a test method to assess the 'inherent' biodegradability of oil products

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Brussels
October 1999

ABSTRACT

This report describes the development of a test method for assessing the potential of oil products to be biodegraded in the environment ('inherent' biodegradability). The method produced was based on International Standard ISO 14593 ('CO_2 Headspace Test') but used a pre-exposed inoculum and an extended test duration. The work of the CONCAWE Biodegradation Task Force is summarised and details are given of an international ring test which demonstrated the practicality of the new method and determined its precision. As a preliminary to submission of the method to the Organisation for Economic Co-operation and Development (OECD) for consideration as a new test guideline for assessing 'inherent' biodegradability, the method has been prepared in 'OECD format' (included as an appendix to this report).

During the course of the Task Force's work, CONCAWE funded a one year project with the University of Hull to evaluate techniques for measuring the incorporation of test substance carbon into new microbial biomass during biodegradation under the conditions of the CONCAWE test method. The report also describes this research and summarises its main findings.

KEYWORDS

oil products, inherent biodegradability, ultimate biodegradation, carbon dioxide evolution, CO_2 headspace biodegradation test, ISO 14593, CONCAWE biodegradation test

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1. INTRODUCTION

There is a growing need for information on the environmental fate of oil products for the purposes of hazard classification and risk assessment. If accidentally spilled or released into the environment, biodegradation may be a significant loss process for petroleum products. Biodegradation is a process by which organic substances are broken down by micro-organisms (principally bacteria and fungi). Microbial attack can result in simply a change in the chemical structure of the substance resulting in the loss of some specific property ('primary' biodegradation) or more extensive breakdown to end-products such as carbon dioxide (CO_2), water (H_2O), inorganic salts and new microbial cells ('ultimate' biodegradation). The susceptibility of a substance to undergo biodegradation is known as its 'biodegradability'.

Currently, the biodegradability of an oil product is often assessed using either the CEC L-33-A-93 test [1] or a test for so-called 'ready' biodegradability. This latter term is applied to a substance which has passed a stringent test for ultimate biodegradability such as the OECD 301 B 'CO_2 Evolution (Modified Sturm) Test'. [2] Although the CEC test was developed for two-stroke outboard engine lubricants only, it is widely used to assess the biodegradabilities of formulated lubricants, base oils and additives. However, the method measures 'primary' biodegradation and CEC test results can sometimes give an overestimation of the role of biodegradation as a removal mechanism for an oil product in the environment. [3] In contrast, 'ready' biodegradability tests such as those detailed in the OECD 301 Test Guidelines, [2] measure the 'ultimate' biodegradation of the test substance. However, these tests were developed to identify substances that would be rapidly and extensively biodegraded in the aquatic environment and their stringency (see Section 2.3) means that the true extent of an oil product's biodegradability can often be underestimated.

In fact, many oil products are not 'readily' biodegradable and information on their potential to be biodegraded (so-called 'inherent' biodegradability) is perhaps more useful when assessing their potential environmental impact. In recent years, there has also been a need for data on the "inherent" biodegradability of the performance-enhancing additives used in formulated lubricants, to meet the requirements of labelling schemes for classifying lubricants as 'environmentally acceptable'. [4, 5, 6]

There are three internationally accepted guidelines for assessing inherent biodegradability. [7, 8, 9] However, two of these tests (OECD 302 A and B) measure biodegradation as the loss of dissolved organic carbon (DOC) and are therefore unsuitable for testing oil products which are usually sparingly soluble in water. The third test (OECD 302 C - 'Japanese MITI II test') measures biodegradation as oxygen (O_2) uptake and could therefore be applied to insoluble substances (see below). However, with the exception of Japan, the test, is little used [10] and has technical limitations concerning the need for an empirical formula for the test substance (to calculate the theoretical oxygen demand) and a complex inoculum. It was against this background that in 1991 CONCAWE formed a task force to develop a test for assessing the 'inherent' biodegradability of oil products.

2. DEVELOPMENT OF THE CONCAWE TEST METHOD

2.1. BACKGROUND

Oil products are typically complex mixtures of (predominantly) hydrocarbons with low water solubilities. They are usually characterised in terms of performance and not chemical composition. The 'ultimate' biodegradation of an insoluble substance can only be measured by respirometric methods. Biodegradation of the test substance results in an increase in respiration of the microbial inoculum, which can be followed by measuring either the net O_2 consumption or CO_2 evolution over that occurring in blanks, which are identical except for the absence of the test substance. The Task Force decided to base their method on the latter approach, as measuring the mineralisation of test substance carbon to CO_2 gives an unequivocal measure of 'ultimate' biodegradation. In addition, only the carbon content of the oil product needs to be known in order to calculate the extent of biodegradation and this can easily be measured, rather than the empirical formula which is usually difficult to derive for oil products.

When interpreting the results from CO_2 evolution tests, it can be useful to know how much of the test substance carbon has been mineralised through to CO_2 and how much has been incorporated into new microbial cells. In order to investigate the importance of this latter process in relation to the CONCAWE biodegradation test method, CONCAWE funded a one year research project, overseen by Prof. Colin Ratledge of the University of Hull with the aims of:

1. Evaluating methods for quantifying microbial biomass in terms of carbon.

2. Estimating the incorporation of test substance carbon into microbial biomass under CONCAWE test conditions for a range of pure hydrocarbons and lubricant base oils.

This work is summarised in **Appendix 1**.

2.2. CO_2 EVOLUTION BIODEGRADABILITY TESTS

Standardised CO_2 evolution biodegradability tests were described over 20 years ago by Sturm [11] and Gledhill, [12] and these methods have since evolved into the test guidelines published by the OECD [2] and US EPA, [13] respectively. Despite their widespread use, there are drawbacks to these tests and improved methods have been proposed. During the course of the Task Force's work, the International Organization for Standardization's Biodegradability Working Group (ISO/TC 147/SC 5/WG 4) produced a method based on sealed vessel CO_2 evolution tests developed independently by Struijs and Stoltenkamp [14] in the Netherlands, and Birch and Fletcher [15] in the UK. The development of the ISO method (ISO 14593) [16] and its successful ring testing in 1995 has been described by Battersby. [17]

2.2.1. ISO Headspace CO_2 Biodegradability Test (ISO 14593)

In ISO 14593, the test substance at a concentration of (usually) 20 mg/l as carbon, is incubated in a buffered, mineral salts medium inoculated with a mixed population of micro-organisms. The test is performed in sealed bottles with a headspace of air that provides a reservoir of O_2 for aerobic biodegradation.

Carbon dioxide evolution arising from the 'ultimate' biodegradation of the test substance is determined by measuring net inorganic carbon (IC) production in test bottles over that occurring in blanks which contain inoculated medium only.

IC can be measured by one of two methods:

1. Acidification of the samples to pH <3 and equilibration at 20^{o}C, so that the concentration of IC in the liquid and headspace phases is the same and only the headspace gas needs to be analysed;

2. Addition of alkali to raise the medium pH to >12 and equilibration at 20^{o}C, so that all the IC is trapped as carbonate in the liquid phase.

The extent of biodegradation is then expressed as a percentage of the theoretical maximum IC production (ThIC), based on the quantity of test substance (as organic carbon) added initially. ThIC is analogous to the term $ThCO_2$ used in the CO_2 evolution (OECD 301 B) "ready" biodegradability test. The ISO test normally uses 160 ml capacity serum bottles, sealed with butyl rubber stoppers and aluminium crimp seals. This test system is less cumbersome than that used in OECD 301 B and the use of sealed bottles enables volatile substances to be tested.

The use of a pre-exposed inoculum (see below) is also permitted in ISO 14593, providing this is stated when the test results are reported. These advantages lead the Task Force to base their test procedure on the ISO method. This allowed the group to concentrate on issues relating to developing an "inherent" biodegradability test for oil products and had the benefit that the CONCAWE method would be compatible with an International Standard.

2.3. 'INHERENT' BIODEGRADABILITY

'Inherently biodegradable' is a term used to classify an organic substance for which there is unequivocal evidence of biodegradation. This could have been determined under a variety of test conditions using either specific analysis (e.g. infra-red spectroscopy, gas chromatography), radiolabelled materials, or by following CO_2 evolution, O_2 uptake or dissolved organic carbon (DOC) removal (water-soluble substances only).

