 7 days, restore to the original volume with distilled water if evaporation has occured. Prepare a flask of fresh medium (G5.2) to contain 20 mg/l of test compound (G7.3) and add 10 ml of turbid 7-day cultures as inoculum. Verify the starting concentration by analysis. The remaining solution in all test flasks is analysed for residual test compound and discarded. In any analytical procedure, the same sample for the blank should be used for the other samples.	
G7.11	Repeat sub-culture and analysis as in G7.10 at each succeeding 7-day interval to result in a total of 3 sub-cultures (note h)	(h) The total time for the test is 28 days; 7 days for the original culture and 7 days for each of the three successive subcultures.

G8 Calculation and Interpretation of Results

After each period of incubation the extent of biodegradability can be calculated:

$$\% \text{ Biodegradation} = \frac{C_o - C_t}{C_o} \times 100$$

where C_o and C_t are the concentrations of test compound determined at the start and completion of the incubation respectively.

By comparing the results of each sub-culture with that obtained for the initial incubation, substances requiring adaptation of the micro-organisms may be identified. Where this has occurred, some indication of the length of the acclimatization period is also gained.

G9 Sources of error

G9.1 As with all biological tests, the inherent variability in living organisms may result in large discrepancies in the data obtained when the same material is tested on different occasions. This problem may be overcome to some extent by simultaneously testing substances which have been subjected to the test many times, (reference substances), or better, substances for which the biodegradability has previously been established in the environment. Ideally both "hard" and "soft" standards should be used and the chemical structure should be similar to that of the test compound. From a comparison of the observed results an assessment of the relative biodegradability of the test compound can be made.

G9.2 As with all biodegradation tests the analytical method used to follow the dissappearance of the test compound determines the meaning and reliability of the results.

G10 Checking the validity of results

The activity of the inoculum is shown by the degree of removal of the reference substances. If this is not removed by the expected amount (usually 80%) another source of sewage should be used for the inoculum.

H Modified semi-continuous activated sludge (SCAS) test for the assessment of inherent biodegradability

Introduction

This method is an adaptation of the American Soap and Detergent Association[36] semi-continuous activated sludge (SCAS) procedure and has been recommended by the OECD Chemicals Group[37]. Only substances not readily biodegradable will be subjected to this test, which, because of the high mean retention time of the sewage (36 h) and of sludge (>100 d) and intermittent addition of sewage, does not simulate conditions experienced in conventional sewage treatment. Since the conditions provided by the test are highly favourable to the selection and/or adaptation of microorganisms capable of degrading a new compound and for co-metabolism, the procedure is most useful as a test of inherent biodegradability and may also be used to produce acclimatised inocula for other procedures (e.g. methods D, E, F).

H1 Performance characteristics

H1.1. Property determined	The inherent biodegradability of organic compounds, expressed as percentage removal of DOC.
H1.2 Type of sample	Organic compounds which are non-volatile, water soluble and not inhibitory at the concentration used in the test: 5–20 mg C/l.
H1.3 Basis of method	Activated sludge, operated on a fill-and-draw basis, is exposed to test chemical and sewage, or sewage alone and biodegradation is assessed from analysis of dissolved organic carbon (DOC) of the two supernatants.
H1.4 Precision	No data available
H1.5 Bias	Because of the high sludge age the conditions of the test are more conducive to acclimatisation than conventional sewage treatment processes.
H1.6 Interferences	Inhibitors of bacterial growth. (see Section H3).
H1.7 Time required for analysis	Total time 3–6 months. Operator time for 10 units: ½ h per day plus 2½ h for analysis.

H2 Principle

The DOC concentration in the effluent of a semi-continuous activated sludge unit which is being dosed with sewage and a known concentration of test substance is compared with the DOC in the effluent from a control unit dosed with sewage alone. Any difference in the concentration of DOC in the two units is assumed to be due to residual test substance.

H3 Interferences

Any chemical substance in the compressed air, or in the sewage which may adversely affect the growth of microorganisms e.g. organic solvents, toxic metals.

The concentration of test substances chosen may also be inhibitory to bacterial growth. If this is the case a lower concentration of test substance should be used. Intermediates or products of biodegradation of the test substances may also be inhibitory.

H4 Hazards

E4.1 **Hygiene**

Since the units contain activated sludge and are fed with domestic sewage, suitable precautions should be taken to avoid infection from the potentially pathogenic microorganisms present in the unknown mixed population.

H4.2 **Chemicals**

If the test substance is toxic or its properties are unknown, it should be handled with care.

H5 Reagents

H5.1 **Deionized or distilled water.**

H5.2 **Settled sewage** from a plant treating predominantly domestic sewage. The sewage should be collected freshly each day.

H5.3 **Mixed liquor** from an activated sludge plant containing about 2–3 g suspended solids/l. This should be freshly collected and aerated if it is not used within ½ h of collection.

H5.4 **Stock solution of test substances**

A stock solution of each test material is prepared to contain 400 mg C/l.

H6 Apparatus

H6.1 **Aeration apparatus**

The aeration apparatus can simply be a 250 ml measuring cylinder fitted with a tube and glass sinter supplying compressed air.

Alternatively, the aeration vessel can be as shown in Figure 10, suitably supported and fitted with a tap so that 50 ml of liquid remain in the vessel after drawing off the supernatant. One such apparatus is required for each test substance, and one for each control (usually two).

H6.2 **Compressed air supply**

H6.3 **Drechsel bottles** to saturate the air with water vapour

H6.4 **Membrane filtration apparatus**

Membrane filters (pore size 0.4 μm)

Membrane filters are suitable if they neither release carbon, nor adsorb the test substance in the filtration step.

Alternatively, centifugation at 4000 g for 15 min may be used.

H6.5 **Suitable organic carbon analyser.**

H7 Test Procedure

Step	Procedure	Notes
H7.1	Place 150 ml activated sludge mixed liquor (note a) in each of the units	(a) see section H5.3
H7.2	Aerate the units with moist air (note b).	(b) This reduces losses by evaporation. The flow-rate should be sufficient to maintain the sludge in suspension, and a dissolved oxygen concentration of at least 2 mg/l.

Step	Procedure	Notes
H7.3	Stop the aeration after 23 h and allow the sludge to settle for 30 min (note c)	(c) A longer settlement period may be necessary.
H7.4	Remove 100 ml of supernatant (note d) if required for analysis in a clean beaker.	(d) It is preferable to discard the first 20 ml if collecting via a tap.
H7.5	Add 100 ml freshly collected sewage (H5.2) to each unit (note e) and the required volume of stock solutions of test substances to appropriate units (excluding controls) to obtain a concentration of 5–20 mg/l. Restart the aeration.	(e) If the supernatant is not clear, a running-in period may be required in which sewage alone is fed to the units until the supernatant becomes clear. In this case it is preferable to mix the individual sludges and add 50 ml of the composite to each unit before starting to add the test substance and sewage.
H7.6	After 23 h repeat the procedure for settling and feeding, collecting samples for analysis, as required. Membrane filter and analyse samples for DOC. (note f)	(f) It is convenient to filter a 50 ml sample through a 25 mm membrane rejecting the first 20 ml of filtrate.
H7.7	Continue the test as appropriate (note g)	(g) For prolonged tests it is not necessary to analyse the supernatant daily. Some compounds have been found to persist for up to 3 months before being extensively degraded, therefore it may be justified to continue the test for at least three months.

H8 Calculation of Results

The dissolved organic carbon concentrations in the supernatant liquors of the test and control units are plotted against time. If the sludge becomes acclimatized and the substance is biodegraded, the DOC of the test supernatants will gradually fall to approach that in the controls. Once the difference between the two concentrations is found to be constant over three consecutive measurements a further three measurements are made and the percentage biodegradation of the test compound is calculated by the following equation.

$$\% \text{ biodegradation} = \frac{C_o - (C_t - C_c) \times 100}{C_o}$$

where C_o = concentration of test compound as DOC added to the settled sewage at the start of the aeration period.

C_t = concentration of DOC found in the supernatant liquor of the test at the end of the aeration period.

C_c = concentration of DOC found in the supernatant liquor of the control at the end of the aeration period.

H9 Sources of error

H9.1 The concentrations of test substance used may be inhibitory to the activated sludge, so that the DOC of the supernatant may rise to unexpectedly high values, the supernatant may become turbid and sludge may be lost from the system on decanting.

If this is the case the procedure should be repeated with a lower concentration of test substance providing that the lower DOC value can be determined with sufficient accuracy.

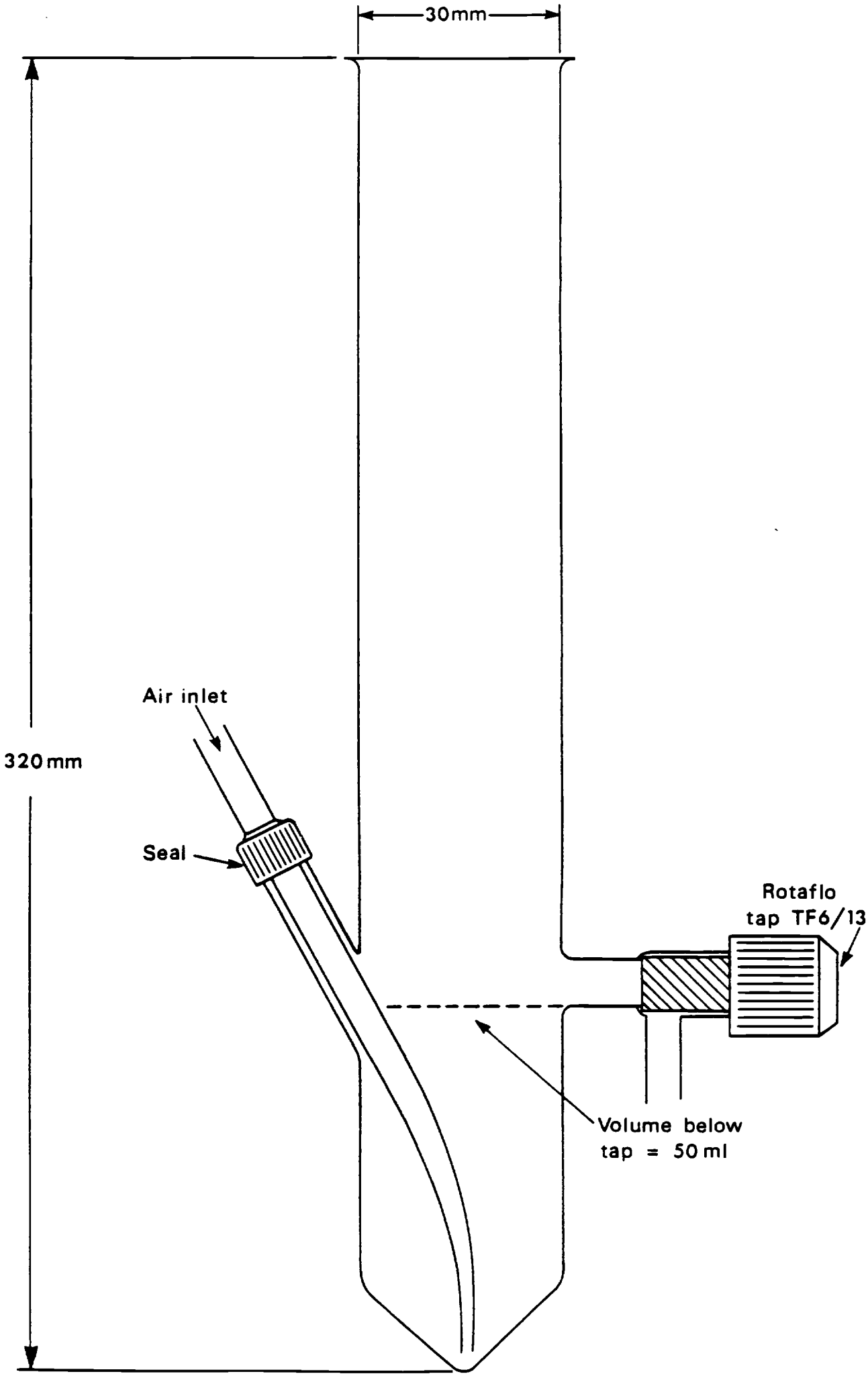
The toxicity of the test substance may be checked beforehand by a suitable method (e.g. method B).

