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Biodegradation Tests for Poorly-Soluble Compounds

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CONTENTS

A. INTRODUCTION

In a previous report (ECETOC, 1983) the applicability, limitations in use, reproducibility and significant technical weaknesses of the aquatic biodegradation test methods in the OECD guidelines (1981) and in Annex V of the 6th Amendment to the European Communities'Council Directive 67/548/EEC (EEC, 1984-a) were assessed. Recommendations were formulated for improving these tests. Subsequently a second ECETOC Task Force was set up :

i. To consider the various improvements to methods for assessing aquatic biodegradation as recommended in the recent report, and to identify those of high priority.

ii. To draw up a programme of experimental work to validate the above improvements. It should be borne in mind that such work could be carried out jointly by industry and outside laboratories.

The present tests for assessing ready biodegradability were developed for water-soluble compounds and were not specifically designed for poorly-soluble compounds. This report considers how these tests can be modified so as to be suitable for poorly-soluble substances.

For abbreviations see Section H, Appendix.

B. CURRENT SITUATION

A variety of screening tests have been developed to determine the biodegradability of organic chemicals. These are designed to measure the potential of a chemical to undergo ultimate biodegradation in the aqueous environment. Such methods were published by the OECD (1981), the EEC (1984-a) and the EPA (1982). They require the incubation of a test chemical at concentrations of 2 - 50 mg/l in a synthetic mineral solution, and measurement of a summary parameter such as dissolved organic carbon (DOC), carbon dioxide evolution or biochemical oxygen demand (BOD). Quite often the test substance concentration, as suggested above, exceeds its solubility in water. Unfortunately, none of these methods offers specific guidance as to how the test must be conducted in such cases. The results obtained, and their reproducibility, may be influenced by the method of adding the poorly-soluble chemical to water.

Most of the published literature on poorly-soluble substances deals with petroleum hydrocarbons and has been reviewed by Gutnick and Rosenberg (1977). They state that, while bacteria can utilise solid and liquid hydrocarbons in the dissolved phase and liquid hydrocarbons directly at the water/chemical interface, there is no evidence that they can utilise a poorly-soluble solid hydrocarbon by growing on the solid phase of the chemical. However, if the solid is first dissolved in a water-immiscible non-biodegradable solvent, microorganisms can then degrade it at the water/solvent interface. This can be explained by the increase of the surface area and/or decrease of the surface tension at the interface. Other examples of testing poorly-soluble substances are given by Ruffo et al.(1984), Gerike (1984), Blok and Booy (1984) and Fogel et al.(1985) where the limitations of existing methods are given and the difficulties in handling poorly-soluble substances are described. Suggestions to modify existing methods so that they become suitable for testing poorly-soluble chemicals have not so far resulted in a generally-accepted procedure.

C. SPECIFIC ASPECTS OF TEST PROCEDURE

1. Water Solubility

The solubility of a chemical in water has to be known for deciding whether it can be tested by any of the current methods or whether a modified approach must be adopted. With the exception of the Closed Bottle Test, in which materials with a solubility of down to 5 mg/l can be satisfactorily tested, all current tests require that a material has a solubility in water of at least 20 mg/l to satisfy the basic analytical and practical limitations of the test. Indeed in practice, substances tested at the limit of solubility can cause problems with the preparation of stock solutions, and precipitation under test conditions, leading to variable results. In this context chemicals with a solubility of 20-50 mg/l should be considered as poorly-soluble, and will require modified test procedures if ready biodegradability (not affected by solubility considerations) is to be reliably assessed.

2. The Choice of Suitable Summary Parameters

The biodegradability of poorly-soluble chemicals cannot be determined accurately by measuring DOC removal since the necessary filtration or centrifugation step to separate biomass from the dissolved chemical would

also eliminate the non-solubilised test chemical. The measurement of Total Organic Carbon (TOC) cannot be used because of the non-homogeneity of the test "solution". Degradation (mineralisation) of such compounds can only be measured only by monitoring BOD or CO_2-evolution.

For the BOD test, the Theoretical Oxygen Demand (ThOD) or Chemical Oxygen Demand (COD) has to be known. For a test based on CO_2 evolution, the carbon content of the test substance must be determined by analysis or calculated from the structural formula.

3. Dispersion Techniques

The Task Force believed that one of the factors affecting the degradation of poorly-soluble compounds would be the limited surface area of the chemical available for attack by enzymes or microorganisms. Thus dispersion methods which would increase the surface area were considered. Several methods such as ultrasonic or simple mechanical dispersion, addition to the water phase with a carrier or dispersing agent and stabilisation with an emulsifier, may be used to achieve a homogeneous and fine distribution of the test chemical. Blok and Booy (1984) have shown that solid carriers are suitable for the testing of oily materials (e.g. di-2-ethylhexylphthalate). Emulsifiers, solid carriers or solvents, at the concentration used, must not inhibit the activity of the bacteria, or be readily biodegraded under the test conditions. It has been demonstrated that e.g. dimethylsulfoxide or zeolites inhibit the bacterial activity (Fogel et al.,1985).