A useful working description of an 'inherent' biodegradability test is one where one or more of the conditions of a 'ready' biodegradability test have been relaxed. [10] This is done to increase the chance of biodegradation occurring and usually includes procedures such as pre-exposure of the inoculum in an attempt to produce an acclimated population of bacteria, an increased test duration and/or a higher micro-organism to test substance ratio.

Microbial inocula in 'ready' biodegradability tests have a low biodegradative potential, so that a substance passing the test is expected to be rapidly and extensive biodegraded under aerobic conditions in the aquatic environment. The stringent test conditions include the presence of only a low concentration of unacclimated micro-organisms, a relatively short test duration (28 days) and a relatively high test substance concentration (2 to 100 mg/l).

2.4. CONCAWE MODIFICATIONS TO ISO 14593 TO PRODUCE A TEST FOR ASSESSING 'INHERENT' BIODEGRADABILITY

Although ISO does not describe its standards as 'ready' biodegradability tests, the recommended conditions given in ISO 14593 are identical to this type of test (as defined by OECD). The Task Force therefore modified the ISO test procedure by following the principles outlined above. These modifications are permissible in ISO 14593 and are summarised below.

2.4.1. Use of a pre-exposed inoculum

In a review for ISO, Painter [18] concluded that whilst many different methods for pre-exposing microbial cultures to a test substance had been described, there appeared to be no comprehensive study that compared the different techniques. When an acclimated inoculum is used to assess 'inherent' biodegradability, it is important that the pre-exposure regime, although intended to increase the biodegradative potential of the inoculum, does not result in a microbial culture that has a biodegradative potential significantly greater than that which could occur in nature. A key factor appears to be the length of incubation, with a pre-exposure period of five to 20 days giving only a slight chance of 'false-positives', whilst periods of a month or longer carry a much greater risk. [18]

After studies by the Task Force, it was decided to use a modified version of the pre-exposure procedure described in US Environmental Protection Agency Test Guideline § 796.3100. [19] This uses a 14 day pre-exposure and a mixed microbial inoculum derived from soil and activated sludge.

As the aim is to increase the biodegradative potential of the inoculum, samples can be taken from sites where previous exposure to oil products may have occurred (e.g. refinery biotreater, contaminated soil, etc.). The inoculum is used at a final concentration of 10% v/v.

2.4.2. Test duration

The ISO test normally runs for 28 days. For the CONCAWE test it was decided to run the test until the biodegradation curve reaches a plateau or up to a maximum of three months.

2.4.3. Test substance dosing

Petroleum substances are normally composed of poorly water soluble, hydrophobic compounds. In an aqueous test system, biodegradation will occur through the metabolism of the small fraction of dissolved material, either through bacterial cell to oil droplet contact or by solubilisation of the oil phase. When assessing the biodegradability of an oil product, steps have to be taken to increase both bioavailability (by increasing its surface area and hence the solubilisation rate) and to facilitate accurate dosing. Commonly used techniques include: emulsification in a poorly biodegradable surfactant; ultrasonic dispersion; the use of a carrier solvent and/or addition via a solid support. [20, 21] For simplicity and ease of use, the Task Force decided that the oil product should be dosed into test bottles (by weight), using a small (21 mm diameter) glass fibre filter as a solid support; the oil product is spread across the surface of the filter to increase its surface area. An accurate test

dose can be calculated from the measured weight dispensed and the total carbon content of the test substance.

2.5. DETERMINING THE PRACTICALITY OF THE CONCAWE MODIFICATIONS TO ISO 14593: SMALL-SCALE RING TEST

2.5.1. Small-scale ring test design

During 1995, a small-scale ring test of CONCAWE modifications to ISO 14593 was conducted by four of the Task Force's laboratories (Castrol International Technology Centre, Exxon Biomedical Science Inc., Shell Research and Zeneca Brixham Environmental Laboratory).

The aims of this exercise were to establish the practicality of the test procedure and estimate its precision.

Three test substances were used in this mini-ring test:

1. Octadecane (CAS no. 593-45-3)

2. Di-isotridecyl adipate, DITA (CAS no. 26401-35-4)

3. 150 SN solvent-refined mineral base oil (CAS no. 64741-88-4).

Octadecane is a 'readily' biodegradable linear alkane, whilst earlier studies by Task Force members had shown 150 SN solvent-refined mineral base oil to be only moderately biodegradable. The dicarboxylic acid ester DITA is the biodegradable reference oil (RL 130) for the CEC L-33-A-93 test, in which it typically achieves 88% primary biodegradation after 21 days. [1] However, the ultimate biodegradability of RL 130 with an unacclimated inoculum is only ~ 30%, expressed in terms of theoretical CO_2 evolution ($ThCO_2$). [22]

In order to study the effectiveness of the pre-exposure procedure, the laboratories collected their inocula from locations where no known exposure to oil products had occurred. Separate pre-exposure cultures for each test substance were prepared and these were then pooled for the test. In this study, the test ran for 28 days only.

2.5.2. Results and discussion

Biodegradation curves obtained by the four laboratories are shown in **Figure 1** and the mean end-of-test results summarised in **Table 1**. As expected, octadecane was extensively biodegraded (with the exception of one laboratory) but there was only moderate biodegradation of the 150 SN solvent-refined mineral oil. Our use of a pre-exposed inoculum led to a significant increase in the biodegradability of DITA, with mean biodegradation in the four laboratories ranging from 42 to 64%, expressed in terms of theoretical inorganic carbon production (ThIC) after 28 days.

It can be seen from **Figure 1** that in many cases biodegradation was still occurring when the tests were stopped and it was clear from this study that incubation for 28 days was too short a time period for biodegradation materials of this type to reach a plateau.

The between-laboratory variability ('reproducibility') of the modified ISO 14593 method, when expressed as coefficients of variation (standard deviation divided by the mean value multiplied by 100) for the overall mean results for octadecane and di-isotridecyl adipate, was < 20% (**Table 1**). This was similar to the reproducibility of ISO 14593 (12 to 16%) and other respirometric biodegradability tests, when easily biodegradable substances have been tested. [17] In contrast, the variability with the mineral base oil was high.

One feature which affects the precision of this type of test is extent of 'background' CO_2 production, as measured by IC levels in the blanks. The four laboratories reported average IC production in replicate blank bottles on Day 28 from 0.20 to 0.27 mg carbon/bottle, with an overall mean of 0.25 mg carbon/bottle which was around 13% of the organic carbon added initially as test substance. This was the same as the overall mean for the blanks found in the ring test of the ISO 14593 biodegradation method. [17]

Figure 1 Biodegradation curves for octadecane, di-isotridecyl adipate and 150 SN solvent-refined mineral base oil in the small-scale ring test of the draft CONCAWE method.