H9.2 Substances present in the sewage may be inhibitory to the biodegradation of the test material and it may be appropriate to use sewage from another source.

H9.3 If the test substance is removed rapidly after addition, and then no further removal occurs during the rest of the aeration period, it is probable that adsorption is

taking place. This can be checked by adding test substance to the sludge, aerating briefly, settling and then analysing the supernatant for DOC. If the expected and determined amounts differ significantly then adsorption is assumed. To prove that biodegradation is occurring, it may be helpful to use the acclimatized sludge as an inoculum for a screening test e.g. in methods D, or F.

FIG.10


30mm
320mm
Air inlet
Seal
Rotaflo
tap TF6/13
Volume below
tap = 50ml

I Continuous Simulation (Activated Sludge) Test for the Assessment of Biodegradability:

Introduction

A simulation test is generally applied to a substance which has failed a screening test for ready biodegradability (e.g. methods D, E or F), but passed an inherent biodegradability test (e.g. method H), or to any substance if more information is required on its effect on activated sludge sewage treatment. Since this is a fairly expensive and time-consuming test, it is resorted to only in the case of substances of economic importance, e.g. those expected to have a large production volume.

Test simulating the activated sludge process have been described by the OECD[38,39], in the EEC directives on surfactants[e.g.40,41] and by the WRC[41]. The international standard is the Husmann apparatus using synthetic sewage, but the WRC porous pot is a permitted alternative for the UK. Both fail to simulate completely the conditions in a sewage treatment works, so that each is a compromise. For example, the Husmann unit has an air-lift pump which returns settled sludge at an abnormally high rate (>12:1 of the sewage flow) and this does not allow anaerobic conditions to develop during settlement. The porous pot has no settlement period at all. Therefore no prediction can be made on the settleability of the sludge.

The standard OECD/EEC method describes assessment of the primary biodegradability of surfactants, whereas the tentative method presented here is extended to other substances and to the assessment of ultimate biodegradability. The implications of the use of DOC analysis are discussed in Section I15.

Strictly, simulation methods determine bioelimination, but ways of distinguishing between true biodegradation and physical adsorption are outlined in Section I10.6.

I1 Performance characteristics

I1.1	Property determined	The primary or ultimate biodegradability of organic compounds, expressed as the percentage removal of substance or DOC.
I1.2	Type of sample	Single compounds which are soluble at the concentration used in the test and non-volatile.
I1.3	Basis of method	A determination of removal of test substance in laboratory-scale plant resembling the activated-sludge process.
I1.4	Standard deviation (within batch)	Primary biodegradation of surfactants.

Surfactant†	Husmann units		Porous Pots	
	%	SD*	%	SD*
Nonidet SH	99	0	99	0.5
Dobane JN	96	0.6	96	1.0
3JN: 1 TPBS	87	1.9	84	1.4
Marlophen 810	86	0.8	83	5.0
3 JN: 2 TPBS	78	1.7	—	—
TPBS	28	1.4	35	1.0

I1.5	Limit of detection	Limited by the analytical method for test substance and by biological variation.

I1.6	Interferences	Inhibitors of bacterial growth. Any substance interfering in the analytical method used. (See Section I3).
I1.7	Time required for determination	A maximum of 9 weeks. Operator time 11 h/ week per 4 units.

† These surfactants are as follows:
Nonidet SH.....a Linear alcohol ethoxylate (C_{12-15},[a] 9 EO units[b])
Dobane JN (or JN) ...a Linear alkylbenzenesulphonate (C_{10-13}[a])
TPBS.....Tetrapropylenebenzene sulphonate
Marlophen 810.....Octylphenolethoxylate (10 EO units[b])
(a) number of carbon atoms in the linear alkyl chain.
(b) number of ethanoxy units in the ethoxylate chain.

*Data obtained at Water Research Centre, Stevenage:– inter unit variation ($v = 3$).

I2 Principle

I2.1 Synthetic sewage (but see Section I13) containing the required concentration of test substance is supplied to activated sludge in a 3 l aeration vessel at a rate of 1 l/h. The mixed liquor is settled in an adjoining vessel and the sludge is continuously recycled, while the separated effluent is collected. Sewage and effluent samples are analysed for test substance either by a specific method or by dissolved organic carbon (DOC) measurements. Control units receiving no test substance are operated in parallel for comparative purposes. These are essential if (ultimate) biodegradation of the test substance is to be assessed by DOC analysis, and biodegradation is assessed from the relative removal of DOC in the two units.

I2.2 The method is based on a continuous simulation test recommended by the OECD[38].

I3 Interferences

Any chemical substance in solution or in the air may adversely affect the growth of sludge micro-organisms. Examples are: organic solvents, toxic metals, strong alkalis, bactericides.

Substances strongly adsorbing onto the walls of the aeration vessels may give false removal values. Substances interfering in any specific chemical methods used may give false results.

I4 Hazards

I4.1 **Hygiene**

Whether sludge growth is started from an airborne infection or by deliberate inoculation of the synthetic sewage, it must be remembered that the population is unknown and some of the micro-organisms present in the sludge may be potential pathogens. It is therefore necessary to take appropriate precautions when carrying out plant maintenance and handling samples.

I4.2 **Mechanical and electrical**

Guards should be fitted on peristaltic pumps to prevent catching fingers in the moving rollers, and electric stirrers and pumps should be guarded from splashes and leaks.

I4.3 **Chemicals**

If the test substance is toxic or its properties are unknown, it should be handled with care.

Mercuric chloride, used to preserve samples, and sodium hypochlorite, used to clean apparatus, should also be handled with care.

I5 Reagents

I5.1 Synthetic sewage

The synthetic sewage is composed as follows for each 1 l of tapwater:– 160 mg peptone and 110 mg meat extract (or 270 mg of commercial peptone-meat extract preparation), 30 mg urea, 7 mg sodium chloride, 4 mg calcium chloride dihydrate, 2 mg magnesium sulphate heptahydrate, 28 mg dipotassium hydrogen phosphate. The chemicals should be of analytical reagent grades.

For convenience, this may be prepared as a 100 × concentrated solution, which can be stored at 1°C for up to 1 week, and the synthetic sewage made daily from this by appropriate dilution with tap water.

After dilution, this synthetic sewage contains approximately: 105 mg C/l 46 mg N/l, 5 mg P/l, and the pH is in the range 7.0–7.5. Other sewages may also be used (see section I13).

I5.2 Stock solution of test substances

Appropriate solution which on addition of a convenient volume (e.g. 50–100 ml) to the synthetic sewage concentrate will, on dilution, result in the required concentration (e.g. 10–20 mg/l) in the final mixture, or if a separate dosing pump is used for the test substance, a stock solution should be prepared which will give the required final concentration of test substance in the synthetic sewage.

I5.3 Mercuric chloride

1.0% w/v solution of A.R. mercuric chloride for preservation of samples when necessary.

I5.4 Lubricant

A lubricant for the rollers of the peristaltic pumps is required e.g. glycerol or olive oil (both suitable for use with silicone rubber tubing).

I5.5 Antifoam

An antifoaming agent e.g. silicone DC antifoam RD emulsion, may be necessary in the early stages when testing surfactants. It is important to ensure that the antifoam does not interfere with the test or analytical method used.

