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TEST GUIDELINES PROGRAMME

Number 2

DETAILED REVIEW PAPER
ON BIODEGRADABILITY TESTING

ENVIRONMENT MONOGRAPH N° 98

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 1995

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Environment Directorate

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Foreword

The OECD is one of the international organisations with a leading role in the promotion of internationally acceptable methods for the testing of chemicals for regulatory purposes. These chemicals include, among others, industrial chemicals, pesticides, food additives and pharmaceuticals.

The development of OECD Test Guidelines in a specific area starts with a Detailed Review Paper (DRP), when it is considered essential that the "state-of-the-art" in the area under review first be assessed. Criteria that apply to DRPs, as well as further details of OECD Test Guideline development procedures, are given in OECD Environment Monograph No. 76, *Guidance Document for the Development of OECD Guidelines for Testing of Chemicals* (1993).

This DRP on biodegradability testing is the second in a new OECD Series on the Test Guidelines Programme, to be published in the form of Environment Monographs. (Environment Monograph No. 76 is being reissued as No. 1 in the series.) The objective of this DRP was to review the area of biodegradability testing in order to identify whether, in the light of scientific developments, there was a need to revise existing OECD Test Guidelines or to develop new Guidelines. An extensive literature review has been performed by Dr Harry Painter of the United Kingdom, as a consultant to the OECD Secretariat, with contributions to section A.9 (Simulations) from Japan.

This Detailed Review Paper has been reviewed, and was greatly appreciated, by OECD Member countries. Their responses to the author's recommendations for further work are included. New work initiated within the Test Guidelines Programme as a result of this DRP is also indicated.

The Joint Meeting of the Chemicals Group and the Management Committee of the Special Programme on the Control of Chemicals recommended that this document be derestricted. It has been made public under the responsibility of the Secretary-General of the OECD.

Detailed Review Paper on Biodegradability Testing

Prepared for the Organisation for Economic Co-operation and Development

Author: **Dr H.A. Painter (UK)**
Freshfield Analysis Limited
Caxton Villa
Park Lane
Knebworth
Herts SG3 6PF
United Kingdom

With contributions to section A.9 (Simulations) from:

Kazuaki Miura	**Lion Corporation, Biological Laboratories**
Yasuhiko Tosima	**Kao Corporation, Biological Science Laboratories**
Susumu Nakamura	**Fuji Photo Film Co. Ltd., Material Safety Test Center, Environmental Protection and Safety Department**
Tadayosi Sigeoka	**Mitsubisi-kasei Institute of Toxicological and Environmental Sciences**
Akira Tanoue	**Sumitomo Chemical Co. Ltd., Environmental Health Science Laboratory**
Mineo Takatsuki	**Chemicals Inspection and Testing Institute, Japan, Chemical Biotesting Center**
Masaru Kitano	**Chemicals Inspection and Testing Institute, Japan, Corporate Technical Department**

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Summary

Literature searches were made to investigate developments and advances in methods for determining biodegradability over the last decade or so. The search was extended to the adjacent fields of microbiology and biochemistry for papers which explain observed phenomena and which might lead to significant improvements in existing testing methods, or to new methods.

The effects of the presence of more than one xenobiotic, nutrients, mixed bacterial populations and other organisms, as well as the concentration of the test chemical on biodegradability, were also included in the review. Other aspects considered were adsorption, inhibition, nitrification and metabolites.

The literature reviewed did not indicate that there was a real need to develop *fundamentally* new OECD Test Guidelines. However, some additional methods which are not addressed in the existing Guidelines could be useful for generating data which are to a great extent not yet available. Some of these data can be obtained using methods with which much experience has already been gained. With respect to further data needs, methods are available but they are unsuitable since the cost and effort involved probably cannot be justified except for chemicals produced at very high tonnages.

Kinetics of biodegradation at environmentally realistic concentrations can be calculated rather precisely by determining mineralization, which requires the use of ^{14}C-labelling, or from primary biodegradation, which requires sensitive compound-specific analytical methods. But a genuine need for these determinations, and at what tier of testing the kinetic data should be obtained, must be clearly established before such methods are considered for inclusion in the OECD and EC sets of methods.

All practical aspects of the present methods, including simulation tests, have been considered, from the composition of the media to the analytical procedures. Their limitations have been examined, and possible improvements have been suggested.

The ability of results from the screening tests to predict accurately the environmental behaviour of the chemicals tested was investigated, and ways of improving confidence in the prediction were considered.

Values of the indicators of biodegradability (% DOC, % ThOD and % $ThCO_2$) indicating essentially complete mineralization were re-examined in the light of experimental evidence: current "pass" values for % ThOD and % $ThCO_2$ are considered to be too high.

The implications of the use of inocula which have been pre-exposed to the test chemical in screening tests were examined.

Changes have been proposed in the strategies for testing to reflect such factors as structure-activity relationships, the very low "pass" rate of "new" chemicals in the screening tests, the proposed use of pre-exposed inocula, and proposed increase in the number of classes of biodegradability. Non-confidential data on biodegradability held by Competent Authorities and the European Commission (EC) might be useful in reviewing strategies and should be made available.

A brief review of literature on structure/biodegradability showed that, though no all-embracing relationship has been established, equations related to small numbers of compounds with common functional groups have been successful.

The recommendations (see section "Author's Recommendations" below) arising from this review can be divided into two groups:

- proposals for new methods, or major changes to existing tests, given in Table R.1, and

- some minor changes, given in Table R.2.

The tables indicate the relevant sections in which the proposal is referred to. In Table R.1, personal priority ratings and proposed actions are indicated. Most of the proposals in Table R.1 are also considered in the section "Discussions and Conclusions".

With respect to proposals for Guideline revision and development (see Table R.1), the following work items were given a clear high priority by Member countries:

(a) development of guidance for testing insoluble substances in aerobic tests;

(b) development of 2-Phase Closed Bottle (BODIS) – suitable for insoluble substances;

(c) revision of Guideline 301B Modified Sturm Test;

(d) development of a Guideline to simulate degradation in aquatic sediments;

(e) development of a Guideline to assess anaerobic biodegradation (methane production);

(f) development of a Guideline to assess inhibition of methane production.

With respect to recommendations for minor modifications to existing tests (see Table R.2), the following work items were given a high priority:

(i) identification of less readily biodegradable reference compounds or sterilization of medium before inoculating;

(ii) identification of alternative sterilizing agents for abiotic controls and sample preservation;

(iii) reconsideration of pass levels and stable metabolites; lowering the levels for ThOD and $ThCO_2$ from 60 to 50%;

(iv) liaison with appropriate ISO working Group on methods for degradation in soil.

Résumé

Une étude bibliographique a été menée afin de faire le point sur les méthodes de détermination de la biodégradabilité développées au cours des dix dernières années. La recherche a été étendue aux domaines voisins de la microbiologie et de la biochimie pour les articles qui décrivent les phénomènes observés et qui peuvent conduire à des améliorations significatives des méthodes existantes ou à de nouvelles méthodes.

Les facteurs suivants qui influent sur la biodégradabilité ont été pris en compte dans l'étude: présence de plus d'un xénobiotique, nutriments, populations bactériennes mixtes et autres organismes, concentration de la substance testée. D'autres aspects ont été considérés: l'adsorption, l'inhibition, la nitrification et les métabolites.

L'analyse bibliographique n'a pas mis en évidence la néccéssité de développer des Lignes Directrices OCDE *fondamentalement* nouvelles. Cependant, il pourrait être utile d'ajouter d'autres méthodes aux Lignes Directrices existantes pour obtenir les données nécessaires qui manquent jusqu'ici. Des méthodes pour lesquelles on a déjà beaucoup d'expérience peuvent permettre d'obtenir ces données. Pour les autres besoins, des méthodes existent mais elles ne conviennent pas, leur coût et l'effort qu'elles impliquent ne pouvant probablement pas être justifiés, sauf dans le cas des substances chimiques produites à haut tonnage.

Les cinétiques de biodégradation à des concentrations environnementales réalistes peuvent être calculées à partir de la mesure de la minéralisation grâce à l'utilisation de composés marqués au ^{14}C, ou à partir de la biodégradation primaire qui nécessite des méthodes d'analyse spécifiques sensibles. Mais le besoin réel d'une telle information et à quel stade des essais séquentiels l'obtenir, doivent être clairement établis avant d'envisager d'inclure de telles méthodes dans les batteries d'essais OCDE et CE.

Tous les aspects pratiques des méthodes actuelles de biodégradabilité, y compris les essais de simulation, ont été considérés, de la composition des milieux jusqu'aux procédures analytiques ; leurs limites ont été examinées et des propositions d'améliorations ont été formulées.

La possibilité de prédire exactement le comportement des produits chimiques dans l'environnement à partir des résultats des tests de screening a été examinée, ainsi que les moyens d'améliorer la confiance dans la prédiction.

Les valeurs des taux de biodégradabilité (% COD, % DThO et % CO_2Th) indiquant une minéralisation complète, ont été ré-examinées à la lumière des observations expérimentales: les valeurs «seuil» pour le % DThO et le % CO_2Th ont été jugées trop élevées.

Les conséquences de l'utilisation, dans les tests de screening, d'inocula préalablement exposés au produit chimique testé ont été examinées.

Des changements dans les stratégies d'essais ont été proposés pour que soient pris en compte des facteurs tels que les relations structure-activité, le très faible pourcentage de substances «nouvelles» qui «passent» les tests de screening, l'utilisation d'inocula pré-exposés et l'augmentation du nombre de classes de biodégradabilité. Les aspects non confidentiels des

données de biodégradabilité détenus par les Autorités Compétentes et la Commission pourraient être utilisés dans l'examen des stratégies et devraient pouvoir être accessibles.

Une rapide revue de la littérature concernant la relation structure/biodégradabilité a montré que, même si aucune relation d'ensemble n'a été démontrée, on pouvait établir des équations reliant un petit nombre de composés ayant des groupes fonctionnels communs.

Les recommandations (cf. la section «Author's Recommendations») issues de cette étude peuvent être divisées en deux parties:

- des propositions pour de nouvelles méthodes ou des modifications importantes des méthodes existantes, tel que présenté dans le tableau R.1, et

- quelques modifications mineures, tel que présenté dans le tableau R.2.

Les tableaux indiquent les sections auxquelles se réfèrent les propositions. Le tableau R.1 présente les priorités et les actions proposées par l'auteur. La plupart des propositions du tableau R.1 sont également analysées dans la section «Discussions and Conclusions».

En ce qui concerne les propositions de révision et de mise au point de Lignes Directrices (cf. tableau R.1), les pays membres ont clairement donné une forte priorité aux points suivants:

(a) mise au point d'un document d'orientation pour les essais de substances insolubles en aérobie ;

(b) mise au point de la méthode en deux phases en flacon fermé (BODIS) – applicable aux substances insolubles ;

(c) révision de la Ligne Directrice 301B, test de Sturm modifié ;

(d) mise au point d'une Ligne Directrice pour évaluer la dégradation dans les sédiments aquatiques ;

(e) mise au point d'une Ligne Directrice pour évaluer la biodégradation en anaérobie (production de méthane) ;

(f) mise au point d'une Ligne Directrice pour mesurer l'inhibition de la production de méthane.

En ce qui concerne les recommandations de modifications mineures des méthodes existantes (cf. tableau R.2), une forte priorité a été donnée aux points suivants:

(i) rechercher des composés de référence moins facilement biodégradables ou stériliser le milieu avant ensemencement ;

(ii) identifier d'autres agents stérilisants pour les témoins abiotiques et la conservation des échantillons ;

(iii) reconsidérer les niveaux «seuil» et les métabolites stables ; baisser les niveaux de 60 à 50 % pour la DThO et le CO_2Th ;

(iv) établir la liaison avec les groupes de travail ISO correspondants à propos des méthodes de dégradation dans le sol.

Author's Recommendations

It is important that any considerations of introducing new methods, or even changes to existing methods, should be made at the same time as an examination of the strategy for testing (see sections E, B.1), since changes in methods could have repercussions within the strategy. This is especially true of the European Union (EU) system of testing. At the same time, consideration should be given to the number of classes of biodegradability (see section A.8.4).

Although any test may be used so long as it can be justified scientifically (a fact not well known), notifiers and testing-houses are conservative and will generally perform the "standard" tests. Thus, it would seem that the possibility of applying other tests should be better advertised. It would also be advantageous to include as many valid points as possible on guidance in performing the tests within the standard guidelines.

A further general point is that at the end of the revision of the methods, more prominence should be given to a new OECD testing strategy than seems to have been given to the previous one (Figure E.2).

The recommendations arising from this review can be divided into two groups:

- proposals for new methods, or major changes to existing tests, given in Table R.1, and

- some minor changes, given in Table R.2.

The tables indicate the relevant sections in which the proposal is referred to and, in Table R.1, personal priority ratings and proposed actions are indicated. Most of the proposals in Table R.1 are also considered in the section "Discussions and Conclusions".

The first three items in Table R.1 are relatively straightforward. The proposals on insoluble, or poorly soluble, chemicals are of high priority. Item 2, BODIS, has been satisfactorily ring-tested by the International Organisation for Standardisation (ISO) and a revised version of the method will be considered at an ISO meeting in Vancouver in October 1994. Volatile chemicals are of less importance than insolubles and item 3 is given a lower priority. The Sturm test (item 4) is a special case; the efficiency of the absorption of CO_2 should be investigated by at least one more laboratory to confirm or deny the present evidence. If the Sturm test is found faulty, the headspace methods (item 5) should be given higher priority and soon subjected to a ring test, since they offer the opportunity to identify the "10-d window" accurately. Even if the Sturm method is found not to be faulty, or can be simply modified by determining dissolved organic carbon (DOC) in the medium, it would still be advantageous to consider the headspace methods since they are less cumbersome and can probably deal with lower concentrations of test chemicals.

The European Commission (EC) version of the activated sludge simulation test (item 6a) contains a variety of options, all treating 10-20 mg DOC/l, and this could easily be accepted into the OECD Guidelines. However, adapting the tests to insolubles and volatiles (item 6b) is of less importance and would require further work; also, the principle of their inclusion may not unanimously be accepted. Methods simulating biofilters (item 6c) are available and have their place, but are of less importance than activated sludge. Operating simulations of sewage treatment

with concentrations in the 10 μg/l region is more environmentally realistic, and methods not involving ^{14}C-labelling are available (item 7).

The "recalcitrant" metabolite method based on the activated sludge simulation test (item 8) is of less general importance. However, there may be some high-tonnage chemicals to which it should be applied, so that any stable intermediates could be quantified. If present, they should then be identified and perhaps tested for ecotoxicity.

The next two items, simulation in natural waters and use of pre-exposed inocula (9 and 10), though diverse, require decisions on the strategy of testing and on the number of classifications of biodegradability. For primary degradation, methods are available not involving ^{14}C-labelling (item 9a); for mineralization (item 9b) ^{14}C-labelling tests are available, and it may now be time to formalize non-mandatory methods using the widespread expertise in the literature. This awaits a decision.

The pre-exposure of inocula to the test chemical (item 10) for use in screening tests, together with the establishment of another class of biodegradability, have attractions, but they should be considered in conjunction with a revised strategy.

The tests for inherent biodegradability could be adapted to coping with insoluble chemicals and with most volatiles (item 11), but this is of low priority.

Turning to anaerobic biodegradability, there are obvious needs for a method for assessing the potential to produce methane plus carbon dioxide (item 12) and for a test to determine inhibition of gas production by chemicals (item 14). Both types of tests are available. The former test has also been ring-tested by ISO, and a revised method will be discussed at the ISO meeting in Vancouver in October 1994. Of lesser importance is the need for a simulation of anaerobic digestion (item 13); such methods are available but are messy in their execution.

There are many minor changes which could be made to the existing methods, some of which are indicated in Table R.2. The items are not all of equal importance, but all should in some way contribute to making the methods more valid, reliable and useful. Individual laboratories could perhaps be invited to investigate the practical topics in Table R.2, and results could be considered at a specially convened meeting and perhaps incorporated into the Guidelines.

Finally, the literature concerning (quantitative) structure-biodegradability relationships ((Q)SBR) should be frequently updated so that any significant development may be quickly absorbed into the strategy for testing.