The most appropriate way of making a chemical available to bacteria depends on its physical characteristics. Each chemical or group of chemicals require a specific dispersion method, and considerable experimentation may be needed before an appropriate technique is obtained.

For the transfer of a chemical into the test vessel, the use of a stock solution in an organic solvent, followed by evaporation of the solvent, appears to be a way to overcome the problems of weighing very small quantities, and mixing the substance with water. However, it has been demonstrated (Gerike, 1984; Boethling, 1984) that even very volatile solvents cannot be completely eliminated, thus yielding high blanks when they are readily biodegradable. The use of too-stringent conditions to evaporate such

solvents completely may lead to an uncontrollable loss of the test compound. This drawback also applies if, as an alternative, a solid carrier is soaked with a solution of test chemical in an organic solvent.

4. Inoculum

Mineralisation is clearly stimulated (or accelerated) by a larger inoculum concentration (Gerike, 1984), and since the biodegradability of poorly-water soluble chemicals can be measured by the degree of oxygen uptake or mineralisation, the inoculum concentration should be as high as possible but not so high as to give rise to high blank values. Recommendations of acceptable high inocula concentrations are given by Blok et al.(1985).

In contrast to the common practice of adding a soluble test chemical to the inoculated mineral solution, it is recommended that for poorly-soluble compounds the test solution be inoculated after dispersing the chemical in the mineral solution. Inoculation after simple dispersion of the chemical is also recommended in cases where ultrasonic dispersion, or distribution of the test chemical using mixers with strong shear forces, is used because the activity of the inoculum may be reduced or completely lost.

D. METHODS AND MATERIALS

1. Required Characteristics of the Test Methods

In developing test methods for poorly-soluble chemicals, the TF took into consideration the aspects discussed above.

1.1. For reasons given in C-2, methods based on TOC or DOC measurements must be excluded from consideration. This leaves only the option of modifying the respirometric methods.

1.2. To avoid settling or flotation of the substance and to ensure maximum contact between the inoculum and test material, agitation or shaking is considered essential.

1.3. Care must be taken that the inoculum is not damaged by the mixing or dispersing operations.

1.4. The inoculation should be to the optimum concentration as recommended by Blok et al. (1985).

2. Review of Existing RBTs and the Possibility of Adapting Them

The most appropriate test should be derived from existing methods but these should not be modified beyond what is essential for testing poorly-soluble chemicals. This facilitates the judgement of the environmental relevance of the results and of their acceptability when compared to current tests for ready biodegradability with soluble compounds. The following tests were considered.

2.1. Sturm-Test (OECD-301B)

In this test CO_2-evolution is used as the test criterion. The guideline permits mechanical agitation and may be modified to allow the use of a high inoculum concentration and carriers or appropriate non-foaming emulsifiers. The analytical procedure for the CO_2 determination should be improved (Gerike, 1984).

2.2. Modified MITI-Test (EEC, 1984-a)

In this test BOD is used as the test criterion. The specified amount of inoculum is sufficiently high. The use of a stirrer is recommended and carriers and emulsifiers can also be used. The determination of suspended matter, the residual chemical or TOC, often used when testing soluble substances, will not give useful results with poorly-soluble chemicals.

2.3. Closed Bottle Test (OECD, 301-D)

The Closed Bottle Test has been used with emulsifiers for testing poorly-soluble substances. However, the Task Force did not use this test in the current evaluation, as it considered that the following disadvantages outweighed the intrinsic advantages (e.g. cheapness). The disadvantages noted were as follows :

- there is no agitation;
- a low inoculum concentration must be maintained because of blank problems;
- it may be extremely difficult to get a test result of the desired accuracy when such low quantities of chemicals are directly weighed in.

2.4. RDA-Test

One variant of this test i.e. where the test chemical is added once at the beginning, and the duration is limited to 28 days, is comparable with the other tests for ready biodegradability and has been proposed to the EEC (EEC, 1984-b). In contrast to the BOD test (BOD_5 or Closed Bottle Test) where the total amount of oxygen available is limited by the quantity which is dissolved in water (and thus limits the amount of test chemical and inoculum to an absolute minimum), this test makes use of a supernatant gas phase as an oxygen reservoir. The test bottles are placed on a shaker, and thus no additional equipment to produce the desired turbulence in the bottles is necessary.

2.5. Choice of Test Methods

The modified MITI-, STURM- and RDA-Test which theoretically, are appropriate for testing the ready biodegradability of poorly-soluble chemicals, were chosen by the Task Force for experimental evaluation.

3. Choice of Test Chemicals

The test chemicals were chosen on the basis of the following criteria :

- ready biodegradability had been established from the literature or previous tests;
- the solubility in water was below 20 mg/litre;
- the substances should represent different morphological forms (e.g. crystalline, amorphous).

On the basis of these criteria, three chemicals, whose characteristics are given in Table 1, were chosen.