I6 Apparatus

I6.1 Husmann Units

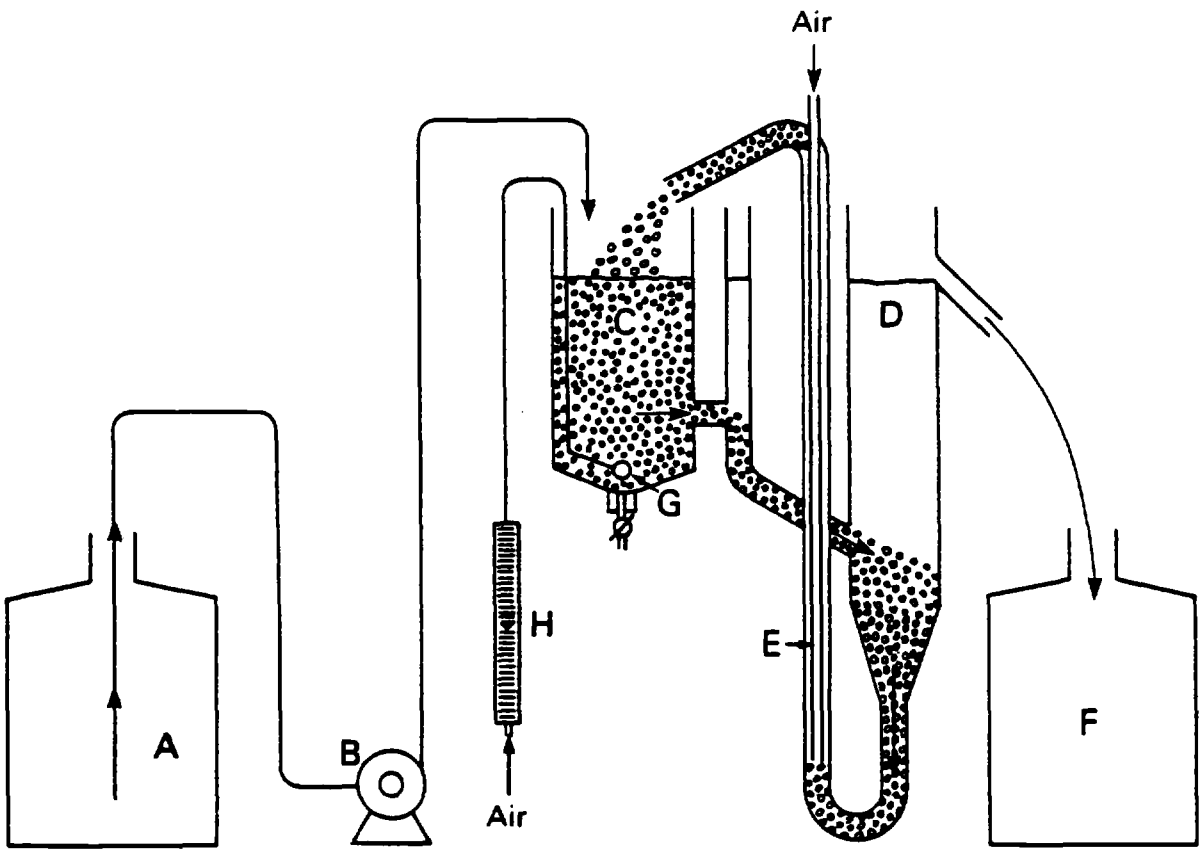
The small activated sludge plants are constructed according to the EEC/OECD specifications (Figs. 11 and 12). They are made from acrylic or glass and consist of a cylindrical aeration chamber (C) of 3–1 capacity with a conical base. The aeration vessel has an outlet at one side which passes into the conical base of a settlement chamber (D), which is also cylindrical and 3/5 of the diameter of the aeration chamber. Near the top of the settlement chamber is an effluent outlet fitted at a level to maintain 3 l in the aeration chamber, while at the base is an air-lift pump (E) for returning settled sludge to the aeration vessel. Temperature control at 18–25°C may be achieved by installation in a constant temperature room.

Filtered compressed air is supplied through a pressure regulator to the base of the air-lift pump and to the aeration vessel. Each air supply is controlled by a needle valve and the aeration chamber supply is metered by a direct reading glass rotameter (H). Two diffusers (G) are placed in the aeration vessel to produce fine bubbles and to achieve nearly-complete mixing (one diffuser may sometimes prove inadequate).

The 24-h supply of synthetic sewage is contained in a polythene vessel (A) of appropriate size and a similar vessel (F) is used for collection of the effluent. Suitable tubing (e.g. silicone rubber) is used to supply the sewage to the aeration chamber by means of a peristaltic pump (B). A length of acrylic or glass tubing notched at the distal end reaches to the base of the influent vessel, and a curved piece of the same tubing at the outlet is clipped to the side of the aeration vessel. A removable curved tube may be fitted on to the outlet of the airlift pump to reach below the rim of the aeration vessel, so that loss of sludge by splashing is prevented.

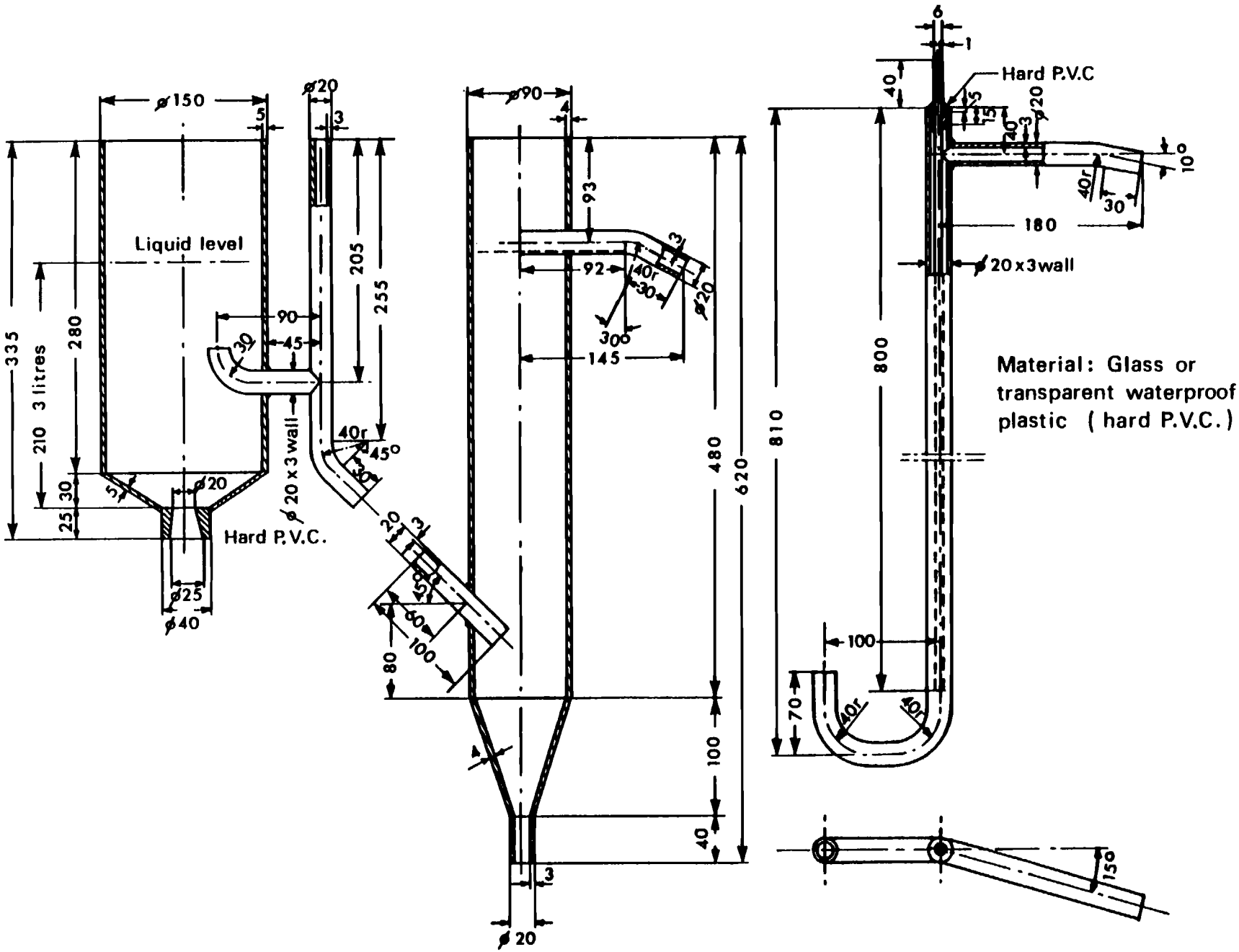
FIG.11 EQUIPMENT USED FOR ASSESSMENT OF BIODEGRADABILITY

(Husmann unit)



A. Storage vessel
B. Dosing device
C. Aeration chamber (3 l. capacity)
D. Settling vessel
E. Air lift pump
F. Collector
G. Aerator
H. Air flow meter

FIG.12 DIMENSIONS OF HUSMANN UNIT



I6.2 Membrane Filtration Apparatus

Membrane filters (pore size 0.4 μm) are suitable if they neither release carbon, nor adsorb the test substance.

I7 Sample Collection and Preservation

Samples of material or effluent to be tested should not have preservative added to them prior to being tested.

A 'spot' sample of synthetic sewage containing the test substance is taken after thorough mixing and, if necessary, preserved, e.g. with mercuric chloride*. It may be necessary to allow for changes in the synthetic sewage over the 24 h period, e.g. by taking a sample at the end of the period and using the average of this and the initial sample. To prevent changes in the sewage-test substance mixture, a solution of the test substance may be dosed to the aeration tank by means of a separate pump. A 24 h composite sample of effluent is collected and preserved if necessary.

Additional spot samples are taken from the apparatus during the course of the test (I8.2.6 and I8.3). If these are not analyzed immediately, they should be preserved as above.

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* 20 mg/l $HgCl_2$ for preserving effluents is recommended by the Harmonized Monitoring Committee of the DOE. Double the concentration is used for sewages. Note that the BOD test cannot be applied to samples thus preserved.

To remove mercury from laboratory waste, first, if present, destroy any organic matter. For suitable digestions see reference 46, and cool the digest, and cautiously pour into at least a 10:1 excess of water. Then proceed as detailed in reference 34.

I8 Procedure

Step	Procedure	Notes
I8.1	**Preparation of synthetic sewage**	
	The synthetic sewage is prepared each day. The required volume of concentrate (I5.1) is measured into a clean influent vessel (note a) and, if appropriate, a measured volume of stock solution of test substance (I5.2) is also added. The volume is made up to 24 l with cold tap-water, and stirred (note b) to mix completely.	(a) Influent and effluent containers should be thoroughly cleaned to remove bacterial (influent) or algal (effluent) growths. (b) e.g. by using an electric stirrer with blade of a suitable length.
	Control plants are operated in the same way, but with no test substance added.	
I8.2	**Operation of activated sludge units**	
I8.2.1	The peristaltic pump is set to deliver 1 l/h synthetic sewage to the aeration vessel (note c). The pump rollers should be lubricated (note d).	(c) A separate dosing pump may be needed for the test substance (see section I7). (d) Glycerol or olive oil may be used for lubricating rollers. Tubing may be moved along when the section under the rollers flattens in use – approximately every two weeks. However, influent tubing will require cleaning regularly (about twice weekly) to remove bacterial growth.
I8.2.2	Fill the aeration vessel with synthetic sewage and inoculate if necessary (note e).	(e) An airborne inoculum may be sufficient in some cases; however, a small volume (e.g. 3 ml) of sewage effluent or soil extract can be used. A larger seed of activated sludge taken from a sewage works treating domestic sewage may be used, but the sludge should be fed with synthetic sewage for several days before adding the test substance to ensure that no unwanted materials are adsorbed onto the sludge.