Table R.1   Author's proposals for Guideline revision and development
(continued on next page)

Proposals for Guideline Revision and Development				
Proposal for work	**Nature of proposal (i.e. revision of existing Guideline, new Guideline, etc.)**	**Relevant section of DRP and leading references**	**Proposed priority (High, Medium, Low) and action**	
1 Aerobic – preparation of insolubles	Guidance relevant to several existing Guidelines	A.5.1(a), C.1 ISO (1990,b)	priority:	H
			action:	accept
2 2-phase Closed Bottle (BODIS) – suitable for insolubles	New Guideline	A.5.1(a), A.7.5, C1 ISO (1990,a)	priority:	H
			action:	await ISO ring test 92[1]
3 Volatile chemicals	Guidance on handling, relevant to several existing Guidelines	A.5.1(b), C.2 ISO (1984)	priority:	M
			action:	accept
4 Sturm test – collection of CO_2	Revision of Guideline 301B – re 10-day window	A.6.1, A.7.4, C4.2	priority:	H
			action:	investigate
5 CO_2 – headspace method	New Guideline for ready biodegradability?	A.7.4, D.3 Struijs & Stoltenkamp (1990) Birch & Fletcher (1991)	priority:	M
			action:	consider after 4 & ring test
6a Simulation of sewage treatment – include porous pot, domestic sewage	New Guideline	A.9.1, C.5.1 Off. J. E. Comm. (1984)	priority:	M
			action:	accept
6b Simulation of sewage treatment – insolubles and volatiles	Include as guidance in 6a?		priority:	L
			action:	consider
6c Simulation of sewage treatment – rotating tubes (biofilters)	New Guideline	Gerike et al. (1980) HMSO (1983)	priority:	L
			action:	consider
7 Simulation of sewage treatment – modified for low concentrations (μg/l)	New Guideline or include as guidance in 6 above?	A.9.1, C.5.1 Nyholm et al. (1992)	priority:	M
			action:	consider
8 "Recalcitrant" metabolite (Husmann units)	Guidance within existing method(s), e.g. 6a	A.8.4, A.8.5, C.5.1 Gerike et al. (1984)	priority:	L
			action:	consider
9a Simulation of natural waters sediments – surrogate tests, specific analysis	New Guideline	A.9.2, A.9.3, C.5.2 Dambourg et al. (1991)	priority:	H
			action:	accept

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[1]   ISO ring test was satisfactory. Revised version to be discussed at ISO meeting in Vancouver in October 1994.

	Proposals for Guideline Revision and Development				
Proposal for work	**Nature of proposal (i.e. revision of existing Guideline, new Guideline, etc.)**	**Relevant section of DRP and leading references**	**Proposed priority (High, Medium, Low) and action**		
9b Simulation of natural waters sediments – surrogate tests, mineralization ^{14}C, kinetics	New Guideline – include in 9a?	A.9.2, B.3, C.6	priority:	M[2]	
			action:	consider after testing strategy decided	
10 Pre-exposure of inocula and establishment of new class of biodegradability	Revision of existing Guidelines to include this possibility?	A.4.4, C.4.1	priority:	M	
			action:	await consideration of testing strategy	
11 Inherent biodegradability – accommodate insolubles and volatiles	Revise existing Guidelines to include guidance on handling insolubles and volatiles	A.5.1a, A.5.1b	priority:	L	
			action:	consider, but less important	
12 Anaerobic – potential for biodegradation; methane production	New Guideline	C.3.1 ECETOC (1988)	priority:	H	
			action:	await ISO ring test (end of 1992)[1]	
13 Simulation of anaerobic digestion	New Guideline	C.3.1 Bruce et al. (1966)	priority:	L	
			action:	would follow 12, but less important	
14 Inhibition of methane production	New Guideline	C.3.2	priority:	H	
			action:	accept, important	

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[2]   Proposal – to formulate a method which will not be mandatory in the EC scheme.

Table R.2   Author's proposals for minor changes to existing Guidelines

	Proposals for minor changes to existing Guidelines	Relevant sections of DRP
1	Seek less readily biodegradable reference compound or sterilize medium before inoculating	A.4.1
2	Use ATP or other methods to check inoculum activity. Use ATP as indicator of growth and biodegradation	A.4.1 D.1
3	Insolubles – try out liposome method of Miller and Bartha (1989)	A.5.1a
4	Use kinetic approach to relate results in screening tests with field/simulation tests	A.6 B.2
5	Sterilizing agents for abiotic controls and sample preservation	A.7.2
6	Investigate endogenous DOC release in blanks, for valid correction of DOC with test chemical	A.7.3
7	Check DOC values for volatile chemicals in various types of analysers	A.7.3
8	Check optimal inoculum size by calculation in respirometer tests	A.7.5
9	Seek improvements in the determination of COD (used for test materials of unknown composition); or seek other methods of determining the theoretical oxygen demand in respirometer methods	A.7.6
10	Consider inhibition tests other than OECD activated sludge respiration test, e.g. ATP, tritiatedthymidine	A.8.2
11	Reconsider pass levels and stable metabolites; lower the levels from 60 to 50% for ThOD and $ThCO_2$	A.8.4
12	Relate results of laboratory tests (surrogates) with field tests. European Workshop on Freshwater Field Tests (EWOFFT), Potsdam, 25-26 June 1992 (see Hill, et al., 1994)	A.9.2 A.9.3 C.5.2 A.4.4
13	Liaise with ISO/TC140/SC4/WG1 on methods for degradation in soil	A.9.5 C.5.4
14	Consider giving guidance on enumeration of bacteria for determining 2nd order kinetic constant	B.3
15	Correlate results of EC oil test (CEC, 1982) with tests for ultimate biodegradation	D.2

Member Country Responses to DRP Recommendations

There was a high degree of consensus from Member countries when they were asked to comment on the recommendations made in the Detailed Review Paper. In general, countries agreed with both the proposed priority given to the various work items and the proposed action.

With respect to proposals for Guideline revision and development (see Table R.1), the following work items were given a clear high priority by Member countries:

(a)   development of guidance for testing insoluble substances in aerobic tests;

(b)   development of 2-Phase Closed Bottle (BODIS) – suitable for insoluble substances;

(c)   revision of Guideline 301B Modified Sturm Test;

(d)   development of a Guideline to simulate degradation in aquatic sediments;

(e)   development of a Guideline to assess anaerobic biodegradation (methane production);

(f)   development of a Guideline to assess inhibition of methane production.

With respect to recommendations for minor modifications to existing tests (see Table R.2), the following work items were given a high priority:

(i)   identification of less readily biodegradable reference compounds or sterilize medium before inoculating;

(ii)   identify alternative sterilizing agents for abiotic controls and sample preservation;

(iii)   reconsider pass levels and stable metabolites; lower the levels from 60 to 50% for ThOD and $ThCO_2$;

(iv)   liaise with appropriate ISO Working Group on methods for degradation in soil.

Work Now in Progress in the Test Guidelines Programme

The development and revision of Test Guidelines for biodegradation is only one of the many areas covered by the Test Guidelines Programme. For this reason, it is not possible to tackle immediately all those areas of biodegradation testing identified by Member countries as having high priority. At their meeting in October 1993, the National Co-ordinators of the Test Guideline Programme agreed that work should focus initially on the development of a Guideline to simulate degradation in aquatic sediments (i.e. item (d) above) and Guideline(s) for assessing fate and behaviour of chemicals in soil (i.e. related to item (iv) above). Work on the development of a Guideline to assess anaerobic biodegradation (methane production) will be initiated once proposals from ISO become available (i.e. item (e) above).

Other areas identified as being of high priority by the DRP and Member countries will be addressed as the work progresses.

A. Conditions in the OECD tests

A.1   Introduction

The conditions under which the OECD tests for ready biodegradability (OECD, 1981) are carried out have been subjected to only minor changes since their inception. These minor changes are included in the latest "harmonized" version (1992). The original guidelines were used in producing the EC methods (Off. J. E. Comm 1984, 1988; revised 1992: L383A, 35, 187-235), and by other countries including the United States (EPA, 1989). The various aspects of the tests will be dealt with under separate headings in the following sections. For convenience, a synopsis of each test is given in Appendix I.

It is first necessary to re-state the objectives of the tests, since the conditions employed in them are a compromise between ideal objectives and what is practical and economic. The methods for assessing ready biodegradability are often criticized in the literature, either directly or implicitly, as being divorced from the "real world". The conditions in the tests for ready biodegradability have been developed to devise screening methods (which can be easily and cheaply carried out in the laboratory) to decide, provisionally, whether a chemical has the potential to be easily biodegraded in the aquatic environment. The objective at this stage is not to predict whether a chemical will degrade in specific environmental situations, and certainly not the extent of biodegradability in these situations.

Failure in such a test does not indicate that the chemical is not biodegradable, but implies that further investigation is necessary. This can take the form of another test for ready biodegradability or a test for inherent biodegradability, the conditions of which are more conducive to biodegradation. The next step for chemicals which pass one or other of the previous tests is to apply a test which simulates a compartment of the environment. It is at this stage that "real world" conditions are applied. More details of the present strategies of testing are given in a later section (E).

In the EU scheme, which is a part of the Directive on "New" Chemicals, the test methods are virtually identical with those in the OECD scheme. The difference lies in what circumstances "trigger" the application of a test in the next higher tier; the OECD scheme is "voluntary", while the EU scheme demands further testing either when certain higher tonnages are attained or if the chemical is particularly toxic to fish or other aquatic organisms. It seems that few, if any, "new" chemicals have yet been tested at the simulation stage under the EU scheme. Since it was this scheme which was the "driving force" in establishing agreed test methods, no simulation tests have been examined or agreed except the OECD Confirmatory Test, which simulates waste water treatment. There are no agreed counterparts simulating rivers, sediments, lakes, etc., although the literature contains many references to tests which in varying degrees mimic environmental compartments other than waste water treatment. Such experiments are not designed for routine use, since either very sensitive methods of analysis for the specific chemical at environmental concentrations (μg/l and less) are required for assessing primary biodegradation or suitably ^{14}C-labelled test chemicals must be available. This is dealt with in a later section (A.9).

A.2   Nutrients

A.2.1 Introduction

The media used in the tests are based on the nutritional requirements of bacteria for optimal growth. Thus they contain the phosphates, sulphates and chlorides of sodium, potassium, ammonium, calcium, magnesium and iron. The work of Gerike (1977) suggested that marginally higher percentage removals were obtained when various trace elements and vitamins (or yeast extract) were added and these were included at an early stage. The AFNOR test already included these minor constituents. In the latest version (OECD, 1992) the minor constituents have been omitted, but may be included if the inoculum is thought to be deficient in them.

The amount of phosphate present far outweighs the nutritional requirement, but is added to serve as a buffer. Pagga (1982) showed that in tests with eight chemicals there were no significant differences in results when the phosphate buffer was raised from 0.375 mM (Mod. OECD test) to 3.75 mM (ISO method). In nature the buffer system is based on carbonate-bicarbonate; no papers have been seen in which buffer systems other than phosphate were used, but there seems to be no reason to suspect that excess phosphate interferes. Although Cassidy, *et al.* (1988) used a salts solution containing 100 mg $NaHCO_3$/l but no phosphate, the solution was sterilized and not inoculated, so that the chemicals tested did not degrade although they did degrade in river water. Similarly, no papers have been seen regarding nutrients in the standard OECD tests with the exception of a study by Richterich and Steber (1989) on the substitution of ammonium by nitrate as a source of nitrogen in the Closed Bottle test. This was investigated to try to avoid the interference caused by the oxygen uptake by nitrification, especially when N-containing chemicals are tested. The substitution was unsuccessful and the authors concluded that, since the use of nitrification inhibitors was also unsuccessful in both the Closed Bottle and Respirometric tests, the only solution was to determine the various forms of inorganic nitrogen at the beginning and end of the test.

Other results of adding nutrients in biodegradation experiments are given below.

A.2.2 Some results

In the study of the water-soluble fraction of gas oil, Kappeler and Wuhrmann (1978) showed that the degradation of about 2 mg/l of hydrocarbons in ground water, containing 0.17 mg N/l, ceased after about 10d, but when NH_4Cl was added further breakdown occurred.

Ward and Brock (1976) examined 25 lakes and found that the counts of oil-degrading bacteria were such that added hydrocarbons should be degraded. In fact, they found a good correlation between $^{14}CO_2$ evolved from 1-^{14}C-hexadecane and the concentration of P in the lake water sample. In many of the samples, addition of 100 μg P/l and 300 μg N/l, together, gave much higher $^{14}CO_2$ evolution and oxygen uptakes, indicating that although competent bacteria are present in a given environment, degradation can be limited by lack of inorganic nutrients.

Rubin and Alexander (1983) appear to have been the first to study the effects of naturally occurring nutrients on the rates of mineralization of organic chemicals at very low concentrations in natural waters. They reported a complex situation, but addition of inorganic nutrients often enhanced the rate of mineralization of phenol and 4-NP at concentrations from 20 μg/l to 200 μg/l. The simultaneous addition of arginine increased the rates still further. By harvesting cells from lake water and adding them back to lake water/distilled water mixtures of different ratios, they showed that a factor (organic or inorganic) was present in the lake water which stimulated the degradation of five concentrations of phenol.

Wang, *et al.* (1984) reported that the addition of a mixture of major inorganic nutrients (K, Na, NH_4, P, Cl, SO_4, Mg, Ca) at 1 mg/l – but not minor elements (Cu, B, Mn, Zn, Mo) – increased the rate of mineralization of 200 ng/l and 400 ng/l isopropyl-N-phenylcarbamate (IPC) in lake water. After 50d the production of $^{14}CO_2$ from 2,4-D rose from 72 to 84% and that from IPC after 60d rose from 69 to 85% $ThCO_2$. Similarly, the addition to lake water of inorganic salts increased the rates of phenol and 4-nitrophenol degradation at low concentrations (20 ng/l to 200 μg/l).

Paris and Rogers (1986) investigated the effect of adding to pond or river water major inorganic nutrients 12h before adding an organic substrate and following its removal. The amended water contained from ten to 100 times as many bacteria as the control at the end of the 12h period. For three pesticides, the lag before removal began was reduced by 30 to 40% and the pseudo-first order rates (k_1) for primary degradation were higher with the salts-amended water. From the number of cells present at the end of the biodegradation period, the second order constant (kb) was calculated and was found to be the same for the amended and non-amended cultures. Paris, *et al.* (1981) had previously reported increases in the rate of transformation of chloropropham to 3-chloroaniline in natural waters, and Lewis, *et al.* (1984) similarly found enhanced biodegradation of 2,4-D-butoxyethyl ester by added nutrients. The latter authors showed that the enhancement was due to an increase in total biomass, while the transformation rate per cell remained constant. This is confirmed by the results of Paris and Rogers (1986).

Inoculation of *Corynebacterium sp.* in samples of lake waters containing added 4-NP at 10 or 26 μg/l gave variable effects which depended on the sample, and apparently related to the rainfall and run-off (Zaidi, *et al.*, 1988). The degradation was enhanced in samples taken after a drought period by the addition of high concentrations of P or N. The addition of 1 g K_2HPO_4/l was more stimulating than 0.1 g NH_4NO_3/l, while concentrations of 10 mg/l of either salt had no effect. At 100 mg/l the phosphate was more effective than the nitrate in degrading 10 μg 4-NP/l and jointly there was a greater effect, namely 82% (P+N), 65% (P), 50% (N) and <20% (unamended) of initial ^{14}C removed from solution after about 3d. The anomalous finding that such high values of P and N are required to degrade extremely low concentrations of 4-NP was tentatively explained kinetically, i.e. the saturation constant (ks) for P may have been high, although the authors agree that a high ks for phosphate and low ks for the C source is surprising. Although stimulating contaminants in phosphate and nitrate were ruled out, the authors postulated that cation imbalance may have been involved.

In another case of inoculating a pure culture, *Pseudomonas cepacia*, at 790 cells/ml into non-sterile lake water, Ramadan, *et al.* (1990) found that 1 mg 4-NP/l was not degraded very well (about 5%) without the addition of 100 mg K_2PO_4/l or 100 mg NH_4NO_3/l (about 30% $DO^{14}C$ removal in 60h). The addition of CaC_{12} (10 mg/l) was not effective. When the organism was inoculated into sterile lake water without addition of inorganic nutrients, mineralization was rapid. The effect was thought to be due either to the uptake of the

inorganic nutrients (already present in lake water) by the indigenous population attacking the natural organic material present in the water, or to the action of grazing organisms.

In a BOD_{20} test, oxygen uptake in the presence of 4-t-butyl-benzene and hexadecane in ground water was increased by the addition of potassium and sodium phosphates plus NH_4Cl, such that the half-life was decreased from infinity to 24 and 77d, respectively (Vaishnav and Babeu, 1987). Addition of acclimated cells as well as the salts further lowered the half-life to 11 and 22d respectively.