4. Work Programme

In earlier sections of the report certain current biodegradability tests have been identified as suitable for use in testing poorly-soluble compounds and methods for dispersing test materials in water have been discussed. The Task Force examined the various options and selected a range of dispersion techniques and tests for experimental assessment. Since manpower and time were both limited, the experimental programme was not comprehensive, but rather covered those variables which were considered to be most likely to optimise the treatment of the three chemicals chosen for test.

The work programme is summarised in Table 2 below.

Table 1
Physical-chemical Data of Test Chemicals

Substance	Solubility in water mg/l (temp. °C)	MP °C	COD g O_2/g	ThOD g O_2/g	$ThCO_2$ g CO_2/g
Calcium stearate $C_{36}H_{70}O_4Ca$	2 (35)	179-80	2.83[2)]	2.74	2.61
Anthraquinone $C_{14}H_8O_2$	0.6 (50)	287	2.30[3)]	2.31	2.95
Beeswax [1)]	<0.2 (22)	60-70	3.0	3.4[4)]	3.1[4)]

1) mixture of esters of C_{18} to C_{36} acids and C_{24} - C_{36} alcohols
2) modified Kelkenberg determination on stearic acid (Gerike, 1984)
3) modified Kelkenberg determination (Gerike, 1984)
4) based on the average of the elemental composition of the beeswax tested

Table 2
Combination of Variables Investigated

Test Type	Agitation	Solvent	Solid Carrier	Dispersing Method/Agent
STURM	+	-	Glass filter	-
	-	-	Glass filter	-
	+	-	-	Ultrasound
	+	-	-	-
	-	-	-	Ultrasound
	-	-	-	-
	+	DCM*	-	-
MITI	+	-	-	Ultrasound
	+	-	-	SPAN 80/TWEEN 80 Ultrasound
	+	-	-	-
	+	-	-	Nonylphenol. 10EO. 5PO
	+	-	Glass filter	-
RDA	+	-	-	-
	-	-	-	-
	+	-	Cellulose	-
	+	-	-	2.4.6 tri-t-butylphenol.8EO
	+	DCM*	-	-

* DCM: dichloromethane

5. Experimental

5.1. STURM-test

Apparatus. Installations and equipment used in the test were as described in the OECD Guideline No. 301 B. The size of the carboys was reduced from 5 to 3 litres and the volume of the solution from 2 to 1.5 litres. A magnetic stirrer with a PTFE-coated rod of 6 cm length rotating at approximately 60 rpm was used for agitation. A constant temperature of 23°C was maintained by immersion of the carboys in a water bath.

Inoculum/mineral solution. Sludge for the preparation of the inoculum was taken from a sewage treatment plant receiving predominantly domestic waste water. The inoculum and mineral solutions were prepared according to the OECD Guideline.

Preparation of the test chemicals. In the method with direct dispersion of the test chemical, beeswax, anthraquinone and calcium stearate (20 mg/l) were added as a powder, or as a suspension in water prepared by ultrasonic dispersion, to the carboys containing the inoculum. No additional treatment was given to the mixture containing the inoculum.

In the method with solid carriers , samples were prepared by melting beeswax and calcium stearate on glass filter papers. Anthraquinone was dissolved in dichloromethane and put onto the filter paper which was subsequently kept in an evacuated desiccator for 24h to eliminate the solvent as far as possible. The glass filter papers were cut into small pieces before putting them into the carboys.

In the method with the test chemical in a solvent, concentrated solutions of beeswax and anthraquinone in dichloromethane were added to the carboys containing the mineral solution 24h prior to inoculation, and were aerated to drive off the dichloromethane.

It should be emphasised that under the test conditions any remaining traces of solvent will not contribute significantly to the CO_2 evolution.

5.2. MITI-test

Apparatus. The test was carried out at 22±3°C in a HACH manometric respirometer according to OECD-Guideline No.301 C.

Inoculum/Mineral solution. The mineral solution was prepared according to the Guideline.

Sludges for the preparation of the inocula were obtained from domestic sewage treatment works, washed twice by centrifugation and resuspension in the test medium. These sludges were dispersed in the test medium to give a final activated sludge concentration of 30 mg/l. Prior to the start of the test the bottles were incubated for 1 week at the test temperature to reduce the endogenous respiration rate of the inoculum.

Preparation of the test chemical. The test chemical was added to the bottles as follows to give a final concentration of 100 mg/l :

a) direct weighing of crushed beeswax in the bottles;
b) prior emulsification in water with different emulsifiers (cf. Table 2);
c) use of glass filter papers (see Sturm test above).

Blank tests were set up with the emulsifiers at the concentration used in case b) to correct for oxygen uptake.

5.3. RDA-test

Apparatus and method. The tests were carried out in equipment as described in the EEC Test Guideline (1984-b), and were performed with one initial addition of the test substance.

Inoculum/Mineral solution. The mineral solution was prepared according to the Guideline. The sludge for the preparation of the inoculum was taken from domestic sewage treatment works and treated according to the Guideline. It was then dispersed in the test medium to give the required final concentration of activated sludge.