Step	Procedure	Notes
I8.2.3	The air flow to the aeration vessel is set to give complete mixing and a concentration of dissolved oxygen of at least 2 mg/l (note f). The temperature should be adjusted to the required value (usually 18–25°C).	(f) For example: an air flow of 2.5 l/min at 21°C will result in a dissolved oxygen concentration of approximately 7 mg/l, but a high rate is necessary to create turbulence and thus to avoid sludge settling in the aeration vessel.
I8.2.4	The air-lift pump should continuously return settled sludge (note g).	(g) The airlift pump gives a high flow of returned sludge. Normally in sewage treatment the return flow of sludge is nearly equal to the inflow of sewage. However, in this design (Fig. 12) the return sludge flow cannot be reduced to less than 12:1.
I8.2.5	Any sludge accumulating in the airlift pump circuit or in the settlement vessel and at the meniscus in the aeration vessel should be returned to circulation as soon as possible e.g. by scraping or brushing – at least twice each day.	
	Sampling	
I8.2.6	Spot samples of synthetic sewage mixtures and 24 h composite samples of effluent are taken (note h).	(h) Samples not analysed immediately should be preserved (see section I7). It may be necessary to filter effluents e.g. using washed glass wool to remove suspended solids before analysis. Samples for DOC analysis should be filtered through membrances of pore size 0.4 μm.
	Checking the performance of the plant	
I8.3	Sewage and effluent samples are taken twice weekly during the 'running-in' part of the test (note i) and the concentration of test substance (note j) and/or DOC is determined (see section I15). COD, NH_3, Oxidized N determinations may also be required for further information on the performance of the plants. The mixed liquor suspended solids (MLSS) are determined (note k) twice weekly and the sludge is wasted when necessary to maintain a concentration of approximately 2.5 g/l.	(i) 'Running-in' is the time taken to reach 2.5 g MLSS/l and steady removal of COD (usually 78–88%) and test substance. In any event this should not exceed 6 weeks. (j) If primary biodegradability is required specific analysis can be used. To assess ultimate biodegradability, DOC analysis of test and control effluents is necessary. (k) Use a standard method[27].
	Steady State	
I8.4	When the concentration of MLSS has reached 2.5 g/l (note l) and the removal of COD and a test substance or DOC has reached a steady level, the concentration of test substance and/or DOC is determined daily (note m) for a period of three weeks. The biodegradation can be calculated from these results.	(l) On some occasions it has proved impossible to maintain a concentration of activated sludge of 2.5 g MLSS/l because of poor settlement of filamentous organisms or by failure to return the sludge because of 'bridging' in the settlement vessel. Settlement can sometimes be improved by the addition of 2 ml of 5% $FeCl_3$ solution to the settlement vessel. (m) At least 14 determinations of percentage removal should be obtained in the 3 weeks.

I9 Calculation of Biodegradation

I9.1 For primary biodegradation, the percentage removal is calculated from the following formula:

$$\text{percentage removal} = \frac{C_s - C_E}{C_s} \times 100$$

where C_S = the concentration of test substance in the synthetic sewage.
and C_E = the concentration of test substance in the effluent.

For ultimate biodegradation the concentration of DOC in the test and control units is compared. Ideally,

$$\text{percentage removal} = \frac{(C_{ST} - C_{SB}) - (C_{ET} - C_{EB})}{(C_{ST} - C_{SB})} \times 100$$

where C_{ST} = the concentration of DOC in the test synthetic sewage
C_{SB} = the concentration of DOC in the control synthetic sewage
C_{ET} = the concentration of DOC in the test effluent
C_{EB} = the concentration of DOC in the control effluent.

I9.2 However, if the concentration (as (DOC) of test substance added cannot be adequately determined (see Section I15) or if the test substance is added separately, its concentration must be estimated; let this be C_T.

Then, percentage removal $= \frac{C_T - (C_{ET} - C_{EB})}{C_T} \times 100$

I10 Sources of Error

I10.1 Operating difficulties

During the weeks in which intensive analysis is done, the plants should be free from operational difficulties. The results are valid only if performance is steady. If any of the difficulties described below are encountered, the analysis should be extended and the results of three weeks continuous steady running should be used. However, the EEC Directive and OECD[38,39,40] method allows no more than six weeks for the 'running-in' period. If a steady state is not reached by this time assume that the test substance is not treatable, or that it has interfered in the removal of synthetic sewage components – or both. These are distinguished by carrying out analysis for both test substance and/or DOC or COD. The concentration of test substance used may be inhibitory to the sludge (see Section I14.3).

I10.2 Loss of sludge in the effluent

If the sludge is allowed to accumulate in the settlement vessel, denitrification may occur causing bubbles of nitrogen gas to be formed which attach themselves to the sludge, resulting in floating masses of sludge, which are washed out in the effluent.

Filamentous growth will also result in poor settling and sludge in the effluent. These solids in the effluent would give rise to high COD values, and therefore effluent samples should be filtered or centrifuged before analysis.

I10.3 Non-return of settled sludge

Sometimes, particularly if the sludge is filamentous, 'bridging' occurs at the base of the settlement vessel, so that sludge accumulates there and is not returned to the aeration vessel, the returned liquid being low in suspended solids. This results in depletion of sludge in the aeration vessel and consequently low removal of COD and test substance. This can be temporarily remedied by dislodging the sludge with a brush.

I10.4 Synthetic sewage

Generally, one batch of peptone and meat extract should be used throughout the test to minimize any changes in constituents which may arise by different states of purity, or from different protein sources.

The concentration of phosphate in the synthetic sewage is insufficient for adequate buffering of the mixed liquor if complete nitrification (an acid producing reaction) occurs and as a result the pH value may fall sufficiently to reduce the degree of removal of COD and perhaps of the substance under test. This may be remedied by adding suitable concentrations of bicarbonate to the synthetic sewage, using the fact that the oxidation of 1 mgN to nitrate produces an acidity equivalent to about 7 mg $CO_3^{=}$.

I10.5 Loss of test substance

Adsorption of test substance onto the sludge, or onto the sides of the plant may lead to apparently high removals at an early stage in the test, although an equilibrium should eventually be reached.

Foaming of the test substance will also result in errors in estimating the removal of test substance. If an antifoaming agent is used, checks should be made to ensure that it does not interfere with the operation of the plant or with the analytical methods used. A similar amount of antifoaming agent should be added to the controls to maintain comparability.

I10.6 Bioelimination

This method measures bioelemination, and so it may be necessary to distinguish between true biodegradation and physical adsorption of test substance onto the sludge. Adsorption, if it occurs, is usually most marked at the start of the test and generally an equilibrium is reached, so it may be identified by careful examination of the course of bioelimination. Biodegradation is usually shown as a steady upward trend of removal of test substance to a constant plateau at the 'steady state'. However, a special investigation is necessary to determine the extent of adsorption.

I11 Checking the Accuracy

The accuracy of the method can be checked by running the plant with standards of known biodegradability, e.g. for primary biodegradability of surfactants a 'soft' and a 'hard' standard may be used, the soft standard being degraded more than 90%, and the hard standard not more than about 35%. If either of the standards fails to meet the requirements, the test should be repeated.

I12 Alternative Apparatus

I12.2 WRC porous-pot units[41]

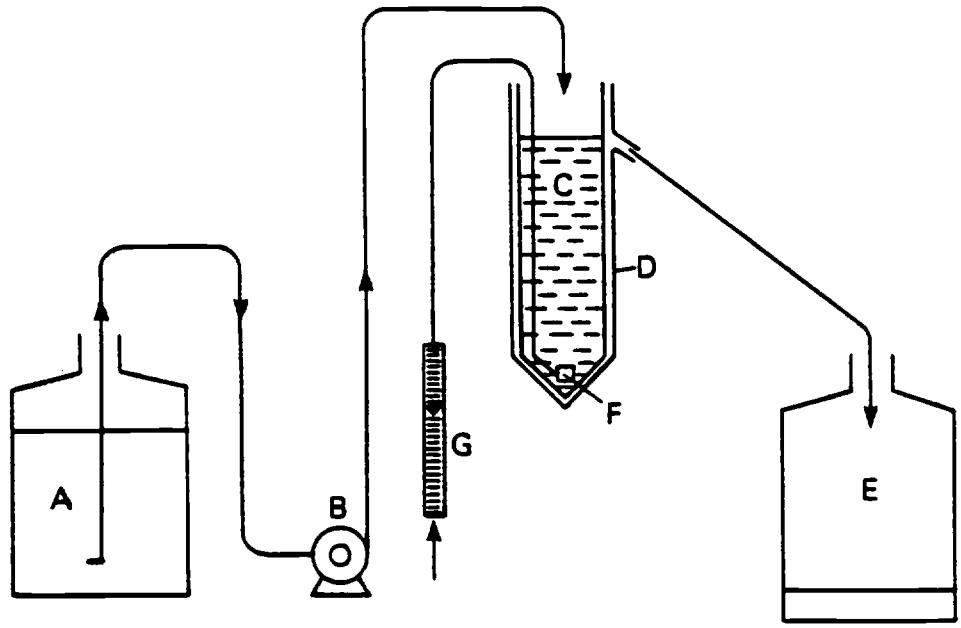
These units differ from the EEC units in one essential point; there is no settlement vessel, most of the solids in the mixed liquor being retained in the aeration vessel by the porous walls.

The porous pots are constructed from sheets of porous polythene (2 mm thick, maximum pore size 95 μm), which are made into cylinders 14 cm in diameter with a conical base at 45° (Figs. 13 and 14). The porous pot is contained in an impervious PVC vessel (D) 15 cm in diameter with an outlet at a height of 17.2 cm on the cylindrical part, which determines the volume of 3 l in the porous pot. There is a PVC supporting ring round the top of the porous pot, so that there is an effluent space (of 0.5 cm) between the inner and outer vessels.

The porous-pot units are set in sockets in the base of a thermostatically-controlled water bath. There is a metered air supply to the apex of the porous pot (G) in which there is placed a diffuser. A second diffuser may be added if necessary to keep the solids in suspension.