Diesel oil or mineral oil added at about 3 g/l to sea water containing about 103 total bacteria/ml, 0.25 mg N/l and 0.02 mg P/l, did not degrade well in Sapromat respirometers unless extra N and P were added (Bilstad, *et al.*, 1987). For example, KNO_3 and Na_2HPO_4 added in stoichiometric concentrations (based on C : N : P mass ratio of 100 : 5 : 1) increased the BOD_{50}/ThOD of diesel oil from 14 to 44% and for mineral oil from 2% to 62%. A novelty was to add oil-soluble sources of N and P (phenylacetyl-urea and octyl phosphate). These sources were not quite as effective as the inorganic sources, the % ThOD being 42% and 49% respectively for the examples given above, after allowing for the oxygen uptake elicited by the N and P sources.

Valo, *et al.* (1985) reported that in a die-away test with a mixed culture adapted to pentachlorophenol (PCP) for one year the rate of degradation of 50 mg PCP/l was increased by the addition of NH_4Cl. With no addition of N (0.8 mg N/l present), 50% $ThCO_2$ took 500h, but with 5.6 mg N/l the degradation time was halved to 250h and with 56 mg N/l it was only 150h. No increase in CO_2 production was observed.

The effect of inorganic nutrients on the degradation of added organic compounds in sub-surface microbial communities was variable and depended on the site of the aquifer and on the substrate (Swindoll, *et al.*, 1988). This was attributed to the existence of a variety of micro-habitats, patchy distribution of microbes in the aquifer, differences in microbial community structure over short distances and different nutrient requirements, and availability in different micro-habitats within superficially uniform aquifer material. At one site in a pristine aquifer the addition of N and P alone or together had very little effect on the degradation of phenol (108 ng/g) or 4-nitrophenol (113 ng/g); the yield of CO_2 was 50% $ThCO_2$ for 4-NP after a lag of about 10d. At a second site in the same aquifer the lag was about 60d without nutrient addition and was reduced to about 15d by the addition of mineral salts (K, Na, Fe, Ca, Mg, chloride, phosphate, sulphate). Addition of a mixture of mineral salts, vitamins and amino acids reduced the lag to <7d. At a third site the lag was reduced from more than 120d to 40d by 0.1 μg NH_4Cl/g and to 20d by 1.0 μg NH_4Cl/g with a yield of about 50% $ThCO_2$. Similar effects were reported for p-cresol and pond micro-organisms (Lewis, *et al.*, 1986).

Unexpectedly, amending samples with N or other mineral salts with toluene or ethylene dibromide as the substrate resulted in a decrease in biodegradation compared with the non-amended samples (Swindoll, *et al.*, 1988). The authors suggested that since the numbers of bacteria were much higher after multiple addition, the additives stimulate the non-degraders rather than those which utilize toluene or ethylene dibromide. They found that addition of mixtures of nutrients usually had greater effects than any single addition.

A few cases have been reported in which N has been supplied in the form of an organic compound. Cook and Hutter (1981) omitted inorganic N from their medium and showed that bacterial isolates could use s-triazines as the sole and limiting source of the element. Bruhn, *et al.* (1987) reported that soil isolates (*Pseudomonas spp.*) could use

nitro-aromatic compounds as the sole source of N; stoichiometric amounts of nitrite were formed from 2,6-dinitrophenol, which was then totally degraded. Arginine often stimulated the mineralization of very low concentrations of 4-NP in lake water (Rubin and Alexander, 1983), but it is not clear whether the amino acid acted as source of N; the effect was also observed in stream water.

In tests simulating the activated sludge process the removal of anionic and non-ionic surfactants and the growth of sludge was found to be low and erratic using the original OECD synthetic sewage (Painter and King, 1978). The effect was traced to a deficiency of phosphate; as a result the recipe for synthetic sewage was modified to contain 28 mg K_2HPO_4/l.

In short-term (12h) die-away tests using activated sludge at 1 g of mixed liquor volatile suspended solids (MLVSS)/l, phenol (at 720 mg C/l) removal was unaffected by the addition of 0 to 162 mg N/l or 0 to 33 mg P/l, but the cell yield was higher when N and P were not added and the cells decayed more rapidly than normal cells (Kim and Armstrong, 1981). The addition of a mixture of salts of Mg, Ca, Fe, Zn and Mn slightly increased the first order decay constant of phenol from 0.053 to $0.055h^{-1}$.

The addition of organic matter can have different effects on the biodegradation of a chemical (see section B). One effect is a stimulation or catalytic increase due to compounds such as those of the vitamin B group, usually added as yeast extract. The addition of 100 mg yeast extract/l to lake water increased the % $ThCO_2$ from 40 mg ^{14}C-caprolactam/l from about 10% to more than 50% in 20d (Fortmann and Rosenberg, 1984). This concentration of yeast extract, however, is much higher than the 0.15 mg/l normally added. The authors suggested that in this case yeast extract enhanced biodegradation by increasing the number of micro-organisms capable of degrading caprolactam; alternatively, yeast extract could have increased the metabolic activity of the caprolactam – degrading species without significantly increasing their numbers – but the mechanism operating was not explained. Rosenberg (1984) found that nutrient broth added to lake water increased the primary degradation of ^{14}C-2,3,6-trichlorophenyl-acetic acid from 5 to 25% in three months. When sewage was used the increase was from 25 to 40%. The evolution of $^{14}CO_2$ showed similar increases.

A.2.3 Conclusions

The inorganic nutrients in the test media are probably optimal for the biodegradation of most organic chemicals and no improvements are envisaged. However, the evidence shows that not only are some compartments of the environments deficient in inorganic nutrients, even when environmentally realistic concentrations of a test chemical are present, but also that a number of complex interactions occur. Also, nutrient and other conditions vary within relatively short distances in some environments. All these factors must be borne in mind when designing and conducting tests mimicking the environment, as well as when interpreting the results.

A.3 Physico-chemical conditions

A.3.1 Dissolved oxygen

In the Closed Bottle test, the medium is saturated with air to give 8-9 mg DO/l. This limits the concentration of test chemical to 2-5 mg/l, but if oxygen were used instead of air to saturate the medium the concentration of DO could reach about 40 mg/l, so that a higher concentration of test chemical could be used, though there is the danger of loss of oxygen when the bottles are opened. No references to this possibility were seen.

In all other tests oxygen is supplied as air by bubbling or shaking to keep the concentration of DO higher than 2 mg/l, which has been accepted as being non-limiting for oxidation of organic chemicals, as well as for nitrification. The need for shaking in the DOC die-away was investigated by comparing the course of degradation in identical flasks, some of which were shaken and others not. With an inoculum of 10 ml effluent/l, 20 mg acetate-C/l was removed after 3d to the extent of 78% DOC in static flasks and 91% in shaken flasks. After 7d 100% was removed in both sets of flasks (Painter, *et al.*, 1981). With 40 mg acetate C/l the 3d values were 50, 73% and 7d value 94, 100%. When similar comparisons were made using a higher cell density (30 mg SS/l), static vessels gave the same removal of acetate, benzoate and aniline as in shaken flasks. The DOC removals were: acetate 95% shaken, 97% static (1d), benzoate 97%, 96% (2d) and aniline 10, 10 (2d), 41, 34 (3d), 88, 94 (5d), respectively. With pentaerythritol, degradation began on day 6 in shaken flasks and on day 7 in static flasks; by 15d, 64 and 42% were removed and by 18d, 83 and 81%, respectively (King, *et al.* 1984). All these tests were made in 2 litre flasks containing initially 1 litre medium of depth 6 cm, and static flasks were shaken just before each sampling. The re-aeration rate would be lower with greater depth of medium. Thus, while it is not absolutely necessary to shake the flasks, even with inocula of 30 mg SS/l, it is prudent to do so to ensure that the concentration of DO is not limiting, especially at the initiation of degradation.

Using the Gledhill (1975 b) closed vessel CO_2 evolution test, Fogel, *et al.* (1985) sparged the medium in the special biometer flasks with 70% oxygen in nitrogen, instead of air, before sealing them and after each CO_2 determination. The dimensions of the flasks were not recorded, but since only 10 mg C/l of test chemical was present, it seems unlikely that adding 70% O_2 was necessary. In their special flasks and in standard Gledhill flasks, the oxygen uptake curves were identical for the first 7d. After 25d the Gledhill vessels showed 82% $THCO_2$ for hexadecane compared with only 67% in the special flasks; similar differences were obtained with sodium benzoate. The authors suggested that the difference was due to the greater agitation, assessed visually, in the larger Gledhill flasks than in the smaller biometer flasks.

Since the concentration of oxygen in air is about 29 times that in water at 20-25° (Blok 1979), the DOC die-away test can be conducted in sealed flasks provided that the headspace to liquid volume is sufficiently high. For example, at 20°C and 760 mm Hg and a volume of air to liquid of 1 : 2, the total oxygen per litre of flask volume is 99 mg, i.e. 93 mg in the gas phase and 6 mg in solution (Blok 1979). Agitation would ensure adequate gas exchange so that the rate of oxidation would not be limited. Indeed, Blok successfully used this method for soluble, insoluble and volatile test chemicals.

Shaler and Klecka (1986) noted that, whereas the effect of DO on microbial growth and respiration has been widely examined, the effect on the biodegradation of individual chemicals has not. They cited Larson, *et al.* (1981) who found that t 1/2 for nitrilotriacetic acid (NTA) in natural water samples increased from 1.3 to 5.8d as the concentration of DO fell from saturation to about 1.4 mg DO/l. They determined the effect of five concentrations of DO from 0.25 to 8.2 mg/l on the rate of removal of 2,4-D by a mixed culture and found little difference between 2.0 and 8.2 mg/l, but there was a marked falling away at 1 mg/l. The KDO was estimated to be 1.2 mg/l, which is about the same as Larson, *et al.* found for NTA. However, it is much higher than KDO quoted for respiration on substrates such as glucose and acetate, in which oxygen is required principally as the terminal electron acceptor for cytochrome oxidase. Thus, a concentration of DO of 1 mg/l or less may be rate-limiting for the microbial degradation of many aromatic chemicals which have a requirement for molecular oxygen as co-substrate; thus it should be ensured that the concentration of DO is >2 mg/l.

A.3.2 pH value/buffer

It is generally accepted that the pH value of media for growth of the majority of aerobic bacteria should be in the region of neutrality, pH 6-8. The original media were intended to be buffered at 7.2 but an error, pointed out during the ring test (OECD 1989), in the specification of the hydrated form of Na_2HPO_4 to be used resulted in a pH nearer to 7.4. The original number of molecules of water of crystallization was seven, but had at some stage been written as 2. It was decided to retain the composition of the medium and to recognize the pH as 7.4 (OECD 1991).

Another problem was caused by the formation of nitrate during the tests if nitrifying bacteria were present in the inoculum directly leading to an increased oxygen uptake. A consequence could be inhibition of growth by a lowering of pH value giving falsely low biodegradation values. In the original modified OECD test the phosphate buffer was present at 0.37 mM and the concentration of NH_4-N was 5 mg/l. It was found (Painter, *et al.* 1981) that the pH fell from 7.2 to 6-6.2 when 3-4 mg N/l was oxidized (no test chemical was added). On the other hand, when 40 mg acetate-C/l was tested the pH rose from 7 to 7.6. Although the changes in pH were relatively small, these and other reports led to the reduction of NH_4-N to 1.3 mg/l in the medium and the increase in the buffer concentration to 3.7 mM in the revised guidelines.

Pagga (1982) had earlier tested eight chemicals at the original and higher buffer strengths and found no reason not to accept the higher buffer concentration.

A.3.3 Temperature

The choice of the test temperature is a compromise between that in the environment and what is practical in the laboratory. For comparative purposes, as well as for convenience and possibly a carry-over from the 5d-BOD test, the prescribed range was 20-25°C. This range has been slightly changed in the revised guidelines (OECD, 1992) to 22 +/- 2°C and for the MITI method, 25 +/- 1°C. For technical reasons the temperature must be kept within narrower limits (e.g. +/- 0.5°C) for oxygen uptake methods.

There is no reason why other temperatures should not be used for special purposes so long as this is reported. The range of temperature at which the simulated activated sludge test is carried out is 18-25°C; the EC version (Off. J. E. Comm., 1988), which is more comprehensive than the OECD version, permits any other temperature provided that the test has already been carried out in the stipulated range. This seems unnecessarily restrictive.

A.3.4 Light

Because photodegradation can occur and algae may unduly interfere in laboratory tests in the presence of light (especially in respirometric methods), it is necessary to carry out biodegradability tests in the dark, or at least in subdued light.

A.4.   Inocula

The source, amount and pre-treatment of the inoculum is probably the most important factor in influencing the result of a biodegradability test. The OECD and EC Test Methods for ready biodegradability make stipulations on all three facets: the sources must be from the environment, the cell density in the test medium is limited to about 10^6/ml, and pre-treatment must not include exposure to the test chemical. The revised Guidelines (OECD, 1992) permit a pre-conditioning which allows the micro-organisms to acclimatise to the conditions of the chosen test (see A.4.4(a)).

The DOC, oxygen uptake and CO_2 production of the blank controls due to the inoculum alone should be as low as possible compared with those with the test chemical, to yield results of greater precision. The custom is to subtract the blank value from the corresponding value in the presence of the test chemical, although it is by no means certain that events occurring in the blank flasks due to the inoculum also take place in the presence of the chemical and, if so, to the same extent.

A.4.1 Source

Material for inocula is taken from sewage, effluents, activated sludge, soil, mud, river water, lake water. It is normally stipulated that when the source is a waste water treatment plant the sewage treated should be domestic in origin, or predominantly so, and free from major specific pollutants. The MITI inoculum, however, is rather different; at least ten sources are sampled – sewage treatment plant, industrial sewage plant (chemical industry), rivers, lakes and inland seas. Sampling is made in areas where a variety of chemical substances may be considered to be consumed and discarded. The mixture of the ten samples is allowed to settle, and the supernatant is used to grow activated sludge on a glucose-peptone-phosphate medium in a fill-and-draw unit.

The ability of specific cultures of common species of bacteria to grow on, or catabolise, a variety of test chemicals has been extensively examined (for example, Payne, *et al.*, 1970). Organisms such as *Aerobacter spp., Pseudomonas spp.* and *Flavobacterium spp.* were used, but it was soon recognized that mixed cultures or consortia of "natural" origin (sewage, rivers, etc.) were superior in their ability to degrade a wider spectrum of compounds, so that the use of pure cultures for the purpose of biodegradability testing has now been largely abandoned.

The potential of various sources has been examined by a small number of workers, and a selection of results is given here. In many cases the main object of the study was not a comparison of inoculum source. Indeed, often the cell densities have not been estimated and inocula from different sources could well have been used at disparate densities.

Activated sludge, grown on "sewage" prepared from human faeces, urine, dishwashing water, starch, etc. containing no synthetic surfactants in any of the constituents, degraded LAS at lower rates than sludge grown on domestic sewage (Painter and Durrant, 1976). Even when the LAS was added to the former sewage, the derived sludge took 5d to remove 50% LAS while the normal sludge took only 3d. Calamari, *et al.* (1980) found activated sludge to have a greater degradative potential than river mud; sludge degraded three of six amines, but mud degraded none. It is well established that unanimous results for all chemicals tested are not achieved in "ring tests" even though steps are taken to minimize differences which could occur. For example, in the second calibration exercise of the Respirometric method (CEC, 1985) only aniline and 2-phenyl-phenol were found to pass (>60% ThOD) by all 25 participants. For five other chemicals about two-thirds of the participants achieved more than the pass level, while for eight other chemicals the proportion recording the pass level varied from one-tenth to one-half. All participants had used an inoculum of activated sludge taken from their locality.

Much attention has been given to 4 nitrophenol (4-NP), which had been tested in the earliest ring tests. Nyholm, *et al.* (1984) found that inocula from four sources – a polluted river, sewage effluent, soil and a pure culture of *Pseudomonas fluorescens* – degraded 4-NP within 7d in the standard test with lags of 3 to 5d. But using inocula from a non-polluted stream, sea water or an effluent from an oxidation basin treating an industrial waste, lags of up to 80d were recorded although degradation was then completed in a few days. Pre-adaptation of the latter three inocula for 15d as in the original Sturm test (1973) produced inocula which degraded the chemical within 5d. Kool (1984) also reported differences in results between sources of inocula; sewage effluent completely degraded 4-NP, but sediment from the River Rhine removed no DOC in 28d, though garden soil resulted in 60% DOC removal in 28d.

In a comparison of the MITI inoculum and activated sludge derived from domestic sewage (Painter, 1985) only two out of 19 chemicals gave conflicting results when expressed as % ThOD. sec-Butylamine was degraded by activated sludge, but not by the MITI inoculum, while diphenic acid "passed" with the MITI inoculum but not with activated sludge. In a further comparison, this time using the ISO DOC die-away test (ISO 7827; 1985), only four out of 54 chemicals gave conflicting results; three passed using activated sludge but not with MITI, while one chemical failed with activated sludge but passed in the MITI test.