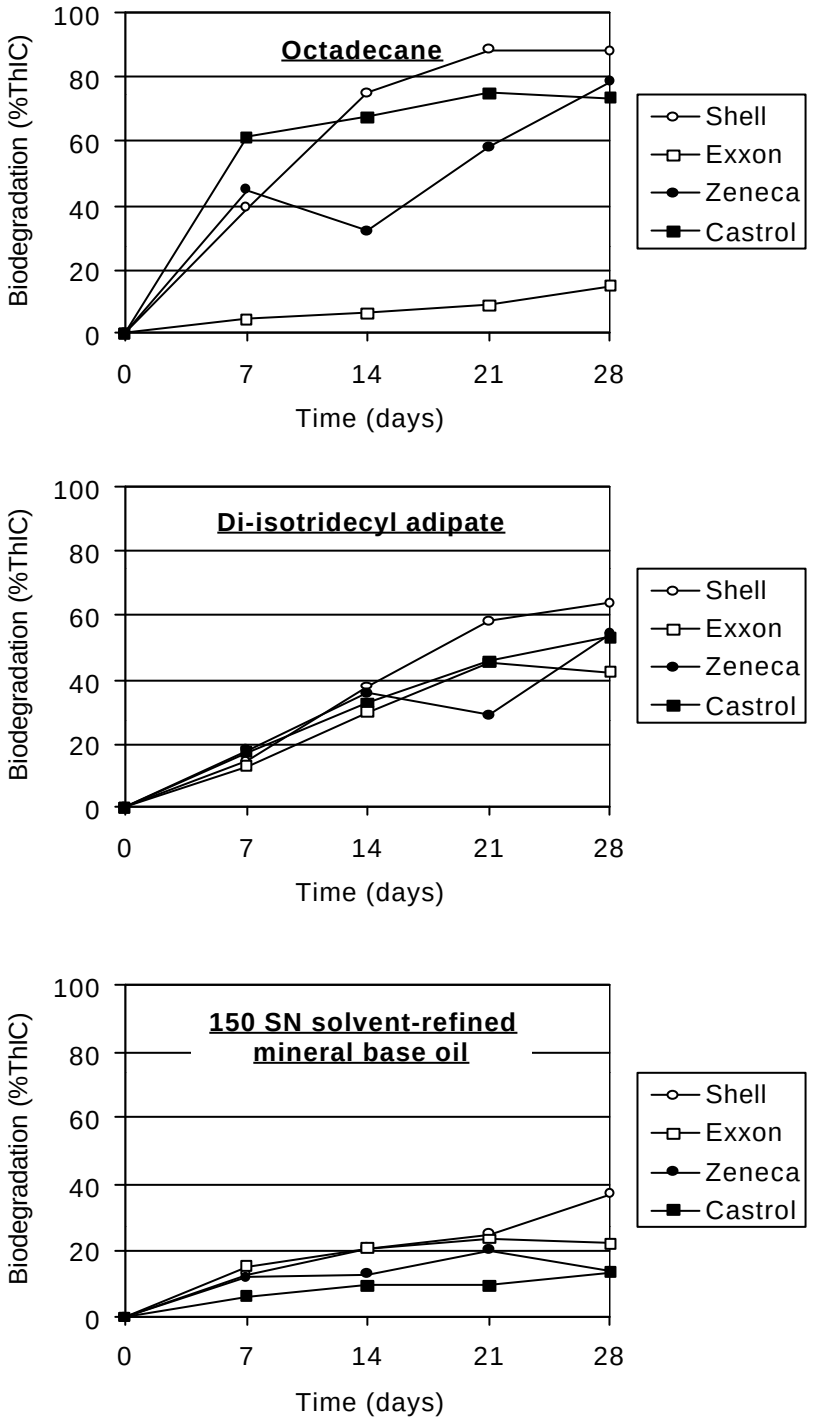

Table 1 Summary of the end-of-test percentage biodegradation values in the small-scale ring test of the draft CONCAWE method (each value is the mean of five replicate bottles).

Laboratory	Mean biodegradation after 28 days (% ThIC)		
	Octadecane	**Di-isotridecyl adipate**	**150 SN solvent-refined mineral base oil**
Shell	88	64	37
Exxon	15 *	42	22
Zeneca	78	54	14
Castrol	74	53	13
Mean biodegradation =	**80** (*n = 3*)	**53**	**22**
Coefficient of variation =	**9%** (*n = 3*)	**17%**	**50%**

* The low result for octadecane in this laboratory was probably due to poor bioavailability as 2½-times the recommended amount of (melted) test substance was added to a smaller, thicker glass fibre filter than that used by the other participants.

2.5.3. Modifications to the CONCAWE test method as a result of the small-scale ring test

This small-scale ring test provided valuable information on the performance of the CONCAWE test and showed that the precision of the method was comparable to that of the ISO 14593 method. Based on experience using the original protocol, clarification and explanation were added, and the decision was made to extend the test duration to at least 56 days (if biodegradation was still occurring). It was also agreed to use *n*-hexadecane as a reference substance, as this is easier to handle than octadecane.

3. 1996/97 RING TEST

3.1. BACKGROUND

The decision was made to conduct an international ring test of the CONCAWE modifications to the ISO 14593 biodegradation method and invitations to participate were sent out from CONCAWE, Brussels in May 1996. Twenty two laboratories expressed a wish to participate and the test protocol, data sheets and test substances were despatched during July. Tests were performed during the second half of 1996 and January 1997. A meeting was held in October 1997 between the CONCAWE Biodegradation Task Force and ring participants to discuss the ring test report and agree changes to the method.

The ring test has been described in detail in a paper, [23] whilst the following sections summarise the main points.

3.2. RING TEST DESIGN

Five test substances were chosen for the ring test (**Table 2**). They were representative of the types of oil product that laboratories would be likely to test routinely and had biodegradabilities that covered the range normally measured (e.g. 10-80% $ThCO_2$, based on OECD 301 B data).

Table 2 1996/97 Ring test substances.

Name	Description	CAS no.	Comments
Hydraulic fluid	Vegetable oil and additives	-	Formulated, 'environmentally acceptable' product
n-Hexadecane	Linear alkane	544-76-3	Proposed reference substance for the CONCAWE test
Hydrotreated slack wax	Hydrotreated slack wax	92062-09-4	Mineral base oil, containing no additives
Di-isotridecyl adipate (DITA)	Dicarboxylic acid ('di-') ester	26401-35-4	Synthetic ester and reference oil (RL 130) for the CEC L-33-A-93 test
White oil	Enerpar M2632	8042-47-5	Mineral base oil and reference oil (RL 110/2) for the CEC L-33-A-93 test

The ring test method was as described in section 2.5.3. Wherever possible, the design and statistical analysis of the ring test data followed International Standard ISO 4259. [24] However, due to the long duration of the CONCAWE test (14 days acclimation, plus 56 days) and the resources required to perform it, minor deviations from the standard were unavoidable (e.g. within-laboratory variability was not quantified).

3.3. RING TEST RESULTS

Data were finally received from 12 laboratories based in five countries (Finland, France, Germany, The Netherlands and the U.K.). A summary of the end-of-test (Day 56) mean percentage biodegradation values for all the laboratories is given in **Table 3**. Test results from Laboratories 11 and 12 were regarded as suspect, since the results for the hydraulic fluid and *n*-hexadecane were spuriously low. Discussions with the responsible scientists from both laboratories could not reconcile these data and in the end, they were not used to estimate the precision of the method.

Table 3 Summary of the end-of-test mean percentage biodegradation values submitted by all the laboratories in the 1996/97 ring test.

	Mean biodegradation after 56 days (% ThIC)				
Laboratory number	**Hydraulic fluid**	***n*-Hexadecane**	**DITA**	**Hydrotreated slack wax**	**White oil**
1	71	58	56	35	15
2	78	91	79	39	35
3	63	50	75	42	-2
4	74	70	50	45	12
5	68	78	44	51	19
6	86	68	75	56	13
7	84	75	63	55	38
8	69	65	66	56	26
9	98	99	88	78	37
10	79	64	58	47	20
Mean biodegradation =	**77**	**72**	**65**	**50**	**21**
Coefficient of variation =	***13%***	***21%***	***21%***	***24%***	***61%***
11 *	49	47	43	35	25
12 *	41	33	55	28	24

* Data set was not used to calculate the precision of the method.

3.3.1. Precision of the CONCAWE method

As far as possible the statistical analysis of the ring test data followed ISO 4259. [24] Estimates of the variability between laboratories ('reproducibility'), and between replicates in the same test run ('replicability') in the ring test are given in **Table 4**.

The reproducibility of the method, according to ISO 4259 was 38%. Put another way, the difference between two results obtained by different operators using the CONCAWE test method, working in different laboratories, on the same oil product

would exceed 38% only in one case in 20. To many this value will seem high but it should be noted that the reproducibility of the CEC L-33-A-93 biodegradation test for two-stroke lubricants is also high at 25%. [1] These differences reflect the variability due to the different inocula used by the different laboratories and are an unfortunate but intrinsic feature of all standard biodegradability tests.

Table 4 Precision of the CONCAWE method based on the 1996/97 ring test results

Parameter	Value from the 1996/97 ring test	Values from other ring tests of biodegradability methods
Reproducibility (coefficients of variation for extensively biodegraded substances)	13 - 21%	15 -20% [17, 25, 26, 27]
Reproducibility (*)	38%	25% [1]
Replicability (coefficients of variation for *n*-hexadecane)	5 - 7%	≤ 10% [17]
Replicability (**)	13 - 25%	No data

* The value equal to or below which the absolute difference between two single test results on identical material obtained by operators in different laboratories, using the CONCAWE method, may be expected to lie with a probability of 95%. [24]

** The value equal to or below which the absolute difference between two single test results on identical material obtained by the same operator at approximately the same time, using the CONCAWE method, may be expected to lie with a probability of 95%.