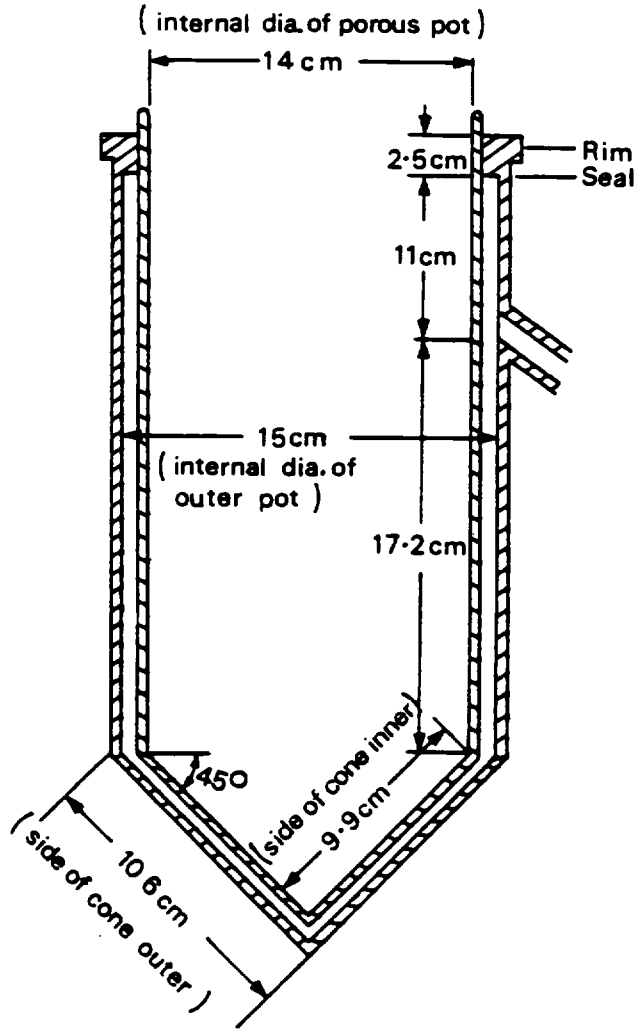
Synthetic (or settled domestic) sewage is pumped from a storage container (A) to the porous pot through suitable tubing (e.g. silicone rubber), using a multi-channel peristaltic pump. The effluent passes through a tube into another polythene container for collection.

FIG.13 EQUIPMENT USED FOR ASSESSMENT OF BIODEGRADABILITY
(Porous pot)



A. Storage vessel ; B. Dosing pump ; C. Porous aeration vessel
D. Outer impermeable vessel ; E. Effluent collection vessel
F. Diffuser ; G Flow meter

FIG.14 DETAILS OF 3 LITRE POROUS-POT AERATION VESSEL



Step	Procedure	Notes
I12.2.1	The method for the porous pot units is similar to that for the EEC units (section I8).	
I12.2.2	The pump should be set to deliver 1 l synthetic sewage per hour to the aeration vessel. The pump rollers should be lubricated (note a).	(a) Glycerol or olive oil may be used for lubrication. Pump tubing should be replaced at the first signs of wear (stretching or splitting) – approximately once a week. Connecting nipples and influent tubing should be cleaned out regularly (about twice weekly) to remove bacterial growth.
I12.2.3	When the vessel is full it may be inoculated, if necessary (note b).	(b) 3 ml of effluent, soil extract or activated sludge may be used (see I8.2.2(e)).
I12.2.4	The air flow to each porous pot is set at about 2.5 l/min, and the temperature is maintained in the required range (usually 18–25° C).	
I12.2.5	The sludge accumulating above the meniscus should be scraped down at least twice daily.	
I12.2.6	The porous pot should be changed at the first sign of blocking of the pores, i.e. when the mixed liquor level rises above the effluent spout. A fresh porous pot is placed in the outer container and all the mixed liquor carefully transferred. Any sludge sticking to the sides of the blocked pot is also scraped off and transferred. The blocked pot is thoroughly cleaned before re-use (note c).	(c) A fine jet of water may be used to remove any remaining sludge from the pots before they are soaked for about 24 h in a 1:1 dilution of industrial grade sodium hypochlorite. The pots should be totally immersed. After 24 h, the pots are removed, rinsed thoroughly with water and then soaked for about 24 h in water. If there is any remaining odour of chlorine the soaking in water is continued until it is removed or it may be removed with thiosulphate.
I12.2.7	The MLSS are determined about twice weekly and the mixed liquor wasted to maintain an approximate concentration of 2.5 g suspended solids/l.	NOTE The use of hypochlorite is hazardous and appropriate eye shields and gloves must be worn when handling the chemical.
I12.2.8	Analyses are carried out as required (see I8.3 and I8.4).	

I12.3 **Advantages of the Porous Pot**

The advantages of the Porous Pot method are that the apparatus is cheaper to construct than the EEC-type unit, easier to place in a water bath for temperature control, and the effluents produced have lower concentrations of suspended solids.

Extensive comparative tests at the WRC using four synthetic detergents have shown that the degree of biodegradation and the precision obtained with the porous pots were not significantly different from those obtained with the EEC units (Section I1.4).

I12.4 **Other alternative apparatus**

In the EEC apparatus alternative pumps have been successfully used. For example, the peristaltic pump can be conveniently replaced by a reciprocating or piston-type pump and the air pump by a peristaltic pump. The test substance may be added from a separate reservoir via a suitable pump if changes are likely to occur in mixing it with sewage and/or standing over the 24 h period.

I13 Type of Sewage

Because municipal sewages vary widely in composition and are not freely available, and for reasons of hygiene, a standard synthetic bacteriological medium (I5.1) was adopted as the "sewage" in the EEC test. This synthetic sewage also has the merit that it should allow more reproducible results to be obtained in various laboratories than if municipal sewages are used. However, it has some limitations; for example, it is not well buffered, it allows only low growth rates, and it gives rise to bacterial communities having a narrower ranger of species than normal activated sludges, since, unlike real sewage, it contains fewer bacteria.

The lack of buffering capacity, which is of special importance for nitrification, can readily be overcome by the addition to the synthetic sewage of suitable concentrations of bicarbonate and carbonate.

An alternative to the EEC synthetic sewage is 'detergent-free' sewage[42]. This sewage is adequately buffered, but again allows only low growth rates of sludge compared with those afforded by domestic sewage[43].

For a number of purposes, domestic sewage, containing little or no industrial wastes, may be successfully used for determining the degree of removal. Sewages containing more than a small proportion of industrial wastes should be avoided, since bacterial inhibitors may be present and spurious results may be obtained. Domestic sewage should be taken daily from the overflow of the settlement tank, and kept aerobic before use.

I14 Modifications in Operation

I14.1 **The dosing rate** of 1l/h in the EEC test gives an average liquid retention of 3 h, which is low by UK standards where retention times of 6–12 h are much more common. The shorter retention time ensures that if the compound is degraded sufficiently in the EEC test, it will also be degraded in practice at longer retention times, provided that bacterial inhibitors are not present in the sewage. Thus, for some purposes it is permissible to increase the retention time, especially if municipal sewage is used for the test.

I14.2 The specified **temperature** range of 18–25°C does not cover the sewage temperatures normally encountered in the UK and it is sometimes necessary to reduce the operating temperature to 10°C to ensure that no difficulties will arise in practice in UK winters. For example, the alkyl phenol ethoxylates are degraded to a much lesser extent at 10° than at 18°.

I14.3 **The concentration of mixed liquor suspended solids** of 2.5 g/l is sometimes reached quicker in the absence of the test compound and the practice in some laboratories is to begin the test without addition of the test substance which may be toxic. It is also sometimes helpful to add the substance initially at a very low concentration and to increase it incrementally over an acclimatization period to the required concentration. However, time may be necessary for the sludge to acclimatize to the test substance, so that this practice may not reduce the "running-in" period. When a concentration of 2.5 g MLSS/l has been reached, constant wasting e.g. of one tenth to one sixth of the mixed liquor per day may result in steadier DOC values.

I14.4 The maximum time allowed for **acclimatization** in the EEC test is 6 weeks, after which time the substance is judged to be not very degradable if less than, say, 80% has been removed. It has been shown, however, that some compounds are eventually well degraded if the acclimatization period is extended to 12 weeks. It is thus advisable to continue the test in cases of compounds of economic importance.

I15 Chemical Tests for Compound

For compounds for which there is no readily available specific analytical method at the concentrations employed and in cases where further information is required, the removal may be followed by the use of a suitably sensitive and precise method of determining dissolved organic carbon (DOC)[23].

The principle of the method is that the DOC of the sewages entering and the effluents leaving control and experimental plants are determined. The difference between the DOC of the two influents equals that of the added test compound and similarly, the difference between the two effluent values equals that of the test compound remaining, from which the degree of removal can be calculated.

It must be borne in mind that DOC of the added compound is usually small (approx. 5–20 mg/l) compared with the DOC of the sewage (EEC synthetic sewage 105 mg/l) so that great care must be taken in making the analyses. If the analytical method is not sufficiently precise, the DOC of the test substance in the sewage may have to be assumed. (See Section I9.2).

For readily biodegradable compounds, the interpretation of DOC removed is unequivocal but when apparent low degrees of removal are obtained the results are difficult to interpret. The compound may truly be removed to only a small extent or it may have been converted to another compound which is not easily biodegradable. There is also the possibility that it has been removed but has interfered with the removal of other compounds present in the sewage. If a 'negative' amount of the compound has been removed – that is, the difference between the DOC of the effluents is greater than that between the influents (i.e. more than the DOC due to be added test substance) – it may be concluded that the substance has not been biodegraded and has interfered with the removal of other substances present in the sewage. This matter is dealt with in the booklet on treatability and toxicity of chemicals[19].

J Continuous Simulation (Biological Filter) Test for the Assessment of Biodegradability

Introduction

Simulation tests, relating to the activated sludge method of sewage treatment, have been described in Method I: this test refers to the biological filter method. It can be applied to a substance which has failed a screening test for ready biodegradability (i.e. methods D, E or F), but passed an inherent biodegradability test (e.g. method H), or to any substance for which additional information is required relating to the biological filter method of sewage treatment.

The method is based on the continuous test originally described by Gloyna[44] and developed by Tomlinson and Snaddon[45].

Since this is a fairly expensive and time-consuming test, it is usually only resorted to in the case of substances of economic importance, i.e. those expected to have a large production, or of local significance.

Much of the initial work on rotating tubes was based on the study of the biodegradability of surfactants, whereas the method described here is extended to other substances. The implications and problems of the use of DOC analysis are discussed in Section J13.