Vaishnav and Babeu (1987) applied the BOD_{20} test to ten chemicals in ground water, river water and harbour water, with no additional inoculation. The lowest % ThOD was given by the ground water, which had the lowest cell density (about 55/ml), while harbour water, with about 310 cells/ml, usually produced higher degrees of removal than river water, containing about 420 cells/ml. Some of the differences were probably due to the low concentrations of N and P in the ground water.

The species composition of the sources of inocula and their biodegradation potential have rarely been reported. Pike and Curds (1971) summarized the species reported in activated sludge, as well as estimates of the numbers of individual bacteria present in sewage, effluent and sludge, but did not link these facts with biodegradation potential. Liu, *et al.* (1981)

used mixtures of lake sediments, silt loam and activated sludge to inoculate their cyclone fermenter for biodegradability assessment. They reported that the numbers and types of bacteria from all three sources were fairly constant and the mixture would thus ensure a reliable source of inocula. Predominant genera were *Acinetobacter, Aeromonas, Alcaligenes, Bacillus, Flavobacterium* and *Pseudomonas,* which were also listed by Pike and Curds (1971).

A more revealing study was reported by Gard-Terech and Palla (1986). They found differences in the populations of eight samples of sewage from two treatment works and of the inocula derived from them by growing on a synthetic medium for 3d. The enrichment modified the genera composition from that present in the original sewage sample. Five of the inocula had similar compositions in which *Acinetobacter, Pseudomonas* and *Flavobacterium* predominated. The other three inocula were deficient in one or other of the three predominating genera. It was found that samples with the three main genera removed a higher proportion of LAS (primary degradation) than those with a deficiency. More importantly, perhaps, inocula with a higher ATP content (about 2×10^{-3} mg ATP/mg protein) removed 90% LAS in 7d, while the "deficient" inocula with an ATP content of about one-tenth of the "non-deficient" removed only about 50% LAS in the same time.

Since the media in biodegradability tests are not sterilized, there is another source of micro-organisms other than the inoculum, namely the medium, in particular the water used in preparing the medium. The ASTM specification for bacteria in water (distilled or deionised) to be used in preparing media calls for not more than 10^3/ml, but the media are, of course, sterilized before use. Only in the original AFNOR DOC die-away test was the medium sterilized before inoculation. In the Closed Bottle test the added one drop of effluent/l results in about ten to 25 cells/ml and in the Modified OECD test the added 0.5 ml effluent/l yields about 50 to 250/ml. Thus, the added micro-organisms are in a minority of one in 100 to one in 40, and one in 20 to one in four, respectively. The effect of the bacteria already present in the deionised water (estimated at 10^3/ml) was determined (Painter, 1985) by adding mercuric chloride at 10 mg/l to some of the replicate uninoculated control vessels in tests on aniline, sodium benzoate, sodium acetate and pentaerythritol. Inoculated flasks contained 30 mg activated sludge solids/l. In the presence of mercuric chloride no DOC was removed from any of the chemicals; neither was pentaerythritol degraded in the uninoculated non-sterilized flasks. However, over 95% DOC was removed within 7d in all inoculated and non-inoculated flasks containing the other three chemicals.

Three chemicals (aniline, benzoate, acetate) are used as reference chemicals with the intention of checking the activity of the added inoculum. If the reference chemical is not degraded by as much as 70% DOC in a given time, the test would be deemed invalid and would have to be repeated. Such an occurrence seems to be very rare; no reports of invalidity have been seen. If, in future, reference compounds are to be used for this purpose, either the medium and flasks should be sterilized before inoculation, or a less easily degradable compound should be used as a reference chemical, a proposal also put forward by others (e.g. Boethling, pers. comm.).

It seems that inocula taken from either activated sludge or sewage effluents have a very wide range of metabolic abilities, but if a chemical "fails" a test it would pay to repeat the test with a mixed inoculum taken from a number of varied sources, as in the case of the MITI inoculum, but without growth on glucose-peptone-phosphate.

A.4.2 Cell density

In the original five methods for ready biodegradability (OECD, 1981) the cell densities (concentration of micro-organisms) were specified in terms of volume of sewage effluent, etc. or concentration of suspended solids and varied widely. The modified AFNOR method specified $5+/-3 \times 10^5$ cells/ml and the modified Sturm test suggested $10^4-2 \times 10^5$ CFU/ml. The cell density was estimated to range from less than 10^2/ml to about 10^6/ml (King, 1981), being derived from one drop (equivalent to 0.05 ml) of filtered effluent/l to 30 mg activated sludge solids/l. A further test, Zahn-Wellens, in which 1000 mg solids/l (equivalent to 10^7 to 10^8/ml) was used, had been rejected as a test for ready biodegradability as a result of OECD and EC ring tests (OECD 1979, 1980). It was found that conditions of this test were not sufficiently stringent to allow all chemicals which degraded in the test to be assumed to be readily degraded in the environment. Sewage effluents should not be sampled during periods of high stormwater flow, neither should the effluent be filtered through fine filters, such as membranes, otherwise the bacterial cell density may be greatly reduced. Also, some effluents taken from what is probably a very efficient sewage treatment plant in Germany yielded cell densities of only about 1/100th of the required density (Voelskow, priv. comm.).

Results were very variable amongst the five accepted tests. Blok and Booy (1984) pointed out that there were 67% passes in the ring test in the Sturm test but only 10% in the Closed Bottle method; similarly their analysis of the results with 44 chemicals of Gerike and Fischer (1979, 1981) gave 52% and 14%, respectively. These differences are not entirely due to differences in cell density, since Gerike and Fischer (1979, 1981) used pre-exposed inocula in the Sturm test.

These differences were probably expected when the test methods were first published, but the Closed Bottle method could not tolerate much higher cell densities because of the low solubility of oxygen in water. The RDA (Blok, 1979) or 2-phase Bottle Test (BODIS, ISO 1990 a) holds out the possibility of using higher cell densities. The modified OECD test, on the other hand, was directly derived from the tests for the primary degradability of anionic and non-ionic surfactants and was a relative method. Very degradable (soft) and poorly degradable (hard) standards were assessed simultaneously with the test chemical. An amount of sewage effluent or soil extract was to be added such that >90% of the soft surfactant was removed but not more than 35% of the hard surfactant in the given time, normally 7 to 10d but certainly not more than 19d. It was usually found that 0.5 ml filtered effluent/l was sufficient to meet these conditions and this cell density was carried over into the DOC die-away test method.

Other comparisons between the test methods are given in reports by Brown and Laboureur (1983), Kitano (1983) and Damborg and Lindgaard-Jorgensen (1990). Of four aromatic amines tested by six laboratories in the modified AFNOR test (10^5 cells/ml) and the modified OECD test (10^2/ml), only aniline was degraded in both tests by all laboratories (Brown and Laboureur, 1983). In the AFNOR test five laboratories reported passes with the remaining three amines; the sixth laboratory failed all three. This last laboratory repeated the test using inoculum from a laboratory filter unit instead of an activated sludge plant, whereupon all three chemicals were degraded. In the modified OECD test there were fewer passes, p-phenetidine not being degraded at all. Half of the laboratories passed o-toluidine, while two out of six passed p-anisidine. However, the laboratory which repeated the test using a filter effluent instead of one from an activated sludge unit reported the degradation of all three amines.

Kitano (1983) tested 31 problematic chemicals by four test methods (MITI test was excluded) and reported the number of passes as:

	no.	%
Closed Bottle	2	6
Mod. OECD	5	16
Sturm	10	32
AFNOR	20	65

An additional five chemicals gave variable % DOC values among the triplicates and are not included in the AFNOR results. The low number of passes with the Sturm test compared with the AFNOR results is surprising, since they have about the same cell densities. The ISO method was applied to 22 of the 31 chemicals (King and Painter, 1985) and there were only four inconsistencies. Pentaerythritol and 2-nitro-anisole degraded in the ISO test but not by AFNOR or Sturm, while the situation was reversed with tributyl phosphate and dicyanobenzene.

Damborg and Lindgaard-Jorgensen (1990) reviewed data on 258 chemicals received from nine laboratories. For only 69 chemicals were data available to compare high and low densities of inoculum. Of the 69 chemicals, 61 were degraded by high inoculum (10^5-10^6/ml and 33 by low inoculum density (10^2/ml). Six chemicals were either not degraded at all or gave inconclusive results; unexpectedly, two chemicals were degraded using low cell density but not at high density.

In all the above cases, the inocula for the high and low density tests were not necessarily taken from the same source nor taken at the same time. There are a few studies in which degradation has been followed by using different amounts of inoculum taken at the same time from the same source. Blok and Booy (1984) reported oxygen uptakes elicited by diethyleneglycol and anthraquinone using from 0.01 to 100 ml sludge/l, equivalent to 0.03 to 300 mg solids/l, to illustrate their mathematical approach. The uptake-time curves generally agreed with theory and there were longer lags with lower cell densities; the % ThOD after 28d was higher with higher cell densities. Haller (1978) also reported shorter lags with higher cell densities in tests with 3-chlorobenzoate (3CB) and 4-chlorophenol (4CP) and inocula varying from 0.5 ml to 20 ml sewage per 40 ml of medium. The lag with 3CB fell from 9 to 6d and for 4CP from 7.5 to 5d.

Higher cell densities in the MITI oxygen uptake test (Urano and Kato, 1986a) gave shorter lags and higher % ThOD values for glutamic acid, aniline and benzene sulphonic acid. Other examples are given by Eden, *et al.* (1968), Painter and Durrant (1976) and Gerike (1984).

Tests with 4-NP often give apparently conflicting results. Generally, inocula derived from effluents at 0.5 to 25 ml/l, or from activated sludge at 3 to 30 mg/l, gave longer lags before the removal of DOC at the lower cell densities but not invariably (Painter, *et al.*, 1981). Replicates of tests with the lower cell densities often gave disparate results. At about 10^2 cells/ml the lag period was 21 to 28d and at 2 x 10^4/ml it was 4 to 7d, but in all cases the removal of DOC was high (90%) and occurred rapidly after the lag period. (Diethylene glycol, unlike 4-NP, degraded slowly once the lag period was over.) Kool (1984) reported a 6d lag at 1 ml activated sludge/l (about 3 mg solids/l) and no lag at 10 ml/l. However, he found only

20% of the DOC of 4-NP removed at the end of the test (14d) with the lower cell density, while at the higher density 100% was removed in 7d. The removal of 4-NP was detected by an increase in the concentration of ATP during the first day of incubation at the higher cell density. 2,-3,- and 4-Methoxyanilines did not degrade with an effluent inoculum, but the 3,- and 4,-isomers degraded well at 1.5 ml activated sludge/l.

Contrary to Kool's findings on 4-NP, Nyholm, *et al.* (1984) concluded that although the lag period tended to decrease with increasing biomass, the rate of biodegradation was not generally proportional to the biomass initially added. They further concluded that the cell density for these tests should be at least 10^4/ml (equivalent to about 5 ml effluent/l). Surprisingly, they rejected soil inocula as having different microbial composition from those characteristic of surface waters and sewage.

The quaternary C-containing compound, pentaerythritol, has also received attention. Its structure suggests that it should prove difficult to degrade and, indeed, it was found not to degrade in four out of five tests for ready biodegradability, nor in the fifth test (Sturm CO_2) using a pre-exposed inoculum (Gerike and Fischer, 1979; 1981). The same authors showed that the chemical was inherently biodegradable and also degraded in "ready" tests using exposed inocula. In contrast, in the two ring tests on the respirometric method (CEC 1982; 1985) six laboratories out of eleven and 16 out of 25, respectively, achieved % ThOD over 60% with non-exposed inocula. Battersby (personal comm.) found that the lag period decreased from 21d with 0.3 mg activated sludge/l (about 10^3/ml) to 14d at 3 mg/l and to 9d at 30 mg/l (10^5/ml). The complete removal of DOC then took about the same time in all three cases. He also found little difference in lag or rate when using 30 mg/l in 100 ml, 500 ml or 1000 ml of culture medium. This fall in lag time and constancy of rate of removal was confirmed by Weytjens (personal comm.). He found the lag to fall from 18d at 0.3 mg activated sludge/l to 7-8d at 30 and 300 mg/l. Birch (personal comm.) inoculated the Sturm test medium with 0.0005 to 50 ml effluent/l (about one to 10^6 cells/100 ml) and found no degradation in 28d with less than 10^3 cells/100 ml (0.05 ml/l). The lag was 24d at 0.05 ml/l to 10 to 12d at 5 and 50 ml/l. In this case the time to reach the plateau of % ThCO$_2$ varied from 4 to 9d after initiation of detectable biodegradation.

To sum up, the effect of cell density is largely on the length of the lag period before initiation of degradation, but a significant difference may not be observed unless the difference between two cell densities is at least three-fold (Eden, *et al.*, 1968). Lag periods appear to be reduced to a minimum with cell densities of about 10^4/ml, i.e. 5 ml effluent/l. Nyholm, *et al.* (1984) have also suggested this density as a minimum to avoid the risk that the concentration of competent micro-organisms becomes critically low and also to avoid variability between replicates. The theoretical approach (Painter and King, 1983; Blok and Booy, 1984) predicts that the cell density should have little effect on the slope of the decay curves, and this has largely been found to be so. These approaches also predict that chemicals which "pass" tests inoculated at 10^5-10^6/ml or less, will be removed in an activated sludge unit provided that the sludge retention time is not less than 5d allowing the competent species to be maintained in the system. This has been examined by applying the activated sludge simulation test (Husmann; porous pot) to a number of chemicals and finding that chemicals classified as "readily biodegradable" in tests using up to 10^5-10^6 cells/ml were in fact removed by 80-95% DOC (e.g. Painter and Bealing, 1989).

A.4.3 Pre-conditioning

In the original version of his repetitive die-away test, Blok (1979) used freshly prepared mixed inocula, but in later versions he limited the inoculum to activated sludge and introduced a pre-conditioning step.

This step was used in a ring test of the method (Blok 1987) and was carried out by aerating the sludge in mineral medium for 7d prior to use. Such prior treatment was said to allow the bacteria to overcome the "shock" of transfer and to reduce the rate of oxygen uptake by the blank controls, without affecting the spectrum or intensity of metabolic activity, and was thought to reduce the variability of results between different inocula. No data confirming these effects seem to have been published. Blok, *et al.* (1985) quote de Waart and Van der Most in an internal report of CIVO/TNO Zeist, which has not been seen. A published paper by de Waart and Van der Most (1986) on a biodegradation test for microbicides describes the "conditioning" of the activated sludge by aerating and stirring for 30 minutes, not one week; no comparisons of results obtained with and without this treatment are given.

Some limited comparisons have been made by others. The degradation of pentaerythritol was followed in 16 flasks inoculated with fresh sludge; pentaerythritol was added to eight flasks on the same day and to the other eight one week after shaking and incubating at the test temperature (Painter, 1986). The lag period was reduced by pre-conditioning from 14.3+/-2.8 to 11.9+/-3.3d. After 18d, DOC removal was somewhat lower with the pre-conditioned inoculum but the variability was not changed, namely 45+/-27% and 54+/-30% pre-conditioned and fresh, respectively. At day 21 the variability was improved – pre-conditioned 87+/-8% DOC removal, fresh 88+/-21%, but at day 28 there was no difference (94+/-1; 97+/-2%). There was no difference in the pattern of DOC values in the blank flasks between the two types of inocula. In the Closed Bottle test (Painter, 1986) there was evidence that pre-conditioned inoculum gave less nitrification (three out of 18 bottles) compared with fresh inoculum (9/18) but in a second experiment the extent of nitrification was the same with both inocula. In the OECD ring test (OECD, 1989) the effect of pre-conditioning on blank oxygen uptake values was equivocal, only a few participants finding a reduction.

Kuenemann, *et al.* (1990a) found no difference in the percentage DOC removed from five chemicals when inoculated with either fresh or pre-conditioned activated sludge. But in the modified Sturm test, although the % DOC values were unchanged by pre-conditioning, the % $ThCO_2$ values were significantly lower for four out of five chemicals (aniline, pentaerythritol, mono-ethanolamine, 2,4,6-trichlorophenol) when pre-conditioned inocula were used. This was interpreted as an increase in the amount of C assimilated, since they were careful to eliminate errors of analysis. They suggested that since C reserves within the pre-conditioned cells had been depleted during the pre-aeration, more carbon would be needed to be assimilated to make good the deficiency.

An explanation for the differences in the results reported in the OECD ring test (1989) could be that a minority of participants had used sludges grown in high-rate treatment plants, while others had used sludges from conventional plants. High-rate plants produce sludges which have higher "endogenous" respiration rates due to the presence of un-degraded insoluble substrates – higher fatty acids, cellulose – which are oxidized during the pre-aeration period to result in sludge with a lower "endogenous" rate of oxygen uptake. The effect on conventional sludge would be much less.