An estimate of reproducibility of the type given by ISO 4259 is not available for OECD, European Union, or most other biodegradation test guidelines. When these methods have been ring tested, reproducibility has usually been expressed as coefficients of variation for the final mean percentage biodegradation of the ring test substances. It can be seen from **Table 4** that the between-laboratory variability for the CONCAWE method using this measure was similar to that of other standard biodegradability tests.

In this ring test, replicability was estimated to lie between 13 and 25%. This perhaps represents a 'worst-case', as some of the laboratories were performing the test for the first time and/or used nominal and not measured test substance doses in their calculations. The within-test variability (expressed as c.v.) for *n*-hexadecane, tested in laboratories known to be experienced in the CONCAWE method and where the alkane was added as a measured weight, lay in the range 5 to 7%. This was comparable to the replicability reported in the ring test of ISO 14593.

3.4. CONCLUSIONS FROM THE RING TEST

The ring test demonstrated that the CONCAWE test was a practical method, with a precision that was comparable to other standard biodegradation tests. There was generally good agreement between the laboratories in ranking the biodegradabilities of the test substances, which decreased in the order:

Hydraulic fluid → *n*-Hexadecane → DITA → Hydrotreated slack wax → White oil

Decreasing biodegradability ——————→

A key factor in determining the precision of this type of test is the extent of the difference between blank IC production and that produced by the biodegradation of the test substance. High blank IC levels reduce precision. The ring test also enabled quality control criteria such as maximum blank IC production limit to be set for 'valid' tests and these were the same as those used in ISO 14593.

As some ring test participants had a problem with achieving low enough IC production in their blanks, guidance is now given in the method on how to address this issue. Other technical improvements were made to the test protocol and guidance given on the interpretation of the test results.

3.5. INTERPRETATION OF BIODEGRADATION RESULTS USING THE CONCAWE TEST

The term 'inherently biodegradable' is used to classify organic substances for which there is unequivocal evidence of biodegradation. [2,10] This may be judged by specific analysis or by following 'ultimate' biodegradation as net carbon dioxide evolution, oxygen uptake or dissolved organic carbon (DOC) removal. [31] The two commonly used existing OECD 'inherent' biodegradability tests both measure biodegradation as the loss of DOC. The OECD Expert Group on Degradation and Accumulation [31] recommended that biodegradation of more than 20% was evidence for inherent primary biodegradability, and biodegradation above 70% was evidence for 'inherent ultimate biodegradability' (70% being the pass level in the DOC ready biodegradability tests). [2] It is therefore proposed that a similar interpretation of CONCAWE test results should be made. However, as the pass level for carbon dioxide evolution in ready biodegradability tests is 60%, [2] biodegradation > 60% of the theoretical inorganic carbon content (ThIC) in the CONCAWE test should be taken as evidence for 'inherent ultimate biodegradability'.

Biodegradation below 20% ThIC is an indication that the test substance is not 'inherently' biodegradable under the conditions of the CONCAWE test. However, this does not rule out metabolism under different circumstances (e.g. using a longer pre-exposure period, or a higher biomass to substance ratio).

These interpretations should strictly only be applied to pure substances, whilst petroleum streams and products are in fact mixtures of different organic compounds. However, for closely related compounds (e.g. homologous series of hydrocarbons) the proposed interpretation of results of the CONCAWE test should allow for an accurate assessment of potential fate in the environment.

3.6. PROMULGATION OF THE CONCAWE TEST METHOD

As was mentioned earlier (Section 2.2.1), the CONCAWE test is based on ISO 14593 and all the modifications made by the Task Force (with the exception of increasing the nitrogen content of the mineral salts medium - **see Appendix 2**) are allowed in the ISO method. The increase in nitrogen was precautionary as biodegradation using the ISO test medium could be nitrogen-limited if test substance concentrations >20 mg/l as carbon were used.

The limitations of current OECD test guidelines for assessing the 'inherent' biodegradability of oil products were highlighted at the start of this report. In fact, there is a need for a new 'inherent' biodegradability test that can be used with any organic substance that has a low solubility in water and/or is volatile. The

CONCAWE test is suitable and accordingly, the final version of our method has been drafted in 'OECD format' (**Appendix 2**). It is intended that this protocol, and supporting material including the ring test paper [23] and this report, are submitted to the OECD as a proposal for acceptance of the method as a new test guideline: 'OECD 302 D, Inherent Biodegradability: CONCAWE Test'.

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5. GLOSSARY

ATP	Adenosine triphosphate
c.v.	Coefficient of variation
DITA	Di-isotridecyl adipate
DNA	Deoxyribonucleic acid
OECD	Organisation for Economic Co-operation and Development
cfu	Colony forming units
fg	Femtogrammes
IC	Inorganic Carbon
ISO	International Standards Organization for Standardization
MITI	Japanese Ministry of Trade and Industry
RNA	Ribonucleic acid
ThIC	Theoretical inorganic carbon production
$ThCO_2$	Theoretical carbon dioxide evolution

APPENDIX 1

EVALUATION OF TECHNIQUES TO MEASURE INCREASES IN MICROBIAL BIOMASS DURING BIODEGRADATION IN THE CONCAWE 'INHERENT' BIODEGRADABILITY TEST

BACKGROUND TO THE PROJECT

The CONCAWE test assesses biodegradability by measuring the extent to which the test substance carbon is mineralised to CO_2. As some of this carbon may also be used to form new microbial biomass, a knowledge of this amount can be useful when interpreting test results. To investigate this further, CONCAWE funded a 12 month research project at the Department of Applied Biology, University of Hull, U.K. The work was performed by Rachel Jones under the supervision by Prof. Colin Ratledge, a recognised expert on the microbial biodegradation of hydrocarbons. The project was managed by the Biodegradation Task Force and ran during 1995-96. The final report on the project [28] is summarised below.

METHODS EVALUATED FOR QUANTIFYING MICROBIAL BIOMASS

A number of procedures are available for measuring microbial biomass under CONCAWE test conditions. The methods investigated ranged from traditional agar plate and microscopical counts of bacteria, through to the use of commercially available automated instruments for counting micro-organisms. The test substances used were linear paraffins (*n*-hexadecane and octadecane), a branched paraffin (pristane), aromatic hydrocarbons (toluene and naphthalene), a mixture of these compounds (*n*-hexadecane, pristane, naphthalene and toluene in the ratio 35 : 6 : 1 : 1 w/w), 150 SN solvent-refined mineral base oil, a hydrotreated slack wax and a synthetic ester. The various methods and their pros and cons are summarised in **Table 5**. The evaluation was based on experimental data but for reasons of brevity the full test results are not reproduced in this report.

In order to be practical, the method chosen would have to have the following characteristics:

1) Sensitive (as there is only ~ 2 mg test substance carbon available in each bottle for metabolism to either biomass or CO_2).
2) Produce results which can easily be converted into 'mg biomass as carbon'
3) Relatively easy and economical to perform.

Based on the findings summarised in **Table 5**, it was concluded that ATP bioluminescence was the best technique for monitoring carbon incorporation into microbial biomass during the CONCAWE test.