Strictly, simulation methods determine bioelimination, but ways of distinguishing between true biodegradation and physical absorption are outlined in Section J10.5.

J1 Performance Characteristics

J1.1 Property determined	The primary or ultimate biodegradability of organic materials, expressed as the percentage removal of substance or DOC.
J1.2 Type of sample	Single compounds which are soluble at concentrations used in the test, and non-volatile.
J1.3 Basis of method	A determination of the removal of test substance in laboratory scale plants resembling the biological filter process.
J1.4 Standard Deviation	*Within test 3.5% Between tests 5.0%
J1.5 Limit of detection	Limited by the analytical method for test substance and by biological variation.
J1.6 Interferences	Inhibitors of bacterial growth of any substance interfering in the analytical method used. (See Section 3.)
J1.7 Time required for determination	A maximum of nine weeks. Operator time for a 6 tube installation (excluding analysis) approximately 12 hours/week.

* Data obtained by the Yorkshire Water Authority.

J2 Principle

Synthetic sewage containing the required concentration of test substance is applied to the internal surface of a slowly rotating inclined tube. A layer of micro-organisms similar to that present on the surface of the biological filter media is built up on the internal surface of the tube. The effluent from the tube is collected and either settled or filtered before analysis for the test substance by a specific method or by dissolved organic carbon measurements (see Section 13). (The settlement/filtration is accepted as in no way simulating the full scale process but is necessary for standardization of technique.) Control units receiving no test substance are operated in parallel for comparative purposes. These are essential if the ultimate biodegradation of the test substance is to be assessed by DOC analysis and biodegradation is assessed from the relative removal of DOC in the two units.

J3 Interferences

Any chemical substance in solution or in the air may adversely affect the growth of sludge micro-organisms. Examples are: organic solvents, toxic metals, strong acids and alkalis, biocides.

Substances strongly adsorbing to the walls of the tubes may give false removal values. Substances which interfere in any specific chemical analytical method used may give false results.

J4 Hazards

J4.1 Hygiene

Whether the microbial film arises from an airborne infection or by deliberate inoculation of the synthetic sewage, it must be remembered that the population is unknown and some of the micro-organisms present in the film may be potential pathogens. It is therefore necessary to take appropriate precautions when carrying out plant maintenance and handling samples.

J4.2 Mechanical and electrical

Guards should be fitted on peristaltic pumps to prevent catching fingers in the moving rollers, and electric stirrers and pumps should be guarded from splashes and leaks.

J4.3 Chemicals

If the test substance is toxic or its properties are unknown it should be handled with care. Mercuric chloride, used to preserve samples, is toxic and should also be handled with care.

J5 Reagents

J5.1 Synthetic sewage

The synthetic sewage is composed as follows for each 1 l of tapwater:–

160 mg peptone
110 mg meat extract
} or 270 mg of commercial peptone-meat extract preparation,
30 mg urea, 7 mg sodium chloride, 4 mg calcium chloride dihydrate, 2 mg magnesium sulphate heptahydrate, 28 mg dipotassium hydrogen phosphate. The chemicals should be analytical reagent grade.

For convenience, this may be prepared as a 100 × concentrated solution, which can be stored at 1°C for up to 1 week, and the synthetic sewage made daily from this by appropriate dilution with tap water.

The synthetic sewage after dilution contains approximately 106 mg/l organic carbon, 46 mg/l nitrogen, 5 mg/l phosphorus.

The pH of the applied sewage should be in the range 7.0–7.5 and any adjustment necessary to achieve this should be done using dilute hydrochloric acid (1M) or dilute sodium carbonate/bicarbonate (1M).

Other sewages may also be used (see Section J12).

J5.2 Stock solution of test substance

An appropriate solution which on addition of a convenient volume (e.g. 50–100 ml) to the synthetic sewage concentrate will, on dilution, result in the required concentration

(e.g. 10–20 mg/l) in the final mixture or, if a separate dosing pump is used for the test substance, a stock solution should be prepared which will give the required final concentration of test substance in the synthetic sewage.

J5.3 **Mercuric chloride**

1.0% w/v solution of mercuric chloride for preservation of samples when necessary.

J5.4 **Lubricant**

Glycerol or olive oil may be used for lubricating the peristaltic pump rollers: both are suitable for use on the silicone-rubber tubing.

J6 Apparatus

J6.1 **Rotating tubes**

The apparatus (see Figs 15 and 16) consists of a bank of perspex tubes 30.5 cm long × 5 cm internal diameter, supported on rubber-rimmed wheels within a metal supporting frame. The tubes (C) have an outside lip approximately 0.5 cm deep to retain them on the wheels, the internal surface is roughened by abrasion with coarse wire wool, and there is a 0.5 cm deep internal lip at the feed end to retain the liquid. The tubes are inclined at an angle of approximately 1° to achieve the required contact time when the test medium is applied to a clean tube. The rubber tyred wheels are rotated using a slow variable speed motor. The temperature is controlled by installation in a constant temperature room. A 24–hour supply of synthetic sewage with added test substance, where applicable, is contained in a 20 litre storage vessel (A).

A storage vessel has an outlet near the bottom and is connected by silicone rubber tubing via a peristaltic pump (B) to a glass or acrylic delivery tube which enters 2–4 cm into the higher end of the inclined tube.

Effluent is allowed to drip from the lower end of the inclined tube to be collected in another storage vessel (D), and is settled or filtered before analysis.

J6.2 **Membrane filtration apparatus**

Membrane filters (pore size 0.4 μm) are suitable if they neither release carbon, nor adsorb the test substance.

J7 Sample Collection and Preservation

J7.1 Samples of material or effluent to be tested should not have preservative added to them prior to being tested.

J7.2 A sample of synthetic sewage containing the test sample (where applicable) is taken after approximately six hours settlement in the feed tank and preserved, if necessary with 40 mg/l mercuric chloride* by adding 2.0 ml of 1% w/v mercuric chloride to 500 ml of sample.

A further sample may be taken 16 hours later if it is considered that the composition may vary in that time. The mean of the two analyses is then used in calculations: this takes account of any changes in the feed during the day.

J7.3 The effluent from the tube is collected over a period of approximately 16 hours starting at the time of the intitial sampling of the feed solution. The bulk effluent is thoroughly mixed before a sample is removed for settlement and/or filtration prior to analysis. If samples are to be preserved, but BOD determinations are not required, the effluent may be collected directly into mercuric chloride solution (2.0 ml of 1% w/v for each 500 ml).

J7.4 To remove mercury from laboratory waste, first, if present, destroy any organic matter. For suitable digestions see reference 46, cool the digest, and cautiously pour it into at least a 10:1 excess of water. Then proceed as detailed in reference 34.

*NOTE Samples for BOD analysis should not be treated with mercuric chloride.