The option of pre-conditioning has been introduced into the revised OECD Guidelines (1992) and in the revised ISO Standard 7827 (1991), so that the facility is available should sludges with higher than normal respiration rates be used.

A.4.4 Pre-exposed inocula

(a)   Introduction

If a chemical is found not to biodegrade in one or other of the screening tests (OECD, 1992; Off. J. E. Comm., 1992), and if it has been shown not to inhibit its own biodegradation, the next step is to apply a test for inherent biodegradability. (ISO methods do not use the terms "ready" and "inherent"). However, time and effort could be saved if another screening test were applied using inocula which had previously been exposed to the test substance. ISO (1988) has defined relevant terms as follows:

> Pre-exposure (or pre-adaptation): the pre-incubation of an inoculum in the presence of the test substance with the aim of enhancing the ability of the inoculum to degrade the test substance.

> Pre-conditioning (or pre-acclimatisation): the pre-incubation of an inoculum under the conditions of the test (to be applied) in the absence of the test substance to improve the performance of the test.

Such a method using exposed inocula is increasingly required, because (a) many chemicals have been shown to be degraded only when exposed inocula are used, (b) only a very small proportion of "new" chemicals are degraded in the accepted screening tests, and (c) interest is increasing in testing those "existing" chemicals on which there are insufficient data.

A number of authors have proposed such a test, e.g. Nyholm, *et al.* (1984), Struijs and Stoltenkamp (1986a). Chemicals "failing" the usual screen tests, but passing the proposed test, would be classified as "readily biodegradable with pre-exposed inocula". Ideally, the specifications of an acceptable method of exposure, such that chemicals which subsequently "pass" may be so classified, are that:

(i)   it should be relatively simple and straightforward;
(ii)   it should be of relatively short duration;
(iii)   it is preferable that the micro-organisms in the inocula for the test should be in the same growth phase as in normal inocula and that the competent species should make up only a minority of the population;
(iv)   the produced inoculum does not consist largely of a mutant(s) which is temporally and spatially rare;
(v)   chemicals which are degraded by a pre-exposed inoculum would also be expected to degrade in tests which simulate waste water treatment, rivers, etc.; and
(vi)   for chemicals which are intermittently discharged, the effects of withdrawal of the chemical and re-exposure should be considered.

In the literature, the terms "exposure", "acclimation", "acclimatisation", "adaptation" and "conditioning", with or with the prefix "pre-", are not always clearly defined and are not always defined in the same way. Although it is often clear from the context what is meant, insufficient information is sometimes given and often details of the conditions of exposure are not clearly stated. The terms as now defined by ISO all relate to treatments given to the inoculum, with stated objectives, but do not imply success or failure in attaining the objective. There is no term to denote a successful outcome. Also, the term "adaptation", as defined by ISO, does not have the same meaning as when used in enzyme adaptation, which implies a successful change in enzyme properties. "Exposure" is a physical process, bringing together the microbial population and the test chemical; "adaptation" is a biochemical or biological process(es) which brings about a change in the properties of a species or population (see Appendix II). An exposed population which did not subsequently degrade the test chemical should not be called an adapted population. Thus, it would have been better to define "pre-exposure" as ISO has done but not to equate it with "pre-adaptation". "Adaptation" could then have been reserved for successful exposures.

Many papers have been seen in which pre-exposed inocula have been used but not all compare the results with non-exposed inocula, and very few with subsequent behaviour in the environment or in tests simulating the environment. In early work, emphasis was on activated sludge but more recently stress has shifted to other parts of the environment – river waters and sediments, estuarine and marine sediments, as well as to anaerobic waste treatment.

Early work, in the 50's and 60's, centred on determining which compounds could be degraded by activated sludges pre-exposed, separately, to simple chemicals such as benzene, phenol and aniline, but these studies have little bearing on the present subject. For example, Malaney and McKinney (1966) found that sludge acclimated to grow on benzene as the sole source of carbon degraded all mono-alkyl substituted benzenes and xylenes tested, but not mono-chloro-,nitro- or sulphonic derivatives; many aliphatics were also degraded. Similarly, Chambers, *et al.* (1963, 1964) adapted mixed cultures to ten phenolic compounds and found that phenol-adapted cultures could degrade more chemicals (78) than cultures adapted to the other nine phenolic compounds. A more recent example is the induction of more than one metabolic pathway of o-cresol in a sludge exposed to phenol (Masunaga, *et al.*, 1986). This sludge became capable of degrading o-cresol not only via the well known intermediate, 3-methyl-catechol, but also via two other intermediates (4-methyl resorcinol and methyl hydroquinone) not reported before.

In reviewing the earlier work, Hartmann and Singrun (1968) found that no method had so far been developed to describe, in a quantitative reproducible way, adaptation phenomena in mixed microbial populations, such as those in activated sludge. They studied the increase in the rate of phenol degradation in Warburg respirometers by activated sludge from laboratory units receiving a synthetic sewage and phenol at concentrations from 20 to 200 mg/l. They calculated a theoretical degradation curve representing complete adaptation by use of Lineweaver-Burke plots and the Henri equation. From this they showed that the time needed to reach full adaptation depended on the concentration of phenol; at 200 mg phenol/l the time needed was 70h. No other similar studies were seen.

The remainder of this section refers to papers relating to the six points given above, but much more information is necessary. Especially required, as pointed out by ECETOC (1984), are studies which throw light on the important feature of general predictability; for example, tests with pre-exposed inocula should not yield "false positive" results.

(b)　　　Methods – conventional tests

Some of the methods taken from the literature, together with the periods of exposure before applying tests for biodegradability, are listed in **Table A4.1**; all methods were probably carried out at around 20°C. Methods for exposing sediments and cores are given towards the end of the section.

In the original AFNOR method (1977) lasting 42d, it was tacitly assumed that adaptation would take place in the first 14d, leaving the remaining 28d for biodegradation. The method used by Sturm (1973) seems to have been used first in a study of nitriloacetic acid degradation by Thomson and Duthie (1968), who in turn modified the method of Bunch and Chambers (1967) with the omission of the weekly culture transfer to fresh medium. Sturm (1973) used 10% settled sewage in BOD dilution water containing 50 mg yeast extract/l and 20 mg test chemical/l. The mixture was incubated for 14d under quiescent conditions and was presumably anaerobic for some part of the time. In the first OECD version of the Sturm method (OECD 1978) the medium was inoculated with 10% settled sewage and was positively aerated by shaking.

Many authors have used the semi-continuous activated sludge (SCAS) method of the Soap and Detergent Association (1969) or versions of it (e.g. Off. J. E. Comm, 1988). Typically, the sludge from a full-scale treatment works was fed daily with synthetic sewage, containing a known concentration of the test chemical. The mixture was aerated for about 23h, then settled and the supernatant was discarded, but no sludge was deliberately discarded, so that slow-growing species were retained and the chances of adaptation were greater. Non-wastage may be of importance since the relationship between ease of adaptation and growth rate, if any, is not known. Larson (1979) used glucose-nutrient broth-salts as the synthetic sewage, while Pitter (1976) used a more concentrated synthetic sewage containing glucose (or starch) and peptone. Pitter (1976) appears to be one of the few workers who deliberately wasted sludge; wastage was one-fifth of the total volume of mixed liquor per day giving a sludge retention time (sludge age) of 5d. Thus, the pressure on the population to produce micro-organisms able to degrade xenobiotic chemicals is less than in Larson's version (1979). Presumably micro-organisms with doubling times greater than about 5d would not be maintained in Pitter's system. It is of interest to note that Pitter (1976) defined a chemical as being "readily decomposable" if it was subsequently removed in his biodegradability test, using the exposed inoculum, at a rate higher than 15 mg COD/g sludge.h, though he did not give reasons for his choice of that value. Both Pitter and Larson introduced the test chemical stepwise, the concentrations being increased to the equivalent of 200 mg COD/l (Pitter) and 20 mg test chemical/l (Larson). While Pitter kept a constant 20d exposure, Larson used inocula after 8 to 13d. Painter, *et al.* (1983) fed the sludge with domestic sewage, containing the test chemical at 2 or 20 mg C/l, without sludge wastage for various periods up to seven weeks.

Table A.4.1   Some methods used for exposing mixed cultures to selected chemicals

Author	Concentration of test substance	Period of exposure	Conditions of exposure
Sturm (1973)	20	14d	10% sewage in BOD dilution water – quiescent
Larson (1979)	4 → 20 in steps	8-13d	SCAS, no wastage of sludge; 300 mg glucose/l, 200 mg peptone/l
Pitter (1976)	increasing up to 200 mg COD/l	20d	SCAS, sludge wasted retention time 5d. 600 mg glucose/starch, 600 mg peptone/l
Painter, *et al.* (1983)	2, or 20	up to 63d	SCAS, no wastage of sludge; domestic sewage DOC – 100 mg/l, BOD – 200 mg/l
Boatman, *et al.* (1986)	2-18 (as DOC) in steps	18d	enrichment, 2d transfers; 1000 mg nutrient broth/l falling to 100 mg/l
Zahn and Wellens (1980)	400 (as DOC)	14d	Zahn-Wellens test, no wastage, 1000 mg solids/l, no other C substrate
Gerike and Fischer (1981)	20 400 20	14d 14d several weeks	Sturm procedure (as above); Zahn-Wellens procedure; OECD Confirmatory test; Husmann unit treating OECD synthetic sewage
Cook (1968)	increasing	several weeks	biological filter recirculation of effluent; feed test substance in tap water

Boatman, *et al.* (1986) used an enrichment procedure making transfers every two days, reducing the nutrient broth concentration from 1000 to 100 mg/l and increasing the concentration of test chemical from 2 to 18 mg C/l over a period of 18d. Their original inoculum was a mixture of activated sludge from a laboratory unit and unchlorinated effluent from an industrial waste water treatment facility. Sugatt, *et al.* (1984) also used a mixed inoculum comprising, per litre of mineral medium, 1 g fresh, rich soil, 2 ml activated sludge and 50 ml raw domestic sewage. The well stirred mixture was filtered and the filtrate was fortified with casamino acids and yeast extract. The test chemical was added at 4 mg/l, and two further additions each of 8 mg/l were made on the 7th and 11th days; aerobic incubation in the dark lasted for 14d.

Zahn and Wellens (1980) reported the exposure of sludge to nine compounds by first applying their test for (inherent) biodegradability (Zahn and Wellens, 1974) using unexposed activated sludge. The latter test was then repeated with sludge from the first test after separation and washing. Gerike and Fischer (1981) in a study of 44 chemicals employed both the Sturm and Zahn-Wellens methods, as well as the OECD Confirmatory (simulation) test, for exposing micro-organisms for testing chemicals which were not readily biodegradable.

Cook (1968) attempted to adapt micro-organisms in sewage effluents by inoculating them onto agar plates containing 10 mg/l of individual surfactants, but she was unsuccessful. However, the use of biological (percolating or trickling) filters through which liquid medium was re-circulated was successful. The medium was the surfactant solution added to sewage effluent, and a decreasing amount of effluent was used each week until after some weeks only the surfactant in tap water was re-circulated. After several weeks the organisms in the filter became fully adapted as tested by the die-away method.

Lund and Rodriquez (1984) used a modified SCAS procedure, increasing the concentration of test chemical and decreasing that of glucose every 2d, the total COD remaining at the very high value of 1000 mg/l, until after 18d no glucose was present. Gledhill (1978) also used the SCAS technique, followed by a die-away in a study of carboxymethyl tartronate, which began to degrade after four to eight weeks. Pawlowski and Howell (1973) used the homogeneous continuous-culture technique in attempts to adapt mixed populations to phenol.

In the Sturm type of method, a second addition of test chemical is often made to the incubated mixture when the test chemical has been removed (e.g. Dojlido, 1979). Blok (1979) has made use of this method in his repetitive die-away method in which three extra amounts of test chemical are added at weekly intervals. In this way it was hoped to be able to build up a non-limiting density of competent bacteria, so that the maximum specific growth rate of the species could be determined in the last part of the test.

(c)   Development of methods

It is not easy now, so long after the events, to trace precisely the development of the methods used for exposure. In particular, the choice of the duration of the exposure periods and the reasons behind them are not clear. No doubt detailed discussions took place at the various meetings of the OECD Group in 1978-1980 and in other forums, but they do not seem to have been recorded. The attempts to produce competent inocula were to a large extent based on methods used by microbiologists to obtain enriched cultures of micro-organisms which would grow on a particular chemical. Since biodegradability testing has its roots chiefly

in the development of degradable synthetic surfactants in the mid-fifties, it was thought that perusal of the literature on detergent testing would give some indication of the rationale behind the methods of exposure reported. Not much was forthcoming, however.

Reasons for using exposure to a surfactant appear to be to retain the relatively short duration of the original die-away tests, which were based on the classic BOD method, and the fact that exposed inocula usually give better reproducibility. This partly contradicts the experience of Lund and Rodriquez (1984), who found that different samples of activated sludge from the same treatment plant did not always behave similarly with respect to their capacity to adapt to a particular chemical. However, this exception is probably due to the extremely high concentration of test chemical (equivalent to 1000 mg COD/l) used to test the resulting inocula.

An SDA report (1969) states that operators usually exposed the microbial populations to the surfactant under conditions similar to those used in the die-away test. They recognized, however, that the exposure period should be limited to preclude development of a predominant, atypical culture. Another aspect of acclimatisation was recognized by Gledhill, *et al.* (1980) who concluded that the need for the use of an exposed inoculum was not a significant issue, provided that the subsequent rate of biodegradation was acceptable and that exposure in the receiving waters is maintained. However, this conclusion may have to be modified in the light of the work of Cook (1968) and Truesdale, *et al.* (1969), especially for predicting whether a chemical will be degraded in sewage treatment rather than in the aquatic environment generally. Both these latter groups showed that the re-circulating filter method can lead to a "predominant atypical culture" when treating the so-called "hard" tetrapropylene benzene sulphonates (TPBS) for many weeks. TPBS degraded by >90% in die-away tests (primary, methylene blue test) using exposed inocula taken from the filters, but by much less (35-65%, primary) in the OECD confirmatory simulation test. The reason for this is presumably that the population was produced over a long period from a medium containing TPBS as virtually the sole source of carbon and was thus "over-acclimatised". Obviously exposure of this sort for many weeks is unsuitable for use in tests for ready biodegradability.

(d)   Tests with sediments and cores

The development of methods of exposure of sediments and cores in fresh, estuarine and marine situations has been different from that of those already described. The reasons for this are the objectives of the studies, the types of chemicals, and perhaps the background of the experimenters.

The object of most of this work is to investigate the actual kinetic rates of removal (k_1, $t_{1/2}$, μg/gh) in "real world" environmental situations, or simulations, as opposed to the OECD Guideline for "ready biodegradability" which indicates whether a chemical has the potential to degrade in the environment. This has necessitated the use of radio-labelled chemicals or specific analytical methods because of the very low concentrations involved; no other method has yet been found of detecting ultimate biodegradation at such low concentrations. Linked with this, the greater importance of high-tonnage products (e.g. surfactants) and the nature of others (e.g. pesticides) have made it economically possible to use radio-labelled versions of such chemicals. It is not economically possible, however, to produce labelled versions on a routine basis of other chemicals which are produced in small amounts.

Many authors used conventional enrichment culture techniques to produce exposed inocula (e.g. Vaishnav and Babeu, 1987; Ruffo, *et al.*, 1984). Neilson, *et al.* (1988) used the method for exposing anaerobic marine sediments to poorly soluble aromatic aldehydes (200-500 mg/l), but found it necessary to modify the usual procedure. To cope with poorly soluble test chemicals and to avoid subsequent precipitation, sulphide and bicarbonate were not added to the medium until the test chemical had been dissolved. Elsewhere, Neilson, *et al.* (1985) indicated that there were essentially no problems associated with the exposure to volatile and insoluble chemicals.

Mikesell and Boyd (1986) used freshly collected anaerobic sludge in 4-litre glass bottles, flushed with 80% N_2-CO_2% mixtures. The test chemical (chloro-phenols) was added weekly as the sole source of C at 25 mg/l. The chlorophenol was monitored weekly or more frequently to avoid accumulation of the chemical or its products. When the sludge was adapted as indicated by faster removal of the test chemical, 75 ml samples were withdrawn anaerobically and transferred to 160 ml serum bottles for assessment of biodegradation of newly added chemicals.