Table 5 Techniques evaluated for measuring microbial biomass in the CONCAWE test

Method	Principle	Advantages	Disadvantages
Total viable counts	Number of colony forming units (cfu) counted after the test medium was serially diluted and a known volume of each dilution incubated on or in nutrient/tryptone soya agar at 22 ^{o}C for 3-7 d.	• Widely-used method • Inexpensive • Simple to perform	• Insensitive • Inaccurate, as competent organisms may not grow on the agar or may not be detected • Difficult to convert cfu into mg biomass carbon
Epifluorescence microscopy	Micro-organisms in the test medium stained with fluorochrome dyes which bind to cellular DNA or DNA and RNA, and fluoresce at wavelengths from 365-470 nm. The cells were counted using a microscope and an estimate of the volume of cells made. This 'biovolume' was then converted to carbon using a conversion factor of 350 fg carbon/μm^3. Incubation in the presence of nalidixic acid and yeast extract also enabled viable (living) cells to be counted.	• Sensitive • Can differentiate between viable (i.e. living) and non-viable cells • Results can be converted into mg carbon • Samples can be preserved and analysed later • Inexpensive reagents	• Accurate sizing of cells is difficult, leading to errors in biovolume calculation • Debris from the glass fibre filter support (used to add test substance in biodegradation test) causes problems • Skilled operator is required • Cost in terms of man hours and equipment is high • Specific conversion factor may need to be derived for CONCAWE test conditions
Macromolecular labelling	The rate of incorporation of radiolabelled material (^{3}H-thymidine and ^{14}C-leucine) into intracellular macromolecules (e.g. DNA, protein) by micro-organisms in the test medium measured. Conversion factors of 2.0 x 10^{18} cells/mole thymidine incorporated and 33.1 fg carbon/cell, and 1,080 g carbon/mole leucine incorporated were used to derive mg biomass carbon.	• Can yield a biomass carbon value	• Uses radioactive compounds • Cumbersome and time-consuming • Expensive • Skilled operator is required • Wide variation in published conversion factors

Table 5 (cont'd) Techniques evaluated for measuring microbial biomass in the CONCAWE test

Method	Principle	Advantages	Disadvantages
Amperometric transducer Sartorius 'MIDAS PRO' system	Micro-organisms in the sample concentrated on a membrane filter. When connected to the 'MIDAS PRO' instrument, the cells reduce an oxidised carrier which then transfers the electrons to an electrochemical cell. Subtraction of background activity gives the activity due to the micro-organisms in the sample and this current (µA) can be correlated with total viable counts.	• Automated system • Rapid (10 minutes) • Relatively inexpensive	• Lack of sensitivity for this application • Debris from the glass fibre filter support (used to dose test substance) causes problems • Calibration curves used are based on total viable counts • Difficult to convert results into mg biomass carbon
Impedance measurement Don Whitley 'RABIT' system	Samples incubated in a medium and change in conductivity (µS) due to growth is measured. This can be correlated with total viable counts.	• Automated system • Relatively quick (24 h) • Simple to use	• Lack of sensitivity for this application • Calibration curves used are based on total viable counts • Difficult to convert results into mg biomass carbon
ATP Bioluminescence Celsis Ltd.	Intracellular levels of ATP (adenosine triphosphate) in the test medium determined by measuring bioluminescence when ATP reacts with the firefly enzyme luciferase . The light produced was measured using a 'Optocomp' luminometer and converted to a concentration of ATP. This was converted to carbon biomass using a multiplication factor of 250.	• Automated system • Simple to use • Sensitive (~ 100 cfu/ml) • Not affected by filter debris • Inexpensive reagents • Widely-used method	• Only measures viable (i.e. living) biomass • Instrument is relatively expensive • The C/ATP ratio can vary widely depending on environmental conditions. Attempts to measure a conversion factor specific for CONCAWE test conditions were unsuccessful but the factor of x 250 used is widely accepted and gave believable results (see text)

CARBON FLOW TO MICROBIAL BIOMASS DURING BIODEGRADATION UNDER CONCAWE TEST CONDITIONS

The second part of the University of Hull project investigated the incorporation of test substance carbon to viable microbial biomass under CONCAWE test conditions. Viable biomass was estimated by ATP bioluminescence as described in **Table 5** and converted to carbon using a factor of x 250. [29] Whilst factors of between 28 and 1,972 have been reported, the value of 250 is considered to be acceptable in most cases [30] and gave realistic results.

The results for a number of model hydrocarbons and two mineral base oils are shown in **Figure 2**. Each column represents the extent of biodegradation as measured by the conversion of test substance carbon to IC (i.e. CO_2) and the increase in biodegradation when the incorporation of test substance carbon into new biomass was taken into account.

It can be seen that the maximum production of viable biomass occurred early in the test and peaked at ~ 10% for easily biodegradable compounds such as *n*-hexadecane and pristane. However, after two to four weeks incubation, this level was down to only 1-2% of the test substance carbon added initially.

As a general conclusion, the proportion of carbon incorporated into new biomass will vary depending on the test substance, microbial population and the environmental conditions. However, the findings from this investigation suggest that under the conditions of the CONCAWE test most of the biodegraded test substance carbon will be respired to CO_2 and only a small fraction will be used to form new microbial cells. These findings are consistent with those reported for the modified Sturm CO_2 evolution test. [13]

Figure 2 Proportion of test substance carbon biodegraded to inorganic carbon (i.e. CO_2) and converted into viable biomass under CONCAWE test conditions

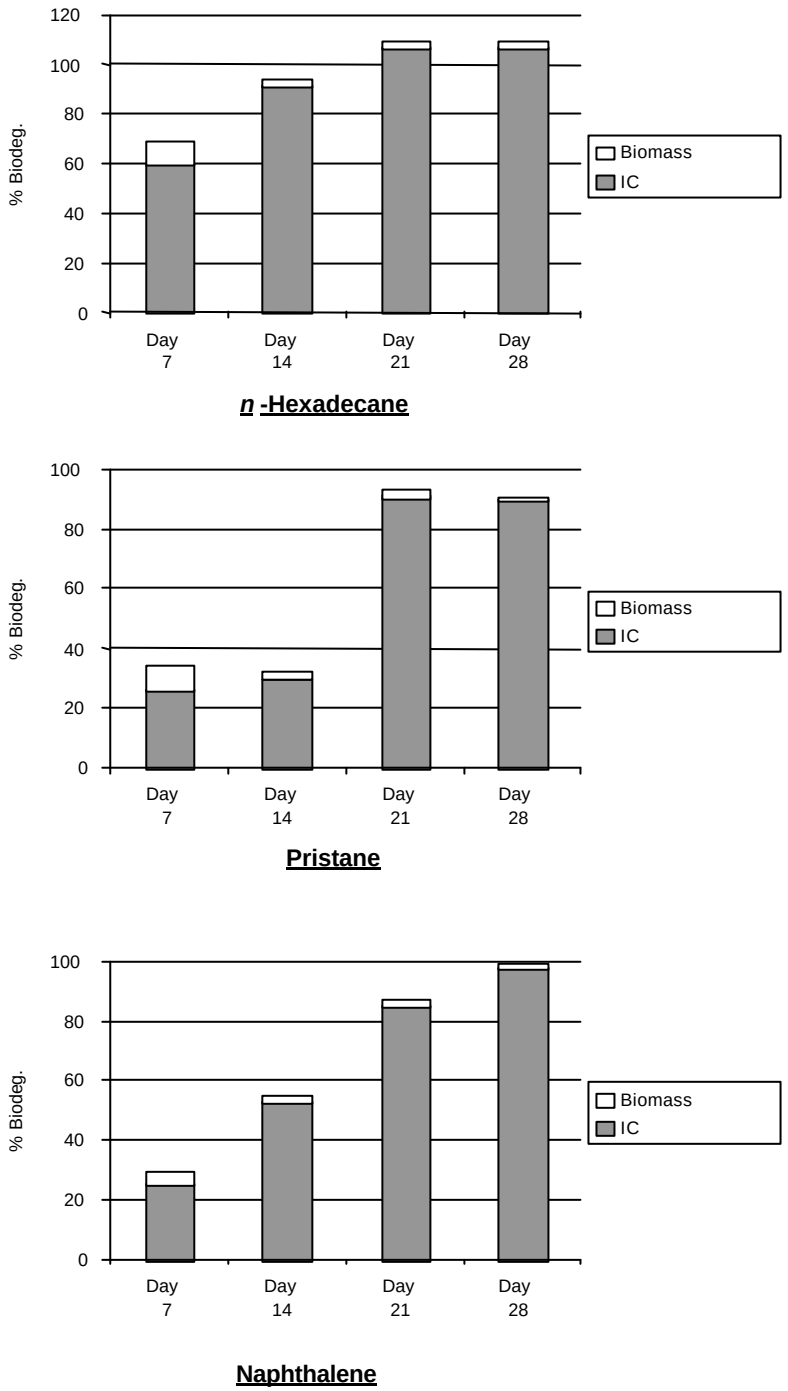
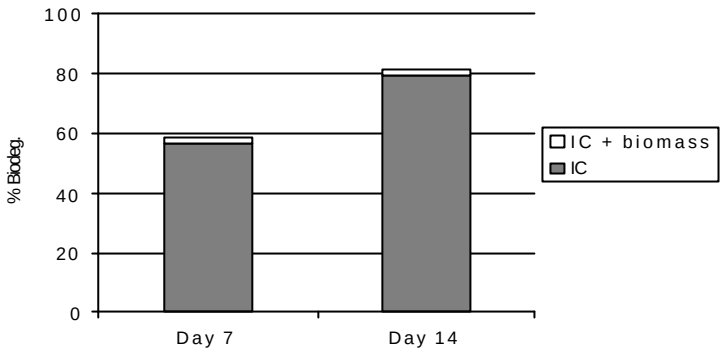
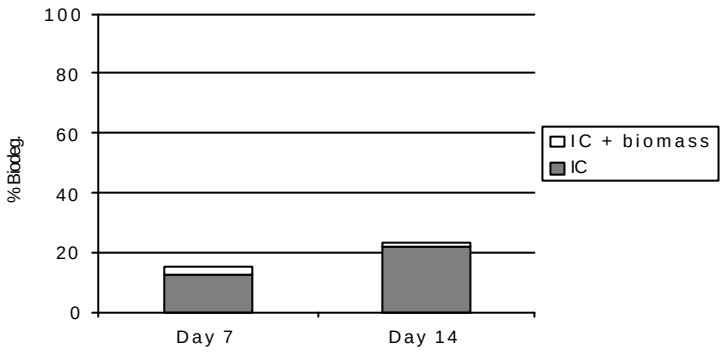