Preparation of the test chemical. Beeswax was added to the test bottles in the following ways :

a) direct addition of small flakes in quantities of 10.5-13.8 mg per bottle without any treatment or auxiliary agent;
b) addition of 2.0 ml of a stock solution of beeswax prepared by mixing
   4975 mg beeswax
   1100 mg 2.4.6-tri-t-butylphenol.8EO

4080 mg nonylphenol.10EO.5PO

in 1000 ml water.

A blank test was set up with the emulsifier at the concentration used in case b) to correct for its oxygen uptake.

E. RESULTS

Results from the experiments carried out by five different laboratories are compiled in Table 3 and shown in Figures 1 - 4.

1. Calcium stearate (Fig.1).

   This chemical was biodegraded under all test conditions, fulfilling the strict criteria of ready biodegradability (60% within 10 days) except in one case where it was applied on a glass filter in a non-agitated container. The solubility of 2 mg/l water was apparently sufficient to ensure the continuous availability of the test chemical to the bacteria. Therefore the modifications introduced to the test method were not necessary in this case. The decreased rate of biodegradation of the sample melted on the glass filter (Fig.1 curve C) can be explained by a reduction in the availability of calcium stearate as a consequence of the melting operation (glazing of the surface) and the absence of agitation.

2. Anthraquinone (Fig.2)

   Anthraquinone was biodegraded in all tests with agitation. Curves A and D indicate that its solubility in water appears to be sufficient to ensure the availability to bacteria only when continuous water turbulence is maintained. The use of solvents, carriers, dispersants or ultrasonic dispersion in addition to mechanical agitation do not further influence the results. The unusually low result shown in curve E compared to curve F is not fully understood, but may be due to a variation in inoculum activity or inadvertent loss of CO_2 from the test vessel. All other curves correspond to rapid and comparable biodegradation rates, after some variation in the initial lag period which may be associated with the use of different inocula.

3. Beeswax (Fig. 3-4)

   All results with emulsifiers and agitation (curves G, I and O), and use of dichloromethane and agitation (curve E), show about the same high

biodegradation rate. All results without agitation (curves A, C and N) show a slow, steady biodegradation rate. When only agitation is used (curves B, H, L and M) only one curve (L) showed an acceptable biodegradation rate. Ultrasonic dispersion without emulsifier (curve F) also gave a low biodegradation rate. The use of glass filter paper as a solid carrier with agitation (curves D and K) gave differing results, one giving a high, the other an intermediate biodegradation rate. This may once again be explained by a glazing effect.

F. DISCUSSION AND CONCLUSIONS

The experiments with calcium stearate, anthraquinone and beeswax demonstrated that the respirometric methods for assessing ready biodegradability as described in the OECD/EEC Guidelines can be adapted for the testing of poorly water-soluble chemicals. The choice of method for introducing a poorly-soluble chemical into the test medium depends on its physical form. In general, agitation clearly accelerates biodegradation. The use of emulsifiers and solvents can achieve a better distribution of the test chemical and improve its availability to bacteria. The magnitude of the effect depends on the physical characteristics of the chemical, such as water solubility, morphology and dispersability.

In this limited study the use of solid carriers for solid poorly-soluble substances produced variable results. This is believed to be due to difficulties in the application of the chemical to the carrier, e.g. glazing. The use of solid carriers might however be suitable for oily materials.

The physical state of the chemical in the test medium can markedly affect the shape of the biodegradation curve. Limited physical availability of the chemical to bacteria, even with the best dispersion techniques, may result in a lower biodegradation rate than that which reflects the intrinsic biodegradability of the substance. The modified methods described above have been shown to be capable of surmounting physical barriers which would normally prevent an assessment of the biodegradability of poorly-soluble materials. More data are needed to appraise the environmental relevance of the modified methods.

Table 3

Results: Biodegradation of Poorly-Soluble Substances

Substance	Curve	Test Method (Lab. No.)	Duration, days (weeks)	Agitation	Carrier / Method of Distribution in Water	Basis of Calc. of Degradation	Degradation %	10% lag time, days (approximate values)	Time to 60% biodegradation (approximate values)
CALCIUM STEARATE	A	STURM (3)	24	-	-	$CO_2/ThCO_2$	91	2	11
	B	STURM (3)	24	+	-	$CO_2/ThCO_2$	99	1	7
	C	STURM (3)	24	-	Glass filter	$CO_2/ThCO_2$	55	5	-
	D	STURM (3)	20	+	Ultrasound	$CO_2/ThCO_2$	72	2	8
	E	STURM (3)	20	+	Glass filter	$CO_2/ThCO_2$	84	2	12
	F	STURM (3)	24	+	Glass filter	$CO_2/ThCO_2$	88	5	12
	G	MITI (5)	32	+	-	BOD/ThOD	91	4	18
	H	MITI (5)	32	+	Nonylphenol.10EO.5PO	BOD/ThOD	93	4	13
ANTHRAQUINONE	A	STURM (3)	24	-	-	$CO_2/ThCO_2$	17	17	-
	B	STURM (3)	24	+	-	$CO_2/ThCO_2$	75	11	19
	C	STURM (3)	20	+	Ultrasound	$CO_2/ThCO_2$	65	7	14
	D	STURM (3)	24	-	Glass filter	$CO_2/ThCO_2$	30	11	-
	E	STURM (3)	24	+	Glass filter	$CO_2/ThCO_2$	51	10	-
	F	STURM (3)	20	+	Glass filter	$CO_2/ThCO_2$	91	7	12
	G	STURM (3)	20	+	Dichloromethane	$CO_2/ThCO_2$	83	7	13
	H	MITI (5)	25	+	-	BOD/ThOD	93	8	15
	I	MITI (5)	25	+	Nonylphenol.10EO.5PO	BOD/ThOD	81	9	18
	K	RDA (2)	14	+	-	BOD/ThOD	70	7	12