The exposure of fresh water, estuarine and marine sediments was conducted in tubes which served both as sampling devices and containers for incubation (Spain, *et al.*, 1980). The sterile glass tube, 40 cm long, diam. 3.5 cm, was inserted vertically into the sediment, taking out the bottom stopper and replacing when the sample had entered. The volume of water above the sediment was adjusted to 175 ml in all cases, and a stopper was placed into the top end. Air was introduced at about 3 cm above the sediment via an 18 gauge needle in the top stopper, so that the sediment is not disturbed and gases exit via a glass-tube outlet, also in the top stopper. The exit gases were collected in NaOH to determine the CO_2 evolved. Cores from a single site were treated in one of three ways; Cores A were spiked with ^{14}C-labelled test chemical immediately, cores B with unlabelled test chemical at the same time and cores C received no addition. When the rate of $^{14}CO_2$ production increased substantially in cores A, cores B and C were spiked with ^{14}C-labelled test chemical, and again $^{14}CO_2$ evolution was followed in the latter cores. If the rate in B was greater than in C, adaptation had occurred.

Shimp and Pfaender (1985a,b) exposed microcosms from lake water in completely-mixed continuous flow 1.5 litre vessels, with a retention time of 3d. They were fed solutions of filter-sterilized distilled water solutions of inorganic nutrients and the test chemical. Usually triplicate vessels were used for each test chemical together with two controls; one received filtered lake water, the other, sterile feed solution of inorganic salts but no test chemicals. After five to seven residence times (two to three weeks) samples of the communities were removed from the vessels for assessment of biodegradability, MPN, etc.

Ventullo and Larson (1986) added various concentrations of the test chemical to lake water held in sterile polycarbonate carboys, suspended at a depth of 1 m in the lake. After 21d, samples were taken from the carboys for biodegradation tests.

Wiggins, *et al.* (1987) exposed the microcosms in lake water by adding 4-NP at 200 μg/l for 10d at 25°C, while sewage was incubated with only 2 μg/l at 28°C for 11d. Fresh and exposed communities of sewage and lake water micro-organisms were collected by centrifugation.

(e)   Period of exposure

Among earlier workers, Hunter and Heukelekian (1964) realised that the time needed before a chemical began to degrade, in laboratory scale activated sludge units, varied from a few hours to several months; as a compromise they used one month in their studies. Experience with a few hundred surfactants over an eight year period led Eden, *et al.* (1968) to adopt a period of six weeks in which to allow sludge in laboratory units to adapt to a surfactant before assessing its removal in a succeeding period of three weeks' settled operation. All surfactants which began to degrade within six weeks were then consistently removed for as long as the units were operated (up to two years in some cases). Six weeks is used in both the EC detergent confirmatory test (Council Directive, 1982) and the OECD and EC Simulation test methods (OECD, 1981; Off. J. E. Comm. 1988).

The sludge retention time in the standard simulation test is around 5d (Off. J. E. Comm. 1988); Stephenson, *et al.* (1984) reported the lag before degradation of nitriloacetic acid to be higher at lower sludge ages. For example, sludge ages of 4 and 12d gave lags of 15 and 6d, respectively.

Struijs and Stoltenkamp (1986a) reported that the length of time of exposure in a modified Pitter (1976) procedure influences subsequent results. With a three-week exposed sludge, 4-nitrotoluene was completely degraded within three weeks in a DOC die-away test, while after four weeks' exposure the time for removal was reduced to one week. This study included two "negative reference chemicals", 2-chloro-4-nitroaniline and 2-chloroaniline, which did not degrade under any conditions applied. This was only an indication, but by no means conclusive evidence that the method of adaptation was not excessively lenient. Gledhill (1978) found that lags of four to eight weeks were needed to adapt sludge to carboxymethyltartronate in a laboratory unit; non-exposed inocula yielded 15-40% $ThCO_2$ in a 28d batch test, while exposed inocula yielded 65-90% $ThCO_2$. Using the SCAS method, 4-chloroaniline and tetra-hydrofuran-tetracarboxylic acid were degraded after lags of two and eleven weeks, respectively (Painter, 1986). Exposed inocula degraded their respective substrates in die-away tests within one and three weeks respectively (>70% DOC) and within 16 and 28d (>60% ThOD); non-exposed inocula did not degrade the chemical. However, in simulation tests (Husmann, porous pot) although 4-chloroaniline was degraded (>90% DOC) after 16-19d exposure (Janicke, 1980) the carboxylic acid was not (Painter, 1986). This indicates that eleven weeks is too long an exposure period.

In a study of 22 chemicals, many of which had been examined in ring tests, the lag period in the EC Respirometric method (Painter and King, 1985) with non-exposed inocula was compared with those observed in the simulation test (**Table A.4.2**). The lag period under simulation conditions was usually much higher, presumably because of sludge wastage and the additional carbon input, not present in die-away tests. With one exception, the 14 chemicals showing lag periods of 14d or less in the screening test were successfully degraded in the simulation test (Painter, *et al.*, 1983). The exception, hexamethylenetetramine, was probably hydrolysed (see discussion under chemical name in Appendix III). The effect of length of exposure on the ability of sludges to degrade eleven of the chemicals was followed in SCAS units over a period of 67d (**Table A.4.3**). The degree of adaptation was assessed by using the exposed sludges, taken at various times, as inocula in the ISO DOC die-away test, using 30 mg solids/l and 20 mg C/l. Two chemicals (BDSA and 2CB) did not degrade at all, while five (PE, 3CB, 3AB, 3AP and 2NP) degraded faster with exposed seed. Two others (SA and DEG) were erratic and have been reported so by others. N-Methylaniline, not degraded by the original sludge, showed >70% DOC removal within 21d with a 10d-exposed

sludge and after 67d exposure only 3d were required to reach 70% removal. The last chemical, t-B, was degraded only after 18d exposure of the inoculum, and the rate of removal increased erratically up to the end of the exposure period. The group of five chemicals and NMA were all degraded with effluent (5 ml/l) inoculum taken from the SCAS units at day 67. Since t-BA did not degrade in the simulation test, 18d exposure may be considered too long a period.

(f)   Retention of acquired ability

In laboratory-activated sludge units receiving surfactant-free natural sewage, a two-week withdrawal of added anionic surfactant after the sludge had reached full activity did not impair the ability of the sludge to remove "soft" surfactants immediately on its re-introduction (Water Pollution Research, 1968). "Hard" surfactants needed about three weeks before they were removed to the previous extent. Pfeil and Lee (1968) reported successful adaptation to nitrilotriacetic acid in one week; withdrawal of the chemical for 2d resulted in no lag on its re-introduction, but a lag of 2d occurred when the withdrawal lasted 5d.

Gledhill (1978) observed better powers of retention of activity by sludges which had taken four to eight weeks before they could degrade carboxymethyltartronate (CMT). Withdrawal of CMT for as long as 35d caused a lag of only 5d before 100% removal was re-achieved on re-introducing CMT. Using ^{14}C-labelled CMT, it was shown that the activity of the non-exposed sludge was only 0.1 μmol$^{14}CO_2$/l.h rising to 27 μmol/l.h on full adaptation. Withdrawal for 35d lowered the activity to 2.8 μmol/l.h, and this rose to 27 μmol/l.hd after reintroduction of CMT.

In the study using SCAS units, described above, sludges which had been separately exposed for 67d to a number of chemicals were then fed for up to 202d with domestic sewage only. Diverse results were obtained. N-MA was still degraded in die-away tests at the end of this period (>70% in 7d), although the original non-exposed sludge could not degrade the chemical in 28d. t-B, on the other hand, was degraded by its sludge after 109d of withdrawal (>70% in 21d) but not after 137d. PE-exposed sludge slowly became less active but could still degrade PE after 202d of withdrawal (>70% in 21d). Lastly, sulphanilic acid and diethylene glycol continued to be degraded erratically throughout the period (King, *et al.*, 1984).

In their study of nitro-toluenes, Struijs and Stoltenkamp (1986a) found that sludge, successfully adapted to 4-nitro-toluene, became somewhat less active after the chemical was withdrawn for two weeks. The adapted sludge took a total of one week to degrade the chemical in a die-away test, while the "de-adapted" sludge showed a lag of 7d with degradation occurring in the following 3d.

Table A.4.2   Lag periods before the onset of degradation of substances in die-away and simulation tests

Compound	Lag before start of degradation (d) O_2 uptake method	Simulation	Degraded in simulation test
2-phenylphenol	2	20	yes
adipic acid	2	0	yes
hexamethylenetetramine	2	>63	no
1-naphthol	4	17	yes
sec-butylamine	5	20	yes
diphenic acid	6	48	yes
3-aminobenzoic acid	7	31	yes
3-chlorobenzoic acid	7	10	yes
thioglycollic acid	8	12	yes
1-naphthoic acid	9	31	yes
tetrahydrofuran	11	46	probably
benzenesulphinic acid	11	>63, 35+	no/yes+
3-aminophenol	13	28	yes
pentaerythritol	14	14, 36	yes
N-methylaniline	>28	46	yes
sulphanilic acid			no
benzene 1,3-disulphonic acid			no
2-chloro-aniline			no
t-butanol	>28	>63	no
cyclopentane tetracarboxylic acid			no
tetrahydrofuran tetracarboxylic acid			no
2-chloro-benzoic acid	>28	>*63, 28	*no/yes

+   = domestic sewage from different sources
*   = synthetic sewage

**Table A.4.3 Effect of exposure (in SCAS test) on the degradation
of selected chemicals in the ISO method (inoculum = 30 mg/l)**

Chemical	Period of exposure of inocula (d)					
	0	**10**	**18**	**26**	**67**	**67+**
	Time required for removal of >70% DOC (d)					
N-methylaniline (NMA)	>28*	21	14	14	3	6
t-butanol (t-B)	>28*	>28*	21	7	15	>28*
pentaerythritol (PE)	28	21	14	7	6	12
3-chlorobenzoic acid (3CB)	14	7	nd	nd	6	12
3-aminobenzoic acid (3AB)	14	21	nd	nd	6	15
3-aminophenol (3AP)	14	21	nd	nd	6	12
2-nitrobenzoic acid (2NP)	7	7	nd	nd	3	6
sulphanilic acid (SA)	>28*	21	>28*	>27*	18	22
diethylene glycol (DEG)	28	21	21	28	24	>28*
benzene 1,3-sulphonic acid (BDSA)	always >28*					
2-chlorobenzoic acid (2CB)	always >28*					

*   = not degraded
+   = effluent inoculum (5 ml/l)
nd   = not determined

Brown and Knapp (1990) grew sludge on a mixture of acetate, salicylate and morpholine with a sludge retention time of 20 to 30d. It degraded the amine at about 8 µg/g.h when fully adapted. In a die-away test this sludge, at the high concentration of about l g/l, removed 80 mg amine/l with little or no lag; 50% was removed in <10h. After 20d of amine withdrawal, the activity fell to zero and remained so for the next 180d. Die-away tests with this sludge showed lags of about 50d, followed by slow degradation reaching 50% removal in a further 120h. When morpholine was re-introduced, there was little or no removal during the next 200d. This was almost certainly due to a change in the nature of the sludge from a flocculent form to one containing largely free-swimming bacteria, causing a loss of sludge and low sludge ages (about 7.5d) falling eventually to only 1.5d. Cech and Chudoba (1988) have suggested that morpholine can be removed completely only if the sludge age is at least 8d; at 3d they calculate that morpholine-degraders would be completely washed out.

The behaviour of many more chemicals would have to be determined before valid conclusions can be made as to the retention by sludge of acquired abilities, and thus how they would behave if discharged intermittently. It is promising that LAS and NTA were degraded after short-term withdrawals, though it may be that no general pattern will emerge.

(g)   Experimental results

(i)   *Aerobic degradation*

In their investigation of 44 chemicals, Gerike and Fischer (1979, 1981) used pre-exposed inocula in the Sturm test and found that 29 passed and these chemicals were also degraded adequately in a simulation test. Eleven of the 44 chemicals, which failed in the Closed Bottle and modified OECD tests, were re-tested using pre-exposed inocula. Pre-exposure caused three chemicals to be degraded in the Closed Bottle test and six in the modified OECD test; these chemicals were also degraded in the simulation test.

The source and time of sampling of organisms used for exposure may play an important part. Lund and Rodriquez (1984) reported variable results in the adaptation to various benzene derivatives of sludges taken at different times from the same treatment works. Struijs and Stoltenkamp (1986a) found the source of the original micro-organisms for the Pitter (1976) fill-and-draw units influenced subsequent adaptation. Activated sludges exposed to 2-nitro-toluene for three weeks degraded the 2-isomer in two weeks and the sludge exposed to the 4-isomer took three weeks to degrade that isomer, while the 3-isomer did not degrade in the 28d test in the presence of sludge previously exposed to 3-nitro-toluene. When a mixture of activated sludge and river mud was exposed for the same time, the 2- and 4-isomers were degraded within shorter times and the 3-isomer was degraded in about three weeks.

In die-away tests with non-exposed inocula of river mud and activated sludge (30 mg/l), benzothiazole-2-sulphonic acid was degraded but only after lags of 15d and 45d, respectively. Degradation was complete in a further 15-20d (Mainprize, *et al.*, 1976). With sludge exposed to the chemical either in the laboratory or in a factory installation, there were no lag periods. The chemical (20 mg/l) was completely degraded in 15d by the laboratory sludge; 100 mg/l took 30d. The factory sludge, at 100 mg solids/l, degraded 20 mg/l in 2d and 100 mg/l in 5d.

Boatman, *et al.* (1986), using 18d exposed sludge, surprisingly found that 4-nitrophenol, N-methylaniline and linear alkyl benzene sulphonate did not degrade (CO_2 production) after 21d. This failure may have been due to the sludge having been exposed using a synthetic medium, though this is unlikely, and to a lesser extent to the die-away test period being only 21d. Another possible reason is that the reaction mixtures were very small, being only 10 ml.

Sugatt, *et al.* (1984) tested 14 esters of phthalic acid by a CO_2 method with pre-exposed inocula and found eleven of them to be degraded by >99% (primary) and 57-95% $ThCO_2$ in 28d. They calculated the ultimate biodegradation rate constants and half-lives by Larson's (1979) method and found rather high intra-set and inter-set variability. Although nitrilotriacetic acid (NTA) degrades well in die-away tests, it was found to require an exposure period of about 4 to 20d when fed to "semi-technical" scale activated sludge units (Nieuwsstad and van't Hof, 1986). The time before complete removal took place increased as the loading on the sludge increased; above $0.46d^{-1}$ considerable amounts of NTA were present in the effluent.

In their study of 4-nitrophenol (4-NP), Nyholm, *et al.* (1984) used the Sturm procedure for exposure. Non-exposed sewage effluent gave lags in the modified OECD test as high as

two to three months; with exposed inocula the lag time was only 3d and was much less dependent on cell density than with non-exposed inocula.

Calamari, *et al.* (1980) found that activated sludge inocula, previously exposed for 15d to various amines, singly, as the sole source of C, were not superior in removing the appropriate amine at 10 and 50 mg/l in die-away tests after 14d as compared with non-exposed sludges. However, at 100 mg amine/l in the die-away test (oxygen uptake) non-exposed sludges were unable to degrade diethylamine, di-n-butylamine and cyclohexylamine, whereas the respective exposed sludges did degrade them. Morpholine was not degraded by morpholine-exposed sludge.

Another effect of exposed inocula was given by Kim and Maier (1986) with 2,4-dichlorophenoxy acetic acid and 3,5-dichlorobenzoic acid in batch reactors. Non-exposed sludge inocula degraded the chemicals after 5 to 8d lag at 10 and 100 mg/l, but at 1 mg/l no degradation occurred after 72d. With exposed sludge, there were shorter lag periods and concentrations of low μg/l were utilized, indicated by the use of ^{14}C-labelled substrates.

The specially prepared MITI sludge was exposed to 100 mg test chemical/l for two weeks in the standard medium for growing MITI sludge (Urano and Kato, 1986a). In the MITI oxygen uptake method, the lag time for 100 mg aniline/l was reduced from 55h (non-exposed) to 15h (exposed) and the net time for degradation was only slightly affected, namely 55h to 45h. There was little effect on the first order constant, 0.046 to 0.049h^{-1}; there was also little difference in the extent of degradation, 49 and 47% ThOD. (Normally, this value is set at >60%). For benzene sulphonic acid the values for lag were 110 and 80h, net-degradation time 70 and 40h, rate constant 0.015 and 0.038h-1 and removal of 26 and 60% ThOD for non-exposed and exposed sludges, respectively. Another example of a readily biodegradable chemical being more readily degraded when an exposed inoculum is used in a die-away was given by Gonsior, *et al.* (1984). Sludge exposed for 7d to 10 mg 2-phenyl-phenol/l in a fill-and-draw unit treating a synthetic medium degraded 50% of the phenol in 3h, compared with 24h using a non-exposed sludge. Similar differences were observed in the production of ^{14}CO$_2$. In the Sturm test, non-exposed inocula produced only 15-40% ThCO$_2$ from carboxymethyl-tartronate (CMT), while exposed inocula (sludge, soil) produced 65-90%. In river waters, ranging from pristine to industrially polluted, four to six weeks were needed before CMT began to degrade (Gledhill, 1978).