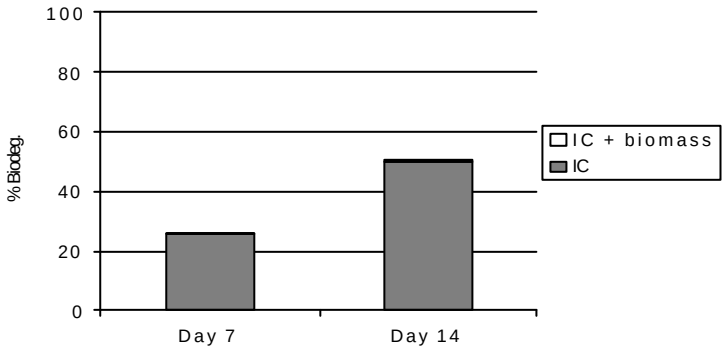
Figure 2 (cont'd)



Octadecane



150 SN solvent-refined mineral base oil



Hydrotreated slack wax

APPENDIX 2

OECD GUIDELINE FOR TESTING OF CHEMICALS PROPOSAL FOR A NEW TEST 'INHERENT BIODEGRADABILITY: CONCAWE TEST' (OECD 302 D)

INTRODUCTION

1. This method is based on ISO [1] 14593: 'Carbon dioxide (CO_2) Headspace Biodegradation Test' (1) and provides a test for assessing the inherent aerobic biodegradability of organic substances. It is particularly useful for testing insoluble and/or volatile materials, and was successfully ring tested in 1996/97 using a formulated hydraulic fluid, hexadecane, di-isotridecyl adipate and two mineral base oils (2).

PRINCIPLE OF THE TEST

2. The test substance is incubated in a buffered, mineral salts medium which has been inoculated with a mixed population of micro-organisms. In order to enhance the biodegradative potential of the inoculum, it is pre-exposed [2] to the test substance using a regime based on methods described in US Environmental Protection Agency Test Guideline § 796.3100 (3). The test is performed in sealed bottles with a headspace of air that provides a reservoir of oxygen (O_2) for aerobic biodegradation. CO_2 evolution from the ultimate aerobic biodegradation [3] of the test substance is determined by measuring the inorganic carbon (IC) produced in the test bottles over that produced in blanks which contain inoculated medium only. The extent of biodegradation is then expressed as a percentage of the theoretical maximum IC production (ThIC), based on the quantity of test substance (as total organic carbon) added initially. ThIC is analogous to the term $ThCO_2$ used in OECD 301 B: CO_2 evolution (modified Sturm) test (4).

3. Dissolved organic carbon (DOC) removal (water-soluble substances only) and/or the extent of primary [4] biodegradation of the test substance can also be measured.

INFORMATION ON THE TEST SUBSTANCE

4. The organic carbon content (% w/w) of the test substance needs to be known, either from its chemical structure or by measurement. For volatile test substances, a measured or calculated Henry's law constant is helpful for determining a suitable headspace to liquid ratio. Information on the toxicity of the test substance to bacteria is useful for selecting an appropriate test concentration and for interpreting results showing poor biodegradability. As this test is usually performed only after failure to pass a test for ready biodegradability, the

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1 ISO: International Organization for Standardization.

2 Pre-exposure is the pre-incubation of the microbial inoculum in the presence of the test substance with the aim of enhancing its ability to degrade the test substance.

3 Ultimate aerobic biodegradation is the breakdown of an organic chemical by micro-organisms in the presence of O_2, resulting in the production of CO_2, water, mineral salts (i.e. mineralisation) and microbial cellular constituents (biomass).

4 Primary biodegradation is the structural change (transformation) of an organic chemical by micro-organisms resulting in the loss of a specific property.

physical and any inhibitory properties of the test substance may have already been ascertained.

APPLICABILITY OF THE METHOD

5. The test is applicable to water-soluble and insoluble test substances. Using the recommended headspace to liquid ratio of 1 : 2, volatile substances with a Henry's law constant of up to 50 Pa m^3 mol^{-1} can be tested as the proportion of test substance in the headspace will not exceed 1% (5). A smaller headspace volume may be used when testing more volatile substances. However, users must ensure that the headspace to liquid ratio and the test substance concentration are such that sufficient O_2 is available to allow complete aerobic biodegradation to occur (e.g. avoid using a high substrate concentration and a small headspace volume). Guidance on this matter can be found in (6).

REFERENCE SUBSTANCES

6. In order to check the test procedure, a reference substance of known biodegradability should be tested in parallel. For this purpose, *n*-hexadecane or a rapeseed oil with a low (≤ 2% w/w) erucic acid content ('Canola oil'), are recommended when testing insoluble substances. Sodium benzoate is recommended for water-soluble test substances. Biodegradation of these substances must reach ≥ 60% ThIC by the end of the test.

7. To demonstrate the increased biodegradative power of the test over a ready biodegradability test, di-isotridecyl adipate (DITA) can be used as a more difficult to biodegrade reference substance. DITA is typically biodegraded by only ~30% ThIC after 28 days with an unexposed inoculum (e.g. in OECD 301 B) but can be mineralised by 40 - 80% ThIC in this test. DITA is a reference oil (RL 130) for the CEC L-33-A-93 biodegradability test and details on how to obtain it can be found in (7).

REPRODUCIBILITY

8. Based on the CONCAWE 1996/97 ring test of the method (2), the difference between two single and independent test results obtained by different operators, working in different laboratories, on the same test substance would exceed 38% only in one case in 20.

9. In the CONCAWE 1996/97 ring test of the method (2), the following results were obtained using the recommended test conditions:

Test substance (Both insoluble)	Mean % D (Day 56)	Coefficient of variation	Number of laboratories
n-Hexadecane	72	21%	10
Di-isotridecyl adipate (DITA)	65	21%	10

For these two insoluble test substances, the variability (as the coefficient of variation) between replicates in the same test run (i.e. replicability) was <10% for laboratories that were experienced in using the method.

DESCRIPTION OF THE METHOD

Apparatus

10. Normal laboratory apparatus and:
    (a) Glass serum bottles, sealed with butyl rubber stoppers and crimp-on aluminium seals. The recommended size is '125 ml' which in fact have a total volume of ~160 ml;
    (b) Carbon analyser or other instrument (e.g. gas chromatograph) for measuring inorganic carbon;
    (c) Syringes of high precision for gaseous and liquid samples;
    (d) Orbital shaker in a temperature-controlled environment;
    (e) A supply of CO_2-free air - this can be prepared by passing air through soda lime granules or by the use of an 80% N_2/20% O_2 gas mixture;
    (f) Membrane filtration device of 0.20 - 0.45 µm porosity (optional);
    (g) Organic carbon analyser (optional).

Reagents

11. Use analytical grade reagents throughout.

Water

12. Distilled or de-ionised water should be used containing ≤ 1 mg/l as total carbon. This represents ≤ 5% of the initial organic carbon content introduced by the recommended dose of the test substance.