Table 3 (./2)

Substance	Curve	Test Method (Lab. No.)	Duration, days (weeks)	Agitation	Carrier / Method of Distribution in Water	Basis of Calc. of Degradation	Degradation %	10% lag time, days (approximate values)	Time to 60% biodegradation (approximate values)
BEESWAX	A	STURM (3)	24	-	-	$CO_2/ThCO_2$	13	17	-
	B	STURM (3)	24	+	-	$CO_2/ThCO_2$	38	12	-
	C	STURM (3)	24	-	Glass filter	$CO_2/ThCO_2$	35	8	-
	D	STURM (3)	24	+	Glass filter	$CO_2/ThCO_2$	77	4	10
	E	STURM (3)	20	+	Dichloromethane	$CO_2/ThCO_2$	82	5	13
	F	MITI (1)	23	+	Ultrasound	BOD/ThOD	10	23	-
	G	MITI (1)	22	+	Ultrasound/Span 80/ Tween 80	BOD/ThOD	58	3	20
	H	MITI (5)	32	+	-	BOD/ThOD	20	25	-
	I	MITI (5)	32	+	Nonylphenol.10EO.5PO	BOD/ThOD	77	3	24
	K	MITI (5)	32	+	Glass filter	BOD/ThOD	29	7	-
	L	RDA (2)	14	+	-	BOD/COD	43	<7	-
	M	RDA (4)	(7)	+	-	BOD/ThOD	38	2	-
	N	RDA (4)	(7)	- *	-	BOD/ThOD	29	3	-
	O	RDA (4)	(7)	+	tri-t-butyphenol.8EO + styrene-phenol.8EO	BOD/ThOD	90	<7	7