FIG.15 ROTATING TUBES

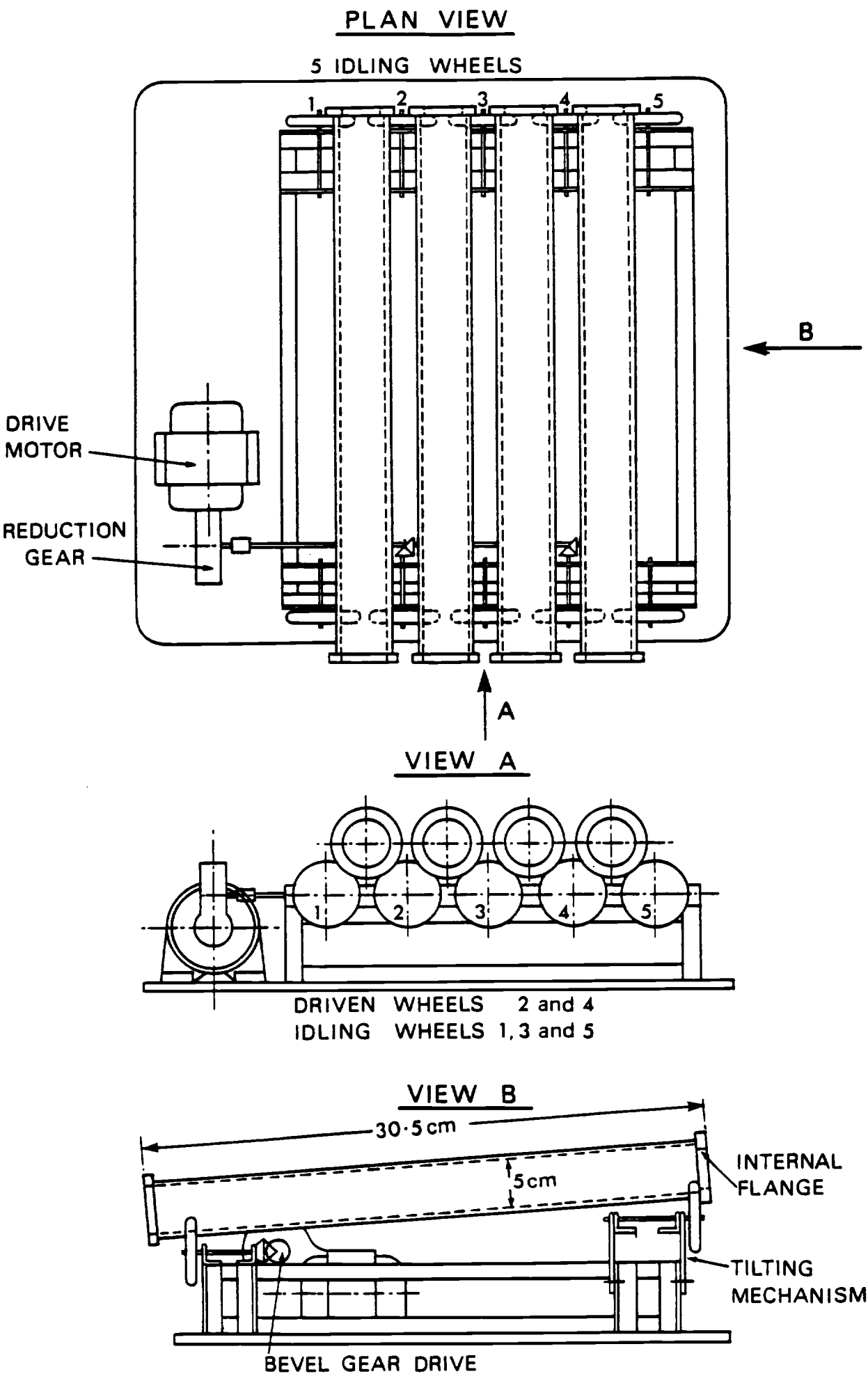
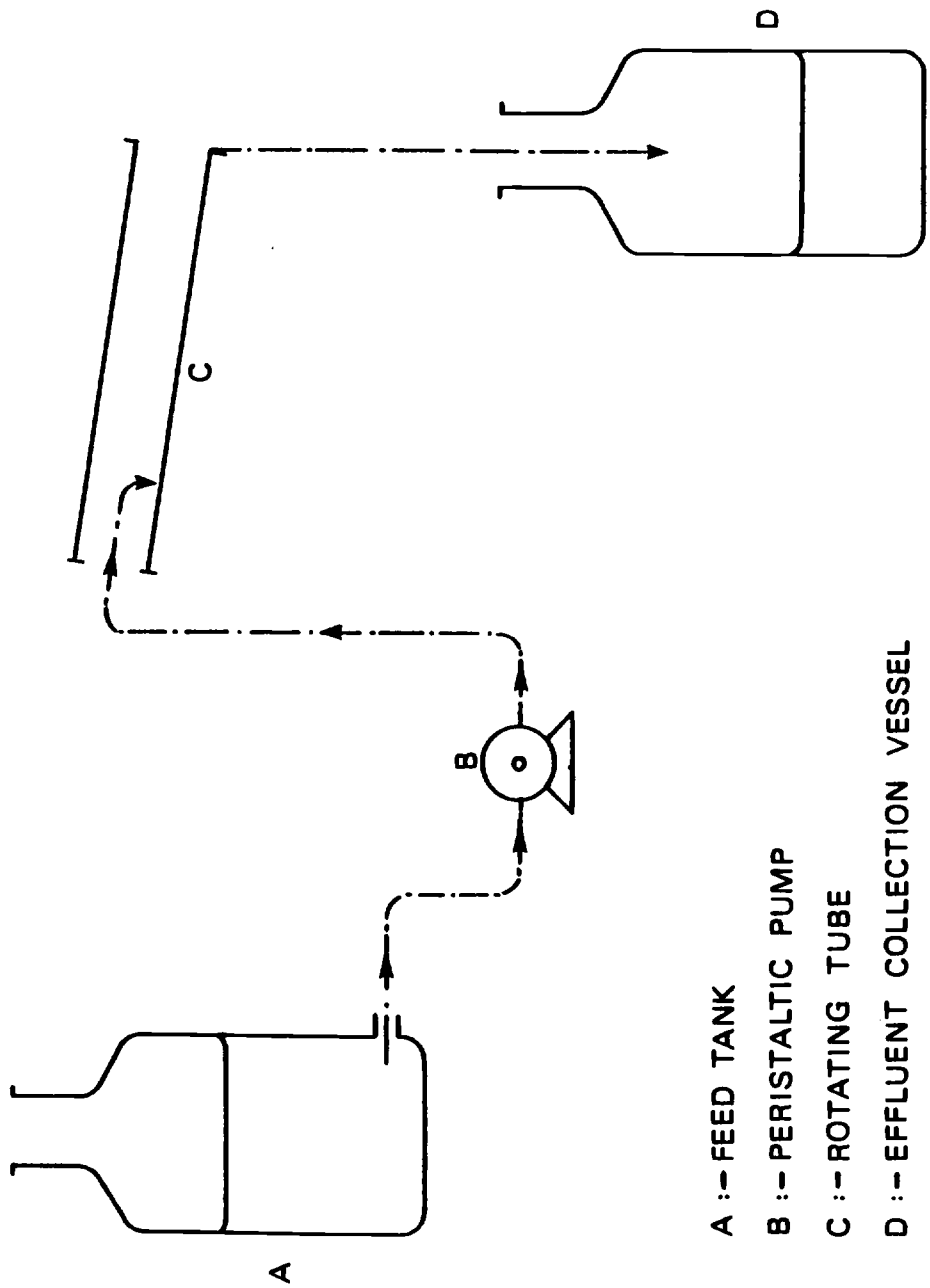


FIG.16 FLOW DIAGRAM



Step	Procedure	Notes
	Preparation of Sewage	
J8.1	Synthetic sewage is freshly prepared each day from a concentrated stock solution (J5.1) by appropriate dilution. The required quantity is measured in a cylinder and added to a clean influent vessel (note a) for the controls. The required volume of solution of test substance is also added to the sewage, before dilution, for the test tubes, (note b).	(a) Influent and effluent containers and feed tubing should be thoroughly cleaned to remove bacterial (influent) or algal (effluent) growths. (b) It may be more convenient to prepare a separate solution of test substance and deliver this to the inclined tubes via a different dosing pump.
J8.2	Operation of Rotating Tubes	
J8.2.1	Two identical pairs of tubes should be used.	
J8.2.2	The peristaltic pump (note c) is controlled to deliver 250 ± 25 ml/h of synthetic sewage into the inclined tube which is rotated at a speed of 18 ± 2 revolutions per minute.	(c) Glycerol or olive oil may be used to lubricate pump tubes.
J8.2.3	The angle of inclination of the tubes is adjusted to produce a mean residence time of 125 ± 12.5 sec for the mixed feed in a clean tube (note d). The temperature is maintained in the range 18–25°C.	(d) The residence time may be estimated by addition of a solution of a non-biodegradable marker (e.g. sodium chloride or an inert dye) to the feed, and the time taken to reach the peak concentration in the effluent is regarded as the residence time.
J8.2.4	An airborne inoculum may be sufficient to start the growth of micro-organisms in some cases, but, if inoculation proves necessary, 1 ml per litre of settled sewage may be added to the feed for 3 days.	
J8.3	Sampling	
	A sample of feed is taken in accordance with J7.2, the volume being dependent on the analysis required (note e).	(e) A minimum volume of 250 ml is collected in 500 ml measuring cylinder.
	A sample of effluent is taken in accordance with J7.3, the volume again depending on the analysis to be undertaken (note e) this sample is either settled (note f) or filtered (note g) before analysis.	(f) For BOD and/or COD analysis the sample is allowed to settle for 60 minutes before removal of sufficient quantity for analysis from below the surface to avoid possible floating solids. (g) For DOC analysis the sample is filtered through a coarse filter (e.g. Whatman No 1), and the first portion of filtrate rejected before collection of the remaining filtrate which is then filtered through a suitable membrane (e.g. polycarbonate, pore size 0.4 μm).
	Checking performance of the tubes	
J8.4	Influent and effluent samples are taken e.g. twice weekly during the 'running-in' period of the test (note h). The required analyses are carried out, e.g. COD, BOD, DOC and specific determination of test substances.	(h) 'Running-in' period is the time taken for the surface film to reach a "steady state". It is usually about 2 weeks, but should not exceed 6 weeks. At this time the COD removal from the control tubes is 80 ± 4%.
	Steady State	
J8.6	Daily analyses (5 times weekly) over a period of 3 weeks are used for the determination of test substance in influent and effluent (note i).	(i) During the 'steady state' period the surface film may slough from the tube surface. If this does occur, the period for collecting of analytical data should cover at least 2 full cycles (see Section J10.4).

J9 Calculation of Biodegradation

J9.1 For primary biodegradation, the percentage removed is calculated from the following formula:

$$\text{percentage removal} = \frac{C_S - C_E}{C_S} \times 100$$

where C_S = the concentration of test substance in the synthetic sewage
and C_E = the concentration of test substance in the effluent.

For ultimate biodegradation the concentration of DOC in the test and control units is compared. Ideally,

$$\text{percentage removal} = \frac{(C_{ST} - C_{SB}) - (C_{ET} - C_{EB})}{(C_{ST} - C_{SB})} \times 100$$

where C_{ST} = the concentration of DOC in the test synthetic sewage
C_{SB} = the concentration of DOC in the control synthetic sewage
C_{ET} = the concentration of DOC in the test effluent
C_{EB} = the concentration of DOC in the control effluent

J9.2 However, the concentration (as DOC) of test substance added cannot be adequately determined (see Section J13) or if the test substance is added separately, its concentration must be estimated, let this be C_T.

$$\text{Then, percentage removal} = \frac{C_T - (C_{ET} - C_{EB})}{C_T} \times 100$$

J10 Sources of Error

J10.1 Operating difficulties

During the weeks in which intensive analysis is done the plants should be free from operational difficulties. The results are valid only if performance is steady. If any of the difficulties described below are encountered, the analysis should be extended and the results of three weeks' continuous steady running should be used. If a steady state is not reached after six weeks, assume that the test substance is not treatable, or that it has interfered in the removal of synthetic sewage components – or both. These are distinguished by carrying out analysis for test substance and COD/DOC. The concentration of test substance used may be inhibitory to the sludge, so it may be necessary to restart the test using a lower concentration, or to extend the test allowing a longer period of acclimatization.

J10.2 Synthetic sewage

Generally, one batch of peptone and meat extract should be used throughout the test to minimize any change in constituents which may arise by different states of purity, or from different protein sources.

The concentration of phosphate in the synthetic sewage is insufficient for adequate buffering of the mixed liquor if complete nitrification (an acid producing reaction) occurs and as a result the pH value may fall sufficiently to reduce the degree of removal of COD and perhaps of the substance under test. This may be remedied by adding suitable concentrations of bicarbonate to the synthetic sewage, using the fact that the oxidation of 1 mgN to nitrate produces an acidity equivalent to about 7 mg $CO_3^=$.

J10.3 Loss of test substances

Adsorption of test substance onto the microbial film may lead to apparently high removals at an early stage in the test.

J10.4 Sloughing

This phenomenon, the sudden removal of large quantities of solids from the tubes, takes place at relatively regular intervals, but to ensure that this does not affect the comparability of results, at least 2 full cycles of growing and sloughing should occur during an experiment. If any adsorption is taking place (J10.5) the 'sloughing' may give a sudden change in removal of test substance.

J10.5 **Bioelimination**

This method measures bioelimination, and so it may be necessary to distinguish between true biodegradation and physical adsorption of test substance onto the sludge. Adsorption, if it occurs, is usually most marked at the start of the test and generally an equilibrium is reached, so it may be identified by careful examination of the course of bioelimination. Biodegradation is usually shown as a steady upward trend of removal of test substance to a constant plateau at the 'steady state'. However, a special investigation is necessary to determine the extent of adsorption.