Stock solutions for the mineral salts medium

13. The stock solutions and the mineral salts medium are similar to those employed in ISO 14593 (1) and OECD 301 ready biodegradability tests (e.g. 4). However, it should be noted that solution (a) in this method contains a higher concentration of ammonium chloride to prevent the possibility of biodegradation becoming nitrogen-limited.

14. Stock solutions should be stored under refrigeration and disposed of after six months, or earlier if there is evidence of microbial growth or precipitation.

15. Prepare the following stock solutions:

    (a)

Potassium dihydrogen phosphate (KH_2PO_4)	8.50 g
Dipotassium hydrogen phosphate (K_2HPO_4)	21.75 g
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	33.40 g
Ammonium chloride (NH_4Cl)	2.00 g

    Dissolve in water and make up to 1 l.

    The pH of this solution should be 7.4. (± 0.2). If this is not the case, then prepare a new solution.

(b) Calcium chloride dihydrate ($CaCl_2.2H_2O$) 36.40 g

Dissolve in water and make up to 1 l.

(c) Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 22.50 g

Dissolve in water and make up to 1 l.

(d) Iron (III) chloride hexahydrate ($FeCl_3.6H_20$) 0.25 g

Dissolve in water, make up to 1 l and add one drop of concentrated HCl.

Preparation of the mineral salts medium

16. Mix 10 ml of solution (a) with approximately 800 ml water, then add 1 ml each of solutions (b), (c) and (d) and make up to 1 l with water.

Inoculum

17. The test uses a composite microbial inoculum, derived from soil and a wastewater treatment plant, that has been pre-exposed to the test substance. In order to maximise the biodegradation potential of the test, samples can be taken from locations where pre-exposure to the test substance or similar materials may have already occurred (e.g. contaminated soil, industrial biotreater).

Soil

18. Clear the soil surface of leaf litter and collect a sample to a depth of up to 20 cm below the soil surface. Transport in a loosely-tied black polythene bag. Remove stones, plant remains and invertebrates from the soil and sieve through a 2 mm mesh (if the soil is too wet to sieve immediately, then partially air-dry to facilitate sieving). If the sieved soil is not required immediately, then it can be stored at 2 to 4 °C in a loosely-tied black polythene bag for up to one month.

Activated sludge

19. Collect activated sludge from a wastewater treatment plant and keep aerated at 20 ± 1 °C until use (this should be within two days of collection).

Pre-exposure procedure

20. To 1 l mineral salts medium, add 1 g prepared soil, 2 ml activated sludge and 50 mg/l yeast extract (e.g. Oxoid, Difco). Adjust the pH (if necessary) to 7.4 ± 0.2 and dispense 100 ml aliquots into conical flasks of 250 to 300 ml volume. Keep the suspension well mixed while dispensing. Add 4 mg/l as carbon of test substance to replicate flasks. The number required will depend on the volume of inoculum needed to perform the test. Close the flasks with stoppers that will allow gaseous exchange (e.g. foam stoppers, aluminium caps) and incubate at 20 ± 1 °C, with shaking or mixing at a rate sufficient to keep the flask contents well mixed and in suspension. Losses of a volatile test substance may occur during this procedure.

21. On or around Day 7 and Day 11 of incubation, make up any evaporation losses of test medium with water, adjust the pH (if necessary) to between 7.2 and 7.6 and add 8 mg/l as carbon test substance to each flask. The final addition of test substance should be made at least three days before the inoculum is used for the test.

22. On the day of the test (normally Day 14 of pre-exposure) coarse-filter the inoculum through a number 4 grade filter paper or glass wool and keep shaken until use. If two or three substances are being tested, the pre-exposed cultures for each test substance can be pooled to give a composite inoculum. This reduces the number of blanks needed and provides a microbial population that has been exposed to more than one substance.

23. The inoculum is normally used within one day. If problems with invalid (i.e. too high) blank inorganic carbon production (see paragraph 49) have been encountered, or are thought likely to occur, then one or both of the following treatments are recommended:

    (a) Aerate the inoculum overnight before use (this reduces its organic carbon content);

    (b) Before use, sparge the inoculum with CO_2-free air for about one hour, while maintaining the pH at 6.5 using orthophosphoric acid. Finally restore the pH to its original value (this reduces its inorganic carbon content).

TEST PROCEDURE

Number of bottles

24. The number of bottles needed for a test will depend on the frequency of analysis and the test duration. It should be noted that the test may have to be continued beyond the 56 days suggested in paragraph 35.

25. It is recommended that at least five bottles from sets ① to ③ (see paragraph 28) are analysed at the end of the test, to enable 95% confidence intervals to be calculated for the mean percentage biodegradation value.

Inoculated medium

26. The inoculum is used at a concentration of 10% v/v (e.g. add 100 ml inoculum to 900 ml mineral salts medium).

Preparation of bottles

27. Aliquots of inoculated medium are dispensed into replicate bottles to give a headspace to liquid ratio of 1 : 2 (e.g. add 107 ml to 160 ml-capacity bottles). Other ratios may be used but see the warning given in paragraph 5.

28. Sets of bottles should be prepared containing the following:

    ① The test substance (held on a glass fibre filter, if necessary - see 30) at a recommended concentration of 20 mg/l as organic carbon;

    ② Blanks containing inoculated medium (and a glass fibre filter, if necessary) only;

    ③ The reference substance at a recommended concentration of 20 mg/l as organic carbon (and a glass fibre filter, if necessary).

    If there is a possibility that the test substance may undergo abiotic degradation, then also prepare sets of sterile controls:

    ④ As ① plus 50 mg/l $HgCl_2$ or sterilised by some other means (e.g. by autoclaving).

If there is a possibility that the test substance may be inhibitory at the test concentration, then also prepare sets of bottles :

⑤ Containing the test substance plus the reference substance at the same concentrations as used for ① and ③.

29. Water-soluble test and reference substances are added from aqueous stock solutions.

30. Insoluble test and reference substances are added to bottles as a measured weight absorbed on a GF/A glass fibre filter (21 mm diameter). This enables an accurate weight to be dosed into each bottle and increases bioavailability. Weighing can be facilitated by preparing a stock solution of the substance in a suitable volatile solvent (e.g. 1,1,2-trichlorotrifluoroethane, dichloromethane) and adding the required volume drop-wise to a tared filter on an analytical balance. An attempt should be made to spread the substance over the filter. The solvent is then allowed to evaporate until a stable weight (≈ nominal test weight) is obtained. For example, 100 µl of a 21.4 mg/ml as carbon stock solution added to a filter gives 2.14 mg carbon/filter (i.e. 20 mg/l carbon when added to 107 ml inoculated medium). The measured weight of test substance on the filter should be used to calculate the test dose (*TOC* in paragraph 44).

31. If the procedure given in paragraph 30 is used, then blank bottles (②) should contain either a filter or a filter from which an equivalent volume of solvent has been evaporated (removal of the solvent should be checked by weighing).

32. Volatile test substances should be injected into sealed bottles using a microsyringe. The syringe should be weighed before and after dosing to enable an accurate test dose to be calculated.

33. All the bottles are then sealed and, if necessary, time zero analyses performed for initial IC concentration (sterile controls ④), DOC concentration, or other determinands.

Incubation

34. The bottles are incubated in the dark or diffuse light at 20 ± 1 °C, with shaking at a rate sufficient to keep the bottle contents well mixed and in suspension (e.g. 150 to 200 rpm).

Sampling

35. Replicate test (①), blank (②), reference (③), and if used, inhibition control (⑤) bottles should be taken for IC analysis periodically to obtain the biodegradation curves. Normally this is done at least once a week. At the end of the test the sterile controls (④), if used, should also be analysed. The test normally runs until biodegradation reaches a plateau - as a guide users should plan for an eight week test. However, the test should be continued if biodegradation is still occurring on Day 56, or if the maximum level of biodegradation is required. In these circumstance, it is not advisable to continue the test beyond three months.

Inorganic carbon (IC) analysis

36. CO_2 production in the bottles is determined by measuring the increase in the concentration of inorganic carbon (IC). There are two methods available for measuring the amount of IC produced in the test, and these are described below. As the methods can give slightly different results only one should be used in a test run.