Fuka and Pitter (1984) were able to degrade four pesticide preparations, containing MCPA and MCPP as the active constituents, in die-away tests with 100 mg sludge/l only when pre-exposed sludge was used.

Moos, *et al.* (1983) succeeded in adapting sludge to pentachlorophenol (PCP) in fibre-wall reactors with no recycle of sludge, resulting in high sludge retention times and maintaining a diverse population. The influent concentration was gradually raised to 4 mg PCP/l and the effluent concentration was only 100 μg/l after six weeks. In batch tests, concentrations of PCP higher than 2 mg/l were inhibitory.

In the adaptation of sludge to NTA, longer lag periods were observed in units treating the chemical in the presence of heavy metals or in soft waters (Stephenson, *et al.*, 1984). The nature of the sewage used also had an effect; in OECD "synthetic" sewage 2-chloro-benzoic acid was not degraded in the full nine weeks of the simulation test, but with domestic sewage it was degraded (Painter 1985, 1986). Similarly, benzene sulphinic acid was degraded in domestic sewage from one source but not from another.

In adapting activated sludge to phenol, which produced a sludge having an activity of about 50 times that of the non-exposed sludge, Urushigawa, *et al.* (1983) showed that the rank order in which a number of other phenols were degraded by the two sludges was different. Also, the adapted sludge produced a yellow colour in solution, indicating a different metabolic pathway.

In a study of 72 problematic "existing" chemicals discharged into the River Thames, Painter, *et al.* (1980) found that only six were readily biodegradable and only three others degraded in the ISO Die-away test using appropriately exposed inocula from the SCAS procedure.

Non-exposed mixed cultures derived from activated sludge removed only 20% of 2-chloro-phenol in 86h; exposed inocula removed 70% in the same time (Pal, *et al.*, 1980). Also, the adapted sludge was less prone than the non-exposed culture to inhibition by 2-chlorophenol. Similarly, sludges exposed for two months to various dyes were less inhibited by the respective dyes than the original sludges were (Ogawa, *et al.*, 1981). For example, 10-4 mol Basic Violet I inhibited the oxygen uptake by non-exposed sludge by about 50%, but showed no inhibition of exposed sludge. Contrary to this, exposure of activated sludges separately for three months, to six inhibitory chemicals, including pentachlorophenol and cetyl pyridinium chloride, did not promote the selection of populations resistant to the inhibitors (King and Painter, 1985).

(ii)   *Anaerobic degradation*

Non-exposed anaerobically digesting sludge degraded the three mono-chlorophenols (CP); 25 mg 2-CP/l was removed in two to three weeks, the 3-isomer took four to five weeks and 4-isomer was removed in five weeks (Boyd and Shelton, 1984). The end-products were CO_2 and CH_4. Of the dichlorophenols, the 3,4- and 3,5-isomers were not degraded. Sludge adapted over a period of four months, separately, to the three mono-isomers degraded their respective substrates much faster than before. Also, the previously non-degraded 3,4- and 3,5-dichlorophenols were degraded by the 3-CP exposed sludge. In further studies (Mikesell and Boyd, 1986), the complexity of microbial adaptation was again illustrated. Anaerobic sludges, adapted separately to the three mono-chlorophenols (25 mg/l), were found to transform pentachlorophenol (PCP) with the accumulation of di-, tri-, and tetra-chlorophenols. When the three adapted sludges were mixed, or when sludge was exposed simultaneously to all three mono-chlorophenols (10 mg/l of each), PCP was completely dechlorinated, though on repeated addition of PCP three chlorinated phenols accumulated. Using uniformly ring-labelled PCP, 66% of the added ^{14}C was shown to be mineralized to $^{14}CO_2$ and $^{14}CH_4$.

(iii)   *Results with natural waters, sediments, cores*

In a series of three papers Wiggins, *et al.* (1987, 1988, 1989) examined possible explanations for the "acclimation" or lag period preceding mineralization of organic chemicals in aquatic environments. They identified eight possible reasons:

- enzyme induction;
- mutation, genetic exchange;
- growth of a minority population;
- lack of nutrients;

- diauxie, prior growth on a secondary substrate;
- "acclimation" to toxins;
- destruction of inhibitors; and
- predation by protozoa.

Their experimental approach was to follow the removal of the test chemical as influenced by a number of factors, such as chemical concentration from 2 µg/l to 100 mg/l in lake water and sewage; filtration of the natural waters to remove larger protozoa; use of selective inhibitors; addition of nutrients and second substrates; addition of competent degraders; observation of within- and between-batch replicate behaviour. Although they reported different results with samples of lake water and sewage taken at different times and from different sources, they concluded that for 4-NP and 2,4-D, the chief mechanism for "acclimation" (lag) was the increase in size of a small population initially present capable of degrading the chemicals. The decrease in concentration of the chemicals could not be detected until about 10^5 degraders/ml were present. In lake water, lack of nutrients (N, P) was also found to be important, while in sewage predation by protozoa could be a minor factor. Most of the other possible explanations were eliminated. For example, the low variability in the lag period between replicates but not between samples militated against mutation. Poor reproducibility in the results in sewage with 3- and 5-nitrosalicylic acid and 4-nitroaniline suggests that mutations may have been occurring, although for 5-nitrosalicylic acid in lake water variability in the lag period was low and only two replicates showed mineralization, indicating that a rare strain was responsible.

They calculated that "acclimation" periods longer than one month are unlikely to be caused by the growth of cells initially present in the sample (Wiggins and Alexander, 1988a). For only one competent cell initially present in 50 ml sewage (the volume normally used in their tests) with a doubling time of 2d, only 26d would be required for the cell to multiply to reach a sufficient density for the mineralization of 2 µg test chemical/l to be detected. Thus, the random occurrence of a mutation or genetic exchange followed by growth of mutants probably accounts for very long "acclimation" periods.

The length of the period before a chemical begins to degrade may also be affected by the concentration of the chemical. At very low concentrations slow growth would lengthen the lag period. At high concentrations the toxicity of the chemical may reduce the number of active organisms, thus increasing the lag period, though it is possible that the longer time may reflect the greater time needed to detect the loss of the chemical.

The presence of a second substrate had varying effects on the length of the lag period, depending on the nature and concentration of the added chemical and also on the particular sample of sewage or lake water taken for study (Wiggins and Alexander, 1988a).

In another series of papers, Spain, *et al.* (1980, 1983, 1984) measured the degradation rate of 4-NP, methyl parathion and other chemicals by non-exposed and 100h pre-exposed ecocores from fresh water, estuarine and marine sources. The rates of removal of 4-NP at 60 µg/l and 24 mg/l were much higher by pre-exposed river sediment/water cores, as measured by evolution of $^{14}CO_2$, than by non-exposed cores (Spain, *et al.*, 1980). The increase in rates with pre-exposed salt marsh cores was much less. Numbers of 4-NP-degrading bacteria increased by four or five orders of magnitude during adaptation. For methyl parathion adaptation occurred only at the high concentration, and only with the river cores. The cores were exposed for 100h; exposure for only 75h at 96 µg/l gave rates of 4-NP removal lower than that for exposure for 100h. Under the conditions of exposure used, the

threshold concentration below which adaptation did not occur was about 55 μg 4-NP/l. The difference in patterns of adaptation to 4-NP at the two sites was probably due to qualitative differences in the population and not to differences in population size. Spain and VanVeld (1983) confirmed that cores from fresh water sites were successfully adapted after exposure, to degrade 4-NP, with no lag period and the maximum effect was found after two weeks exposure, the threshold concentration being 10 μg/l. Lags in non-exposed cores were as long as 100h. The rates in pre-exposed cores were much higher than in non-exposed cores and were proportional to the concentration of the test chemical over the range 20 to 100 μg/l. Adaptation was maximal after two weeks' exposure, but was not detectable after a further four weeks; about 50% activity was lost in two weeks after peak activity. Adaptation of the ecocores to 2,4-D was similar to that of 4-NP; o-cresol was rapidly mineralized by both non-exposed and pre-exposed communities, while trifluralin was not degraded under any conditions. No degradation occurred with estuarine and marine cores; neither could 4-NP degraders be isolated.

Spain, *et al.* (1984) extended the study to a field system (a pond) and found that the results of exposure in the laboratory tests fairly well reflected the result in the field sites. Such information increases the confidence that the time required for adaptation and subsequent biodegradation of at least some pollutants can be predicted from appropriate laboratory tests. For example, the lag period in the pond treated with 4-NP was 130h compared with 110h in a flask test with sediment, 88h in ecocores and 160-170h in small and large microcosms in the laboratory. Sediments were obviously important, since in flask tests with pond water without sediment the lag period was as high as 460h. The initial rates of removal of the chemical in adapted material were 10.4 $\mu g/l.h^{-1}$ in the pond and about 6 (range 2-17) $\mu g/l.h^{-1}$ in laboratory systems. One reason for the lower values in laboratory tests was thought to be interactions between sediment and radio-activity from the ^{14}C-labelled 4-NP. In general, increases in biodegradation rates correlated well with increases in numbers of 4-NP-degrading bacteria. Since the lag periods, population changes and specific bacteria present were similar among the various test systems and among replicates of the same test system, the adaptation was probably not the result of a random event. This would rule out mutation or recruitment of plasmid genes, suggesting instead that the mechanism was selection of organisms able to grow at the expense of 4-NP.

Swindoll, *et al.* (1988) also studied 4-NP but used sub-surface soil from a pristine aquifer. Slurries were incubated with ^{14}C-labelled chemical and the course of degradation was determined by measuring $^{14}CO_2$ evolved from replicate reaction mixtures sacrificed at time intervals. The activities of the samples varied considerably within the aquifer; for example, the lag period before the degradation of 4-NP varied from 10 to 120d and the extent of removal varied from 50% $ThCO_2$ in a further 10d to only 10% $ThCO_2$ in a further 30d. The addition of nutrients (N and P) and vitamins greatly decreased the lag period where this was greater than 10d. These results indicated the lack of nutrients in the sub-surface samples. In general, the addition of glucose or amino-acids inhibited the mineralization of ethylene dibromide, toluene and 4-NP, presumably because of preferential utilization of the more easily degradable carbon additions. Cell counts (acridine orange) were $2.5 \times 10^7/g$ solids initially, increasing by five to seven-fold after 15d when ethylene dibromide or toluene (-100 ng/g) was added. When a mixture of nutrients was added, the numbers increased by about 100-fold compared with the initial numbers.

The effects of exposure of three estuarine ecosystems on NTA degradation was studied by Pfaender, *et al.* (1985). The estuarine samples, which had different histories of exposure to NTA, were treated with 10 and 2000 μg ^{14}C-NTA/l. The low concentration was

below the level commonly thought to be suitable for microbial growth (Pirt, 1965) while the higher concentration was high enough to support growth. The sample from the estuary exposed to NTA for about ten years showed no lag in removing NTA at 10 μg/l and only short lags at 2000 μg/l; degradation was complete in 15d at the higher concentration. Thus, an NTA-adapted community existed in this estuary even though it had apparently been exposed to (unmeasured) concentrations below those usually assumed to be necessary for growth. This estuary sample had about 650 NTA-degraders/ml; at the other two sites fewer than 10/ml were detected. Samples from these two relatively unpolluted sites either removed NTA at a very low rate or exhibited lags of 50 to 60d before degradation was completed in a further 20 to 30d at the higher concentration. Spain, *et al.* (1980, 1983) had concluded that where no competent degraders are found, the chemical is either not degraded or is degraded very slowly and adaptation is not observed. Pfaender, *et al.* (1985) disagree with this view, but indicated that the problem may be the sensitivity of the method used for counting bacteria.

The exposure of estuarine communities to NTA was also investigated by Hunter, *et al.* (1986). They used a laboratory "estuarine interdependent microcosm", consisting of five compartments in series, each held at a different salinity. Each compartment was inoculated simultaneously from fresh water and saline sources. At concentrations of 0.7 to 1 mg NTA/l biodegradation was incomplete at salinities higher than 9.2% and at 7 to 10 mg NTA/l the salinity limit was lower, that is, greater than 5%. Thus, adaptation was dependent on the concentration of NTA and on salinity; the authors called the phenomenon a "salinity stress-induced failure" of NTA catabolism.

No evidence was reported by Johnson, *et al.* (1984) for any increase in the rate of degradation of four phthalic acid esters by pre-exposed fresh water sediments. However, acetone used as a carrier for the esters was found to lower the degradation rate of two of the esters.

Cripe, *et al.* (1987) used shake flasks with river water-sediment slurries to examine the primary biodegradability of di-butylphthalate (500 μg/l). After about 30h degradation began and the ester was completely removed in a total time of 2d; the flasks were then re-spiked with the ester and some of the control flasks were spiked at the same time. The ester in the re-spiked flasks was removed within a few hours with no lag, while in the others a lag of about 30h was followed by rapid removal. The authors took this as evidence of adaptation, presumably because it eliminated any changes in the 30h lag period within the sediment and water, unconnected with the ester, as being the cause of the lag, such as removal of a toxic substance.

Working with m-cresol, 3-aminophenol and 4-chlorophenol in lake water, Shimp and Pfaender (1985a) found that pre-exposure, in long residence chemostats, to naturally occurring substances (amino-acids, glucose or fatty acids) enhanced the ability of the microbial community to degrade all three phenols. However, the degradation of glucose and naphthalene was also enhanced, suggesting a stimulation of general microbial metabolism. Later, they exposed microcosms from lake waters to 1 mg phenol/l as the sole C source in chemostats with a retention time of 3d (Shimp and Pfaender, 1987). After two to three weeks of exposure, the rate of degradation of the three phenols by the exposed culture was found to be markedly higher than by the non-exposed culture; the first order rate constants increased by five- to 20-fold. The MPN of competent degraders increased by at least ten-fold over that in the control community.

The rate of $^{14}CO_2$ production from the cationic compound dodecyltrimethylammonium chloride (DTMAC) by microbial communities in lake water, measured over a period of 3h, was much higher in samples previously exposed to the chemical for 21d than for samples which had not been exposed (Ventullo and Larson, 1986). Maximal biodegradation rates increased as the concentration to which the communities had been exposed was increased and were two to five orders of magnitude higher than the controls. The number of DTMAC-degraders increased from about 10 to 50/ml in unexposed controls to about 2×10^3/ml in communities exposed to 10 mg/l. At this level, not only were more DTMAC degraders present, but they were more active per cell. It was also shown that exposure to DTMAC did not lower the number of heterotrophs present but did lower the heterotrophic activity (^{14}C-glucose turnover) at 0.5 mg/l and above. Chronic exposure, however, had no effect on heterotrophic activity up to 10 mg DTMAC/l.

Vaishnav and Babeu (1987) found that four of ten chemicals tested did not degrade in the BOD_{20} test using unamended natural waters containing, 55 cells/ml: (ground water), 420 (river water) and 310 (harbour water). Inocula, produced by an enrichment procedure to each of the four chemicals separately, added at about 300 cells/ml, degraded two of the chemicals (4-tert-butylbenzoic acid and hexadecane) in ground water with $t_{1/2}$ of 87 and 16d respectively. Naphthalene was degraded in the (adapted) river and harbour waters, with $t_{1/2}$ of 53 and 43d respectively. These half-lives were significantly lowered when nutrients (N, P) were added with acclimated inocula. Benzene was degraded in harbour water ($t_{1/2}$ = 8d) and naphthalene degraded in ground water ($t_{1/2}$ = 28d) only in the presence of added exposed inocula and inorganic nutrients.

(h)   Conclusions

Waste water treatment

The evidence indicates that, providing the pre-exposure period is not more than 18d, no chemical will pass the subsequent screening test with a pre-exposed inoculum without also being adequately removed in the activated sludge simulation test. The number of chemicals so far found in this category is admittedly not large, so that for confirmation of the procedure more chemicals should be tested. Even so, it would be worthwhile considering using the procedure in the near future.

Natural waters

The situation regarding pre-exposure in natural waters, with or without sediments, differs from that in sewage treatment. The experiments reported for pre-exposing and assessing degradation, have been carried out under conditions much more nearly approaching "real" environmental conditions than the OECD screening methods resemble sewage treatment. Where tested, the ability to degrade the chemical was retained for some weeks after withdrawal of the chemical. These tests, which used low concentrations (µg/l) of the test chemical, are really simulation tests or at least surrogates for such tests.