J11 Checking the Accuracy of Results

The accuracy of the method can be checked by running the plant with standards of known biodegradability, e.g. for primary biodegradability of surfactants a 'soft' and a 'hard' standard may be used, the soft standard being degraded by more than 90% and the hard standard not more than about 35%. If either of the standards fails to meet the requirements, the test should be repeated.

J12 Type of Sewage

Because municipal sewages vary widely in composition and are not freely available and for reasons of hygiene, a standard synthetic bacteriological medium (J5.1) was adopted as the "sewage" in the test. This synthetic sewage also has the merit that it should allow more reproducible results to be obtained in various laboratories than if municipal sewages are used. However, it has some limitations; for example, it is not well buffered, it allows only lower growth rates, and it gives rise to bacterial communities having a narrower range of species than normal activated sludges, since unlike real sewage it contains fewer bacteria.

The lack of buffering capacity, which is of special importance for nitrification, can readily be overcome by the addition to the synthetic sewage of suitable concentrations of bicarbonate and carbonate.

For a number of purposes, domestic sewage, containing little or no industrial wastes, may be successfully used for determining the degree of removal. Sewage containing more than a small proportion of industrial wastes should be avoided, since bacterial inhibitors may be present and spurious results may be obtained.

Domestic sewage should be taken daily from the overflow of the settlement tank and kept aerobic before use.

J13 Chemical Tests for Compound

For compounds for which there is no readily available specific analytical method at the concentrations employed and in cases where further information is required, the removal may be followed by the use of a suitably sensitive and precise method for determining dissolved organic carbon (DOC).

The principle of the method is that the DOC of the sewages entering and the effluents from control and experimental plants are determined. The difference between the DOC of the two influents equals that of the added test compound and similarly, the difference between the two effluents values equals that of the test compound remaining, from which the degree of removal can be calculated.

It must be borne in mind that the DOC of the added compound is usually small (approx. 5–20 mg/l) compared with the DOC of the sewage (EEC synthetic sewage 105 mg/l), so that great care must be taken in making the analyses. If the analytical method is not sufficiently precise, the DOC of the test substance in the sewage may have to be assumed (see Section J9.2).

For readily biodegradable compounds, the interpretation of DOC removed is unequivocal but, when apparent low degrees of removal are obtained, the results are difficult to interpret. The compound may truly be removed to only a small extent or it may have been converted to another compound which is not easily biodegradable. There is also the possibility that it has been removed but has interfered with the removal of other compounds present in the sewage. If a 'negative' amount of the compound has been removed – that is, the difference between the DOC of the effluents is greater than that between the influents (i.e. more than the DOC due to the added test substance) – it may be concluded that the substance has not been biodegraded and has interfered with the removal of other substances present in the sewage. This matter is dealt with in the booklet on treatability and toxicity of chemicals[19].

K Isolation of Bacteria Capable of Degrading Specific Chemicals

Introduction

It may be that some compounds which have been found to be biodegradable, especially those which exhibit only primary degradation require further study, e.g. identification and investigation of intermediate metabolic products. In these cases it is useful to be able to work with enriched or even pure cultures of the relevant micro-organisms, since this will yield higher kinetic rates of degradation and higher concentrations of intermediates: such a method is described here.

K1 Performance Characteristics

K1.1 *Purpose*	The isolation of bacteria capable of degrading a test compound.
K1.2 *Type of sample*	The method is suitable for soluble, non-volatile compounds which are not toxic to micro-organisms at concentrations of 100 mg/l.
K1.3 *Basis of method*	Exposure of a mixed population of micro-organisms to a test compound as a sole source of carbon and energy for growth followed by enrichment of competent micro-organisms by transfers to fresh media.
K1.4 *Application*	The method is suitable for aerobic fresh-water bacteria but not for the isolation of fungi or marine bacteria.
K1.5 Time required	Up to 3 months. Operator time is about 4 h/week for screening up to 5 compounds.

K2 Principle

The test is based on the conventional microbiological method of enrichment culture in which a mixed population of micro-organisms is exposed to a test compound as a sole cource of carbon and energy. By transference of small aliquots of culture to fresh media at regular intervals those micro-organisms which cannot utilize the test compound are diluted out and competent micro-organisms, if present, are concentrated or 'enriched'.

In order to achieve simplicity, bacterial growth is estimated visually and hence a substrate concentration sufficient to support visible growth is used – this is about 100 mg/l with a readily utilizable substrate. Toxicity of the compound should be checked (e.g. by using Method B) and the highest concentration not giving toxic effects should then be used.

K3 Limitations

K3.1 The pH of the medium makes it unsuitable for the isolation of fungi.

K3.2 The medium described is unsuitable for the isolation of bacteria from marine environments.

K3.3 Insoluble compounds or compounds which are toxic to bacteria at the recommended concentrations cannot be used.

K4 Hazards

K4.1 **Hygiene**

Micro-organisms isolated may be potential pathogens and therefore appropriate precautions should be taken.

K4.2 **Test substance**

The properties of the test substance may not be fully documented and samples should thus be handled with care in case they are hazardous.

K5 Reagents

Analytical grade reagents should be used.

K5.1 **Distilled or de-ionized water.**

K5.2 **Stock salts solutions**

(a) 1 M potassium dihydrogen orthophosphate adjusted to pH 7.0 with 5 sodium hydroxide.

(b) 10% W/v ammonium sulphate.

Store both solutions at 4°C in glass bottles in the dark and discard at the first sign of biological growth or other sediment.

K5.3 **Trace elements solution**

For each litre of solution required, take about 800 ml of distilled water and add the following: 1.25 g calcium carbonate, 0.25 g zinc oxide, 3.38 g ferric chloride hexahydrate, 0.62 g manganese chloride tetrahydrate, 0.11 g copper chloride dihydrate, 0.15 g cobalt chloride hexahydrate, 0.04 g boric acid, 63.5 g magnesium chloride hexahydrate and 8.28 ml hydrochloric acid d_{20} 1.18, stir until dissolved, make up to 1 litre with distilled water and store in a glass bottle at 4°C in the dark.

K5.4 **Stock solution of test substance**

10 g/l in distilled or deionized water.

K5.5 **Source of micro-organisms**

Any source of aerobic micro-organisms may be used, e.g. soil, mud, sewage, etc.

K6 Apparatus

K6.1 **Wide-necked conical flasks** of 100 ml capacity.

K6.2 **Shaking machine** to accommodate flasks, either having automatic temperature control or used in a constant temperature room.

K6.3 **Cotton wool plugs.**

K6.4 **Pipettes** plugged loosely with cotton wool.

K7 Procedure

K7.1 To 800 ml distilled water add 40 ml potassium dihydrogen phosphate stock Solution (K5.2(a)), 12 ml ammonium sulphate stock solution (K5.2 (b)), 2ml trace elements solutions, 50 ml of test substance solution.

Make up to 1 litre with distilled water.
Repeat this for as many substances as are to be tested.

K7.2 Dispense 20 ml aliquots of the appropriate medium into the requisite number of 100 ml flasks. The number of flasks required will depend on the number of sources of inoculum used — for each source quadruplicate flasks should be used.

K7.3 Inoculation

Add approximately 1 g of the material to be used as inoculum to each of the replicate flasks and loosely plug the flasks with cotton wool.

K7.4 Incubation

Place the inoculated flasks on a suitable shaker operated at approximately 200 rpm and incubate at 20–25°C.

K7.5 Sub-culturing

At approximately 7–day intervals remove 0.5 ml aliquots from each flask and transfer to fresh media of the same composition. Incubate the freshly inoculated flasks as described above. Repeat the transfer of culture aliquots to fresh medium every 7 days until termination of the experiment.

K7.6 Estimation of growth

After the first 2–3 transfers of material to fresh medium the inoculating material (i.e. mud, sludge, soil, etc.) will be diluted out and the medium will be comparatively clear thus allowing visible assessment of bacterial growth. Growth is indicated by a cloudiness of the medium and can be scored visually as described below:–

Medium	Scoring
Clear	–
Slightly opaque	+
Cloudy	++
Very cloudy	+++

K7.7 Duration of the experiment

Cloudiness of the medium is indicative of bacterial growth providing that any colour or opacity due to the incoculum has been diluted out. When an aliquot of cloudy medium is transferred to fresh medium the cloudiness should re-develop after a few days incubation if the cloudiness is due to bacterial growth. At this time the clouded medium should be used to provide an inoculum for a biodegradation test.

If no cloudiness is observed in media over a period of 1–3 months, i.e. 4–12 transfers of material, then the test should be scored as negative and more complex isolation procedures should be adopted.

K7.8 Biodegradability testing

Cloudy medium, where this has developed, should be used to provide an inoculum for one of the biodegradation tests described earlier in this booklet.

Alternatively, a simple biodegradation test may be performed in the same medium after scaling up, as follows:–

Inoculate 250 ml medium in a 1 litre Erlenmeyer flasks with 20 ml of cloudy medium from the enrichment experiment and incubate as described above. At 1–2 day intervals remove samples from the flask, filter through a membrane filter (0.2 μm) and determine the organic carbon content of the filtrate.

K8 Calculation of results

(for the alternative in K7.8)

The percentage biodegradation of the sample is calculated from the following formula:

$$\% \text{ biodegradation} = \frac{(C_o - C_t)}{C_o} \times 100$$

where C_o = mean initial organic carbon concentration in culture filtrate
C_t = mean concentration of organic carbon in culture filtrate at time t.

In this way a curve of % biodegradation against time can be constructed and the experiment should be terminated when the curve reaches a plateau.

K9 Interpretation of results

If, after a number of transfers the medium becomes cloudy within 4–5 days after inoculation this is indicative of microbial degradation of the test compound. The degree of degradation effected can be determined by using the culture as an inoculum into a standard biodegradation test. If appreciable biodegradation is then observed this is indicative of the presence of competent bacteria in the material from which isolations were made. It does not necessarily indicate that the test compound will degrade in the environment but simply that micro-organisms capable of degrading the test compound were present in that environment from which the inoculum was taken.

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The compilation of these methods was originally proposed by the Standing Technical Committee on Synthetic Detergents. The work of selecting, drafting and testing these methods was started by the Analytical Working Group of the Technical Sub-Committee of the STCSD, but on the formation of Working Groups 7 and 8 of SCA in 1975 was transferred to panels 7.5 and 8.4 dealing with waters and sludges respectively. These were soon amalgamated as panel 7.5 reporting to Working Group 7, Biological Methods.

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