* agitation only for 1 hour per day

FIGURE 1

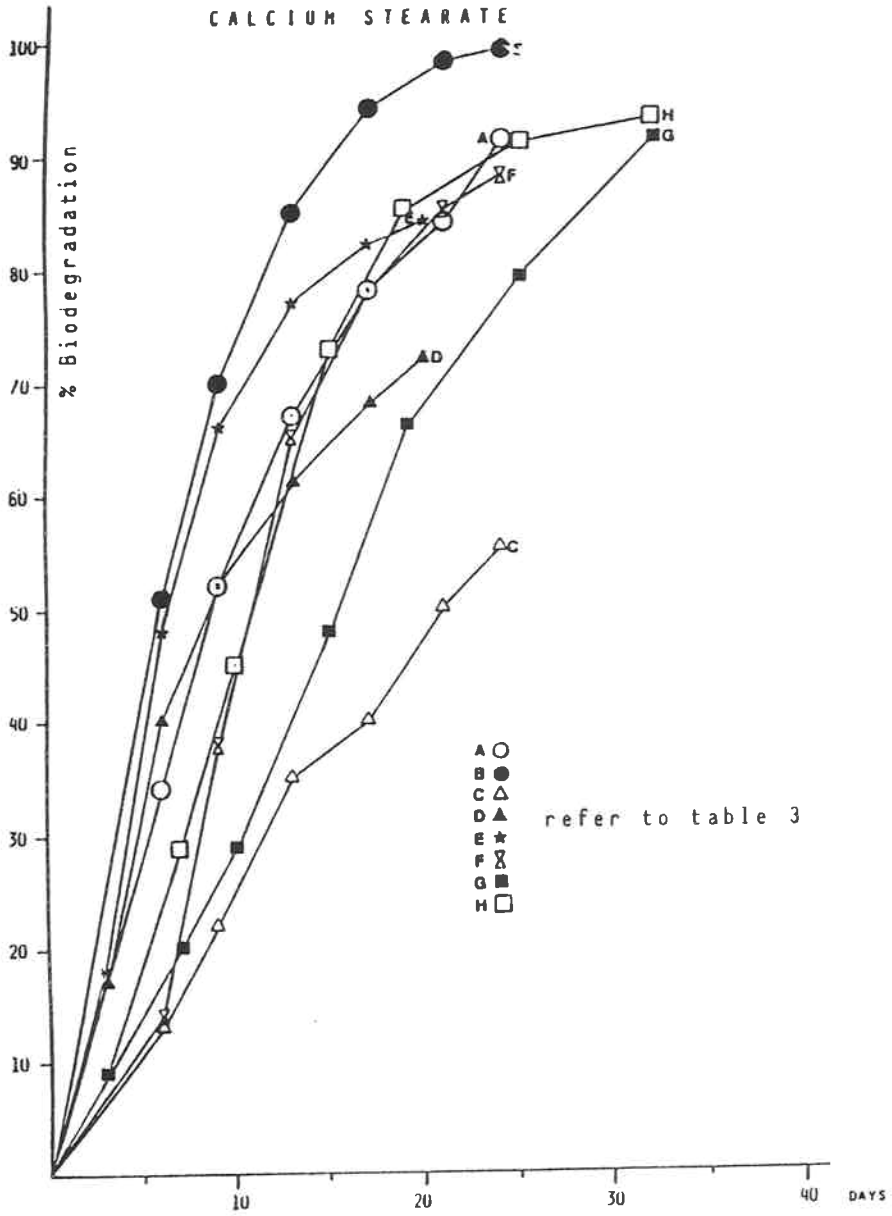
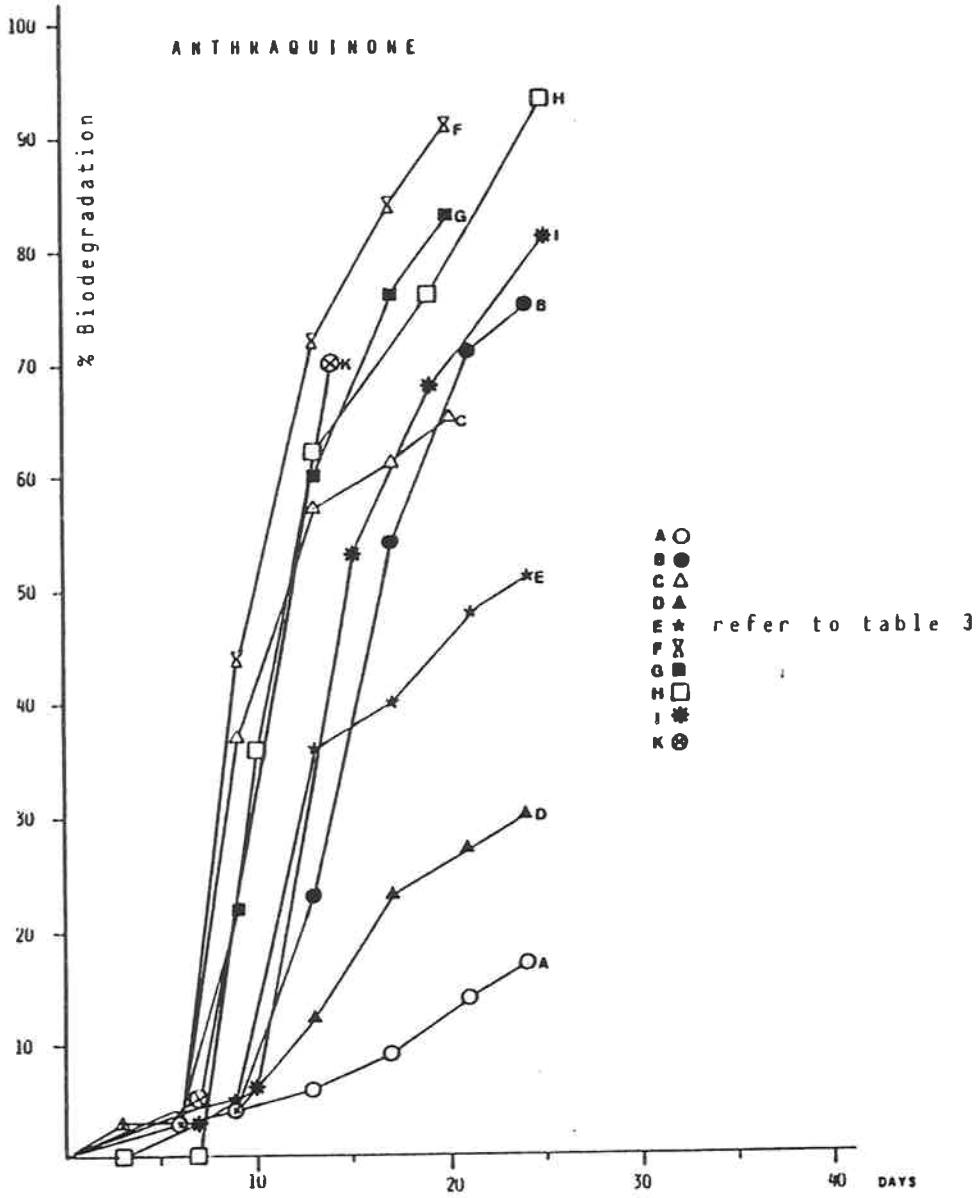