A.5   Test chemical

The choice of the concentration of the chemical to be tested depends largely on the limits of the sensitivity and precision of the analytical procedures for the determination of DOC, CO_2, oxygen and dissolved oxygen and on the blank control values given by the inoculum alone. For DOC, the present analysers are such that at least 5 mg DOC (test chemical)/l, but preferably 10 mg/l, should be used. In the Sturm test the same concentration can be used by virtue of having 3l of culture medium to produce easily detectable differences in CO_2 evolution between control and test vessels. Rather more test chemical (50 mg/l) is needed in the respirometric methods and normally 100 mg/l is used. For the Closed Bottle test, the limitation depends on the solubility of oxygen in water and the endogenous metabolism of the inoculum; this limits the concentration of test chemical to 2 to 5 mg/l.

Another limitation is that the test chemical may be toxic to the micro-organisms in the inoculum and for this reason the concentration tested should be kept as low as possible. A test for inhibition may be made before starting the biodegradability test or simultaneously with it.

The concentrations stipulated in the tests will inevitably be much higher than those found in the environment for all chemicals, except a very small minority of compounds produced in very high tonnages. A discussion on tests on environmentally realistic concentrations is deferred to section C5.

This section deals with the methods of adding the test chemical and some results illustrating the effect of concentration of the chemical on its biodegradation. The kinetics of degradation at various concentrations is dealt with in section B.3.

A.5.1 Method of addition

Very water-soluble chemicals present no problem and can be added as a relatively small volume of a strong solution (e.g. 1000 mg/l). Less soluble chemicals can be added directly to the medium with stirring until solution is complete.

The standard methods (OECD, EC, ISO) do not specify how insoluble chemicals and volatile chemicals should be prepared for introduction into the reaction mixture. Indeed, one view is that there is no point in testing insoluble chemicals for aerobic biodegradability; since they probably find their way into suspended solids, sludges and muds, they should be tested for anaerobic biodegradability. However, as pointed out by, for example Gerike (1984) until more is known about the form in which poorly soluble chemicals are found in the environment, it is sensible to determine a "biodegradability spectrum" for poorly soluble chemicals, that is, from using simple mechanical turbulence to an optimum distribution by ultrasound dispersion and stabilisation by inert emulsifiers. Fogel, *et al.* (1985) complained that, for the relatively small numbers of insoluble chemicals for which biodegradability had (by then) been determined, the techniques for introducing test chemicals were both numerous and poorly described. They quote Liu's (1980) finding the rates of polychlorinated biphenyls (PCBs) were much higher if the PCBs were dispersed in water by sonication, after adding sodium ligninsulphonate to stabilise the emulsion. Yet Schoor (1975) had found that different methods of preparation led to PCB-water emulsions varying in degree of dispersion and stability - hence the need to be specific in the details of preparation.

Similarly, the need for assessing the biodegradability of volatile chemicals is sometimes questioned e.g. ECETOC (1984) since they are thought to escape rapidly to the atmosphere. As with insoluble chemicals, the standard tests pay little attention to these chemicals.

Poorly soluble chemicals and volatile chemicals are dealt with in sections A.5.1 (a) and (b), respectively.

(a)   Poorly soluble chemicals

It is not known in what physical form insoluble chemicals are present in the aquatic environment so that it is not clear how such chemicals should be presented to inocula in laboratory tests: perhaps they should be subjected only to anaerobic tests. Because of this difficulty some workers are of the opinion that no emulsifying agents should be used to disperse insolubles in tests and that no insoluble chemical can be classified as readily biodegradable. In any case when assessing results the dispersion method applied must be taken into account.

In early work, insoluble or sparingly soluble chemicals were added directly, for example, 2 to 3 mg calcium salts of higher fatty acids were added per Warburg flask (Loehr and Roth, 1968) or by dissolving the chemical in a solvent, for example, 4,41-dichlorobiphenyl was dissolved in ether and evaporated to dryness in the flasks in which the test was to be carried out (Tulp, *et al.* 1978). In both cases the techniques appeared to be successful. However, Gerike (1984) found that the solvent technique was unsuccessful when the Closed Bottle test was applied, the control bottles containing solvent only having unacceptably high blanks. The last traces of solvent were difficult to remove.

Fogel, *et al.* (1985) also used the solvent technique with acetone and applied the CO_2 evolution method of Gledhill (1975b), with 200 mg test chemical-C/l and a headspace of, unusually, 70% oxygen in nitrogen. They were successful in that the rate and extent (67% $ThCO_2$) of CO_2 evolution were the same in "single lump" controls of hexadecane and in the flasks to which the acetone solution had been added. They suggested that a non-volatile but biodegradable contaminant in the 3ml or so of solvent used by Gerike could have caused the high control blank oxygen uptake values compared with the much smaller volume, 0.1ml, used in their own experiments. Fogel, *et al.* (1985) also used dimethylsulfoxide, at 22 and 220 mg/l, but this solvent appeared slightly to inhibit oxidation yielding 56% $ThCO_2$ rather than 69% with the "single lump", no solvent addition. These authors also coated a zeolite molecular sieve with hexadecane, a process said to promote microbial utilization and to avoid the need for agitation. However, the zeolite decreased the rate of CO_2 evolution, although by 28d the same amount (55% $ThCO_2$) was attained in both zeolite and "single lump" flasks.

An altogether different method has been used, for example, by Kiyohara, *et al.* (1982). A pure bacterial species was inoculated onto the plates of agar-salts medium, then an ethereal solution of a hydrocarbon (phenanthrene, anthracene, etc.) was sprayed onto the surface and the plates were incubated for 2 to 3d. Clear zones around colonies indicated degradation. This attack on a solid surface is contested by other workers (see below).

The problem of insoluble chemicals was brought into focus by Boethling (1984), by a more detailed and practical paper by Gerike (1984) and by a report by ECETOC (De Morsier, *et al.*, 1987). Boethling (1984) stressed the need for adequate methods and stated

that arising from studies on petroleum hydrocarbons with which most previous work had been done (along with studies with carbohydrates), insoluble liquids can be degraded at the water-liquid interface. Solids cannot apparently be attacked directly (but see Kiyohara, *et al.* above) but can be attacked at the solvent (plus dissolved solid)/water interface. The interfacial rates can be enhanced by increasing the surface area and by lowering the surface tension at the interface. Some bacteria secrete their own emulsifying agents to achieve an increase in surface area. His strategy for testing liquid insolubles is to emulsify by ultra sound treatment and stabilize with an emulsifying agent, for example, sodium lignosulphonate; solids would first be dissolved in an inert, water-immiscible solvent, for example, heptamethylnonane, and then emulsified. The choice of agent is critical; some are toxic (for example, Triton X-100), while biodegradable agents complicate the situation. Emulsifiers used to enhance oil recovery operations are likely candidates.

Gerike (1984) discussed problems of analysis (e.g. COD) of insoluble chemicals as well as methods of getting the test chemical into intimate contact with the inoculum. Dispersing agents suggested were nonylphenol ethoxylates, additionally propoxylated with 3,5 or seven propylene oxide units, and tetrapropylene benzene sulphonate, or a mixture of these. He warned that not all chemicals were amenable to dispersion. Adding solutions of a test chemical in solvents (ether, methylethylketone, n-hexane) to BOD bottles, followed by evaporation for 6h under vacuum for assessment by the Closed Bottle method was unsuccessful. The blank controls were up to three fold higher than was acceptable. Gerike dismissed the TOC die-away method of Muller and Tittizer (1979), since C assimilated into cells could not be separated from the insoluble chemical and because of the possibility that CO_2 retained in the enclosed system may be transferred to cells by autotrophic bacteria (though there is no evidence of this). Both these factors would give removal values which are lower than the true values.

Gerike (1984) considered the Closed Bottle suitable only if a stable stock dispersion of the test chemical could be obtained, but direct weighing of 0.5 to 1.0 mg is probably not practical as a routine procedure. He did not consider the respirometric (oxygen uptake) method but thought the Sturm method to be suitable, though he preferred to collect CO_2 in NaOH solution and determine DIC in a carbon analyzer. On average, the % $ThCO_2$ values for sodium stearate, C8/18 fatty acids and biphenyl were increased when dispersed with nonylphenol 10EO+5PO from 53 to 62, 51 to 52 and 23 to 42% respectively. However, the author favoured a modified form of Blok's method (1979), although he had reservations about some aspects. With the above three chemicals, he found the effect of NP10+5PO to be small and mainly negative, namely 102 to 85, 103 to 100 and 74 to 77% DOC, respectively. In previous work he had found a small, but positive, effect of the presence of the emulsifier. The modified Blok method was considered the most promising. Indeed, the method has been revised and presented (BODIS) as a draft to the ISO working group (ISO, 1990a).

The ECETOC report (De Morsier, *et al.*, 1987) went over some of the same ground as the previous two papers but in addition gave results of a ring test. The group rejected the Closed Bottle test on the grounds of lack of agitation, low cell density and accuracy and precision if direct weighing is used, though they realised that better results could be obtained if emulsifiers were used. Solvents, glass filters and cellulose as carriers and dispersion agents were used in the Sturm, MITI and modified Blok methods. Beeswax and calcium stearate were transferred to solid carriers by melting on glass filter papers, while beeswax and anthraquinone were dissolved in methylene dichloride, added to the mineral medium followed by aeration for 24h to drive off the solvent before inoculation. The preparations were not described in much detail.

Calcium stearate, tested by the Sturm and MITI methods, was degraded adequately, >70%, under all conditions, except when "carried" on a glass filter paper - 55% ThCO$_2$ and gave the longest lag of 5d. The reason for this low value was the reduced availability of the chemical to the bacteria as a result of a "glazing" on the surface produced by the melting and setting. Another reason was the absence of adequate agitation, since when agitation was applied 84% ThCO$_2$ was recorded. Anthraquinone was degraded (>51% ThOD or ThCO$_2$) in all cases when agitation was applied in all three types of test. For example, direct addition alone gave 17%, with agitation 75% ThCO$_2$, and glass filter carrier, no agitation, gave 30% while agitation increased the yield to 51 to 91% ThCO$_2$. In the MITI test, the use of NP10+5PO reduced the degradability from 93% to 81% ThOD, while in the normally stirred Blok test 70% ThOD was recorded. Beeswax gave more variable results.

Emulsifiers (Span 80, Tween 80, NP10EO5PO, tri-t-butylphenol-8EO and styrene-phenol 8EO) plus agitation and use of methylene dichloride plus agitation gave 58-90% removal, but tests without agitation usually showed a slow, steady rate and did not reach 50% in the four to seven weeks of the tests. Carriers (glass filters) gave variable results for probably the same reasons as with the stearate, but it was thought that oily substances could be successfully tested with carriers, though more data were required.

Other evidence was collected by the ISO group. For example, French laboratories showed that ultra sound had little effect on the rate of degradation of anthraquinone compared to the control but by day 28 the treated medium reached 62% ThOD compared with 55% in the control. Dispersion with an agent and adsorption onto an inert medium increased both the rate and extent. These treatments reached about 50% in 8d, compared with only 5% in the control, and by day 28 the treatments had given around 70% ThOD and the control only 55%. Similar relative results were obtained with di-iso-octylphthalate, the extent of degradation varying from 80 to 98% ThOD.

Nyholm and Seiero (1989) and Nyholm (1990) used the same chemicals in the EC Respirometric method, comparing direct addition, adsorption on silica gel or glass filters via solution in methylene chloride, Tween 80, branched chain alkyl benzene sulphonate and ultrasound. Direct addition (27.5 mg on Al foil ~0.3g) was a difficult but feasible operation. For adsorbing on to silica gel, 5.5ml of a solution of 5 g/l in methylene dichloride was added to 15g silica gel in a Petri dish. After allowing the solvent to evaporate for 3h, the entire contents of the dish were transferred to the test vessel. Adsorption onto glass fibre filters was by adding the same volume of the test chemical solution to eleven filters (only about 0.5ml solvent could be absorbed by one filter of 4.5cm diam.) and all eleven were transferred to the test vessel. Dispersion with emulsifiers was carried out by mixing 5g Tween 80 and 2g test chemical in a beaker placed in an ultrasound bath at 50°C. The mixture was then diluted, after cooling, to 500ml with distilled water in a graduated flask, and shaken vigorously. With the branched chain anionic surfactant, the ratio of test chemical to agent to CaCl$_2$ was 1 : 5 : 2.5 and two short periods of ultrasound treatment are given. In both cases the phthalate produced stable emulsions but anthraquinone was not emulsified.

Some results agreed with those reported by the French laboratories, namely, that ultrasound treatment had little effect and adsorption onto an inert solid (silica) increased the % ThOD for anthraquinone though not for the ester. However, dispersion with emulsifying agents lowered rather than increased the extent of biodegradation, namely 46 to 24-42% ThOD for anthraquinone and 65 to 51% ThOD for the phthalate.

The biodegradation of anthraquinone was enhanced – 52 to 69% ThOD – when the chemical was emulsified with Tween 85, after allowing for the small oxygen uptake by the agent, which was less degradable than Tween 80 (Painter 1985). The treatment was without effect on sodium stearate. Tween 80 was without effect in isopropyl palmitate and isopropyl myristate, both of which degraded well – 75 and 93% ThOD respectively (King and Painter, 1985).

Discussion of these and other data has resulted in a draft paper (ISO, 1990b) which gives general practical guidance in preparing insoluble and sparingly soluble chemicals for biodegradability assessment. It is known that no single method is applicable to all such chemicals but the aim is that, for any given technique for dispersion, the same working method will be used by all laboratories so making it easier to compare results. Even so it is probable that variations in duration of treatment, concentration of emulsifying agent, etc. will have to be permitted so that chemicals of diverse properties may be successfully dispersed.

It is instructive for understanding the mechanisms of degradation of insoluble chemicals to consider a study by Thomas, *et al.* (1986) on the rates of dissolution and biodegradation of such chemicals. They showed that the rate of dissolution was directly related to the surface area presented to the micro-organisms and they quantified these rates. Their approach was to measure concentrations in solution of chemicals in inoculated (with enriched mixed cultures) and sterile suspensions and also cell densities. In a naphthalene suspension (50 mg unsized particles/l) the rate of solution in the first hour was 2.8 μg/ml.h in both sterile and inoculated vessels. After this the concentration in solution in the inoculated suspension fell from 12 mg/l to 0.1 mg/l and stayed at this level for the remainder of the test (70h); in this time the cell density rose from ~10^4/ml to 3×10^8/ml. In the uninoculated suspension, however, the concentration of naphthalene rose to 17 mg/l. The pattern with 4-chlorobiphenyl (4-CBP) was similar, except that growth was slower, and the rate of dissolution in the first hour was much lower at 87 ng/ml.h and the final concentration in the sterile vessels was 0.6 mg/l. When single crystals of 4-CBP (5 mg) were added, separately, to portions of mineral medium, no 4-CBP was detected in solution at any time in inoculated vessels. Bacteria grew, but growth stopped abruptly at about 10^7 cells/ml. In uninoculated medium none was detected after 24h and the concentration after 14d was only 0.21 mg/l, that is, dissolution was very slow. When a 36 mg crystal was added the concentrations in the uninoculated medium were 0.18 mg/l at 24h and 0.39 mg/l at 14d. In the inoculated medium cells grew rapidly initially but then the rate declined.

Four graded sizes of the chemicals (from 0.1 to 3.5mm) obtained by sieving were added, separately, at 25 mg/l. The rates of dissolution were highest with the smallest particle size and lowest with the largest size, that is, the rate was proportional to surface area. The concentration of 4-CBP in uninoculated solution reached about 1 mg/l after 5d, but this concentration was not reached with the other sizes in that time. When 4-CBP was no longer detectable in the inoculated medium, the rate of cell growth declined abruptly; naphthalene behaved similarly. From the data on increase in cell density and the amount of substrate dissolving, the authors were able to calculate the amount of substrate required to produce each cell. They concluded that it was likely that the bacteria were obtaining more carbon than the amount predicted from the dissolution rates determined in the absence of cells. Thus, either the bacteria enhanced dissolution or they assimilated the substrate directly from the solid phase. For naphthalene and 4-CBP if the bacteria used only substrate in solution, the mineralization rate would be limited by the dissolution rate when the substrate in solution was depleted. But the fact that maximal concentrations of naphthalene and 4-CBP in the inoculated samples were correlated with surface area suggests that the dissolution rate initially

exceeded the bacterial demand. Thomas, *et al.* (1986) quote other workers including Fogel, *et al.* (1985), as finding increased degradation with increased surface area but this is not what Fogel, *et al.* (1985) say. Neither