37. Method (a) is recommended if the liquid medium is likely to contain the remnants of a GF/A filter and/or insoluble test substance. This analysis can be performed using a gas

chromatograph if a carbon analyser is unavailable. It is important that the bottles should be at or close to the test temperature of 20 ± 1°C when the headspace gas is analysed. Method (b) can be easier for laboratories using carbon analysers to measure IC. It is important that the sodium hydroxide solution used to convert CO_2 to carbonate is either freshly prepared or its IC content is known so that this can taken into account when calculating the test result (see paragraph 45).

(a) Acidification to pH <3

38. Calibrate the IC analyser using an appropriate IC standard (e.g. 1% w/w CO_2 in N_2). Inject concentrated (≥ 85% w/v) orthophosphoric acid through the septum of each test bottle sampled to lower the pH of the medium to < 3 (e.g. add 1 ml to 107 ml test medium). Replace the bottles on the shaker. After shaking for one hour at the test temperature (20 ± 1°C), remove the bottles from the shaker, withdraw aliquots (e.g. 1 ml) of gas from the headspace of each bottle and inject into the IC analyser. Record the measured IC concentration (mg/l carbon).

39. The principle of this method is that after acidification to pH < 3 and equilibration at 20 °C, the equilibrium constant for the distribution of CO_2 between the liquid and gaseous phases in the test bottles is 1.0 (2, 5, 8). This should be demonstrated for the test system at least once (e.g. at the end of the test) as follows: set up bottles containing 5 and 10 mg/l as IC using a solution of anhydrous sodium carbonate (Na_2CO_3) in CO_2-free water (prepared by acidifying water to pH 6.5 with concentrated orthophosphoric acid, sparging overnight with CO_2-free air and raising the pH to neutrality with alkali).

    Ensure that the ratio of the headspace volume to the liquid volume is the same as in the tests (e.g. 1 : 2). Acidify and equilibrate as described in paragraph 38, and measure the IC concentrations of both the headspace and liquid phases. Check that the two concentrations are the same within experimental error.

40. If DOC removal is to be measured (water-soluble test substances only), take samples of the liquid phase from separate (unacidified) bottles, membrane filter and inject into the DOC analyser. These bottles can be used for other analyses as necessary.

(b) Conversion of CO_2 to carbonate

41. Calibrate the IC analyser using an appropriate standard - for example, a solution of sodium bicarbonate ($NaHCO_3$) in CO_2-free water (see paragraph 39) in the range 0 to 20 mg/l as IC. Inject 7 M sodium hydroxide (e.g. 1 ml to 107 ml medium) through the septum of each test bottle sampled and shake for one hour at the test temperature (20 ± 1°C). Remove the bottles from the shaker, allow to settle and withdraw, by syringe, suitable volumes (e.g. 50 to 200 µl) from the liquid phase in each vessel. Inject the samples into the IC analyser and record the concentration of IC.

42. The principle of this method is that after the addition of alkali and shaking, the concentration of IC in the headspace is negligible. This should be checked for the test system at least once (e.g. at the end of the test) by using IC standards, adding alkali and equilibrating, and measuring the concentration of IC in both the headspace and liquid phases (see paragraph 39).

43. If DOC removal is to be measured (water-soluble test substances only), take samples of the liquid phase from separate bottles (no alkali) and inject into the DOC analyser. These bottles can be used for other analyses as necessary.

DATA AND REPORTING

Calculation of results

44. Assuming 100% mineralisation of the test substance to CO_2, the theoretical maximum IC production (*ThIC*) in excess of that produced in the blanks equals the amount of total organic carbon (*TOC*) from the test substance added to each bottle at the start of the test. For insoluble substances, *TOC* should be calculated from the measured weight of test substance absorbed on the filter and its organic carbon content. For volatile substances, *TOC* should be calculated from the measured weight of test substance dispensed by microsyringe and its organic carbon content.

The total mass (mg) of inorganic carbon (TIC) in each bottle is:

$$TIC = (\text{mg C in the liquid} + \text{mg C in the headspace})$$

$$= (V_L \times C_L) + (V_H \times C_H) \qquad \text{Equation 1}$$

where:

V_L = volume of liquid in the bottle (l)

C_L = concentration of IC in the liquid (mg/l as carbon)

V_H = volume of the headspace (l)

C_H = concentration of IC in the headspace (mg/l as carbon)

45. The calculation of *TIC* for the two analytical methods used for measuring IC in this test are described below in paragraphs 47 and 48. Percentage biodegradation (*% D*) in each case is given by:

$$\%\ D = \frac{(TIC_t - TIC_b)}{TOC} \times 100$$

where:

TIC_t = mg TIC in test bottle at time t

TIC_b = mean mg TIC in blank bottles at time t

TOC = mg TOC added initially to the test vessel.

Determine *% D* for the test (①), reference (③) and inhibition control bottles (⑤), if included, from the amounts of TIC produced up to each sampling time.

46. If there has been a significant increase in the TIC content of the sterile controls over the test period, then abiotic degradation of the test substance has occurred.

Acidification to pH <3

47. Acidification to pH <3 and equilibration results in the equalisation of the concentration of IC in the liquid and gaseous phases. Hence only the concentration of IC in the gas phase needs to be measured as $C_L = C_H$. Therefore $TIC = (V_L + V_H) \times C_H$ in Equation 1.

Conversion of CO_2 to carbonate

48. In this method calculations are performed as described in Equation 1 but the negligible amount of IC in the gaseous phase is ignored (i.e. $(V_H \times C_H) \approx 0$ in Equation 1).

Validity of results

49. The test is considered as valid if:

    (a) The mean percentage biodegradation of *n*-hexadecane, the low erucic acid-containing rapeseed oil ('Canola oil') or sodium benzoate reaches at least 60% ThIC by the end of the test.

    (b) The mean amount of IC produced from the blanks at the end of the test is ≤ 15% of the organic carbon added initially as the test substance to the test bottles.

50. If these criteria are not met, repeat the test with another inoculum and/or review procedures. For example, if high blank IC production is a problem then follow the procedures given in paragraph 23 .

51. If inhibition controls were included in the test, it can be assumed that the test substance is inhibitory if the mean percentage degradation in these bottles is < 25% by the end of the test. In this case, the test can be repeated with a lower concentration of test substance (e.g. 10 mg/l as carbon). However, it should be noted that this will reduce the precision of the method. If lower test substance concentrations are used, it is important that blank IC production is as low as possible and that the smaller amount of test substance can be dosed accurately.

Interpretation of results

52. Biodegradation ≥ 60% ThIC in this test demonstrates that the test substance is inherently and ultimately biodegradable under aerobic conditions.

53. Biodegradation ≤ 20% ThIC is an indication that the test substance is not inherently biodegradable under the conditions of this test. However, this does not rule out metabolism under different circumstances (e.g. longer pre-exposure period, higher biomass to test substance ratio).

54. Biodegradation is > 20% ThIC is an indication that the test substance has inherent, primary biodegradability.

55. If more information is needed on the potential fate of the test substance, then further investigations such as DOC analysis to detect water-soluble metabolites may be useful.

Test report

56. Compile a table of *% D* for each test (①), reference (③) and, if included, inhibition control bottle (⑤) for each day sampled. If comparable results are obtained for replicate bottles, plot a curve of mean *% D* against time. Record the amount of TIC in the blanks (②), and in the sterile controls (④), if included in the test. If determined, also record the levels of DOC and/or other determinands and their percentage removal.

57. Determine the mean value of *% D* in the plateau phase, or use the highest value if the biodegradation curve decreases after peaking, and report this as the 'extent of biodegradation of the test substance'. It is important to ensure that in the latter case the highest value is not an outlier.

58. The test report should include the following:

    (a) A reference to this Test Guideline;

    (b) Name and characterisation data for the test substance;

    (c) The concentration of the test substance used an the amount of carbon dosed into each test bottle;

    (d) The name of the reference substance used, and the amount of carbon dosed into each reference bottle;

    (e) Details of the inoculum, its pre-exposure to the test substance and any pre-conditioning (e.g. overnight aeration);

    (f) Details of the headspace to liquid ratio used;

    (g) Validation of the principle of IC analysis;

    (h) The main characteristics of the IC analyser employed (and any other analytical methods used);

    (i) Tabulated results and degradation curves;

    (j) Reasons for any rejection of the test results;

    (k) Any other facts that are relevant to the procedure followed.

LITERATURE

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