FIGURE 2



BIODEGRADATION CURVES OF CALCIUM STEARATE AND ANTHRAQUINONE

FIGURE 3

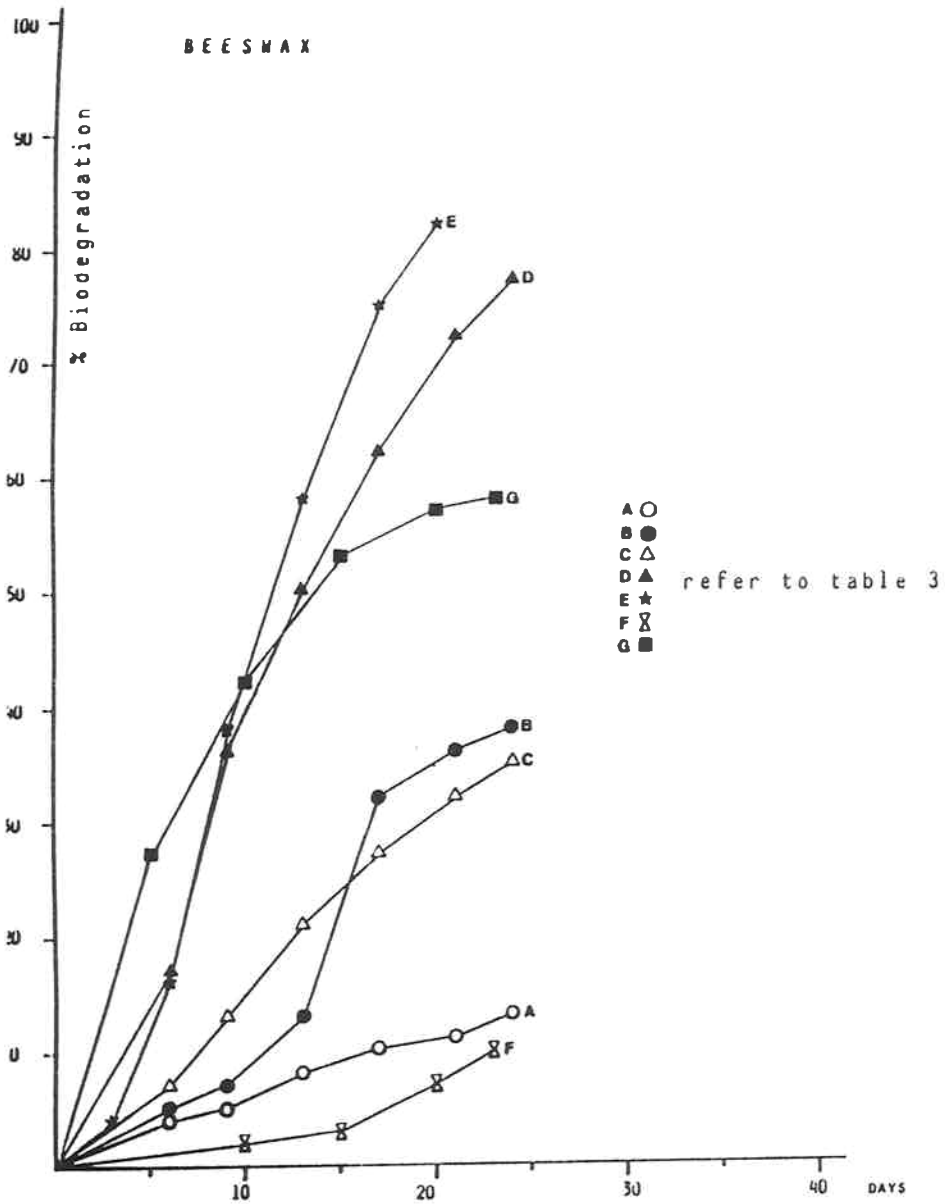
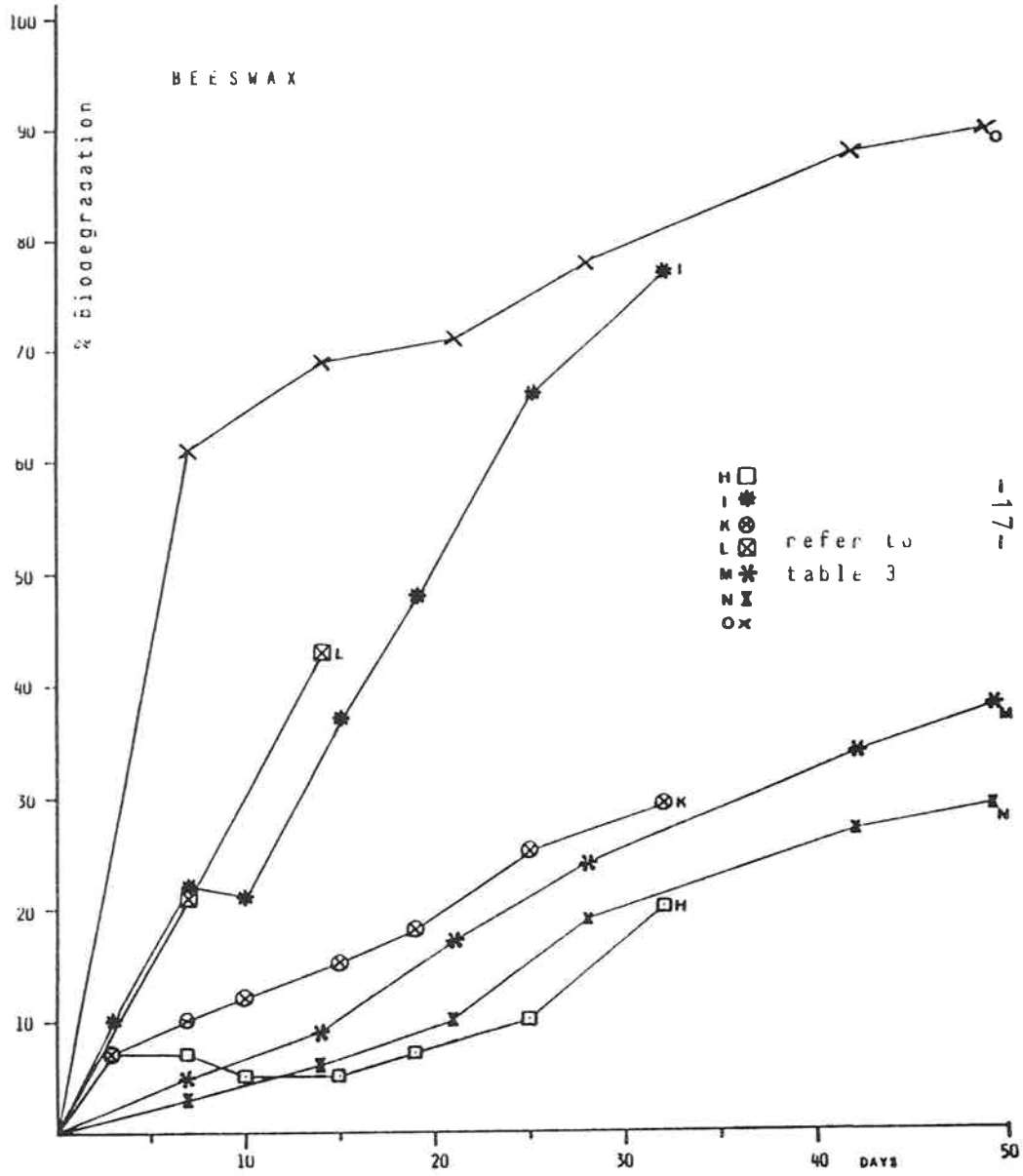


FIGURE 4



BIODEGRADATION CURVES OF BEESWAX

G. RECOMMENDED TEST PROCEDURE

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

1. For sampling and weighing substances in quantities of 10-50 mg a balance must be available allowing accurate weighing in the range of ± 0.5 mg. While homogeneous liquids will seldom present sampling problems, it is recommended that solid materials be homogenised by appropriate means to avoid errors due to non-homogeneity. When testing mixtures prepared by mechanical blending, or substances with large amounts of impurities, sampling of an homogeneous and representative quantity of a few milligrams of the substance requires great attention and skill.

2. ThOD must be calculated from the structural formula of the test chemical as in the OECD-Guideline No.301 D, and $ThCO_2$ from the carbon content. For substances of unknown composition or with a high level of impurities the COD must be established by e.g. the modified determination described by Gerike (1984). The $ThCO_2$ of substances of unknown composition must be derived from the carbon content determined by elemental analysis.

3. Various forms of agitation may be used. Care should be taken to use only sufficient agitation to keep the chemical in dispersion, and to avoid overheating, excessive foaming and excessive shear forces.

4. An emulsifier (e.g. those used in this study) which gives a stable dispersion of the chemical may be used. It should not be toxic to bacteria, and should not be biodegraded or cause excessive foaming under test conditions.

5. The same criteria apply to solvents as to the emulsifiers. Although reservations about the use of solvents were made above (cf. chapter B), the tests with dichloromethane showed that under strict conditions some solvents can be recommended for use.

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

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

8. Any of the three respirometric tests modified as above (Chapter B.4) can be used to study the biodegradability of poorly-soluble compounds.

The recommended test procedure described above could form the basis of a general chapter in the existing Guidelines setting out special requirements for the testing of poorly-soluble chemicals.

H. APPENDIX

Abbreviations

BOD	: Biochemical oxygen demand.
COD	: Chemical oxygen demand.
DOC	: Dissolved organic carbon.
EEC	: European Economic Communities.
EPA	: Environmental Protection Agency (USA).
ISO	: International Standards Organisation.
MITI	: Ministry of International Trade and Industry (Japan).
OECD	: Organisation for Economic Co-operation and Development.
PTFE	: Poly-tetrafluoro-ethylene
RBTs	: Ready Biodegradability Tests.
RDA	: Repetitive Die Away.
ThOD	: Theoretical oxygen demand.
$ThCO_2$	: Theoretical carbon dioxide production after complete mineralisation.
TOC	: Total organic carbon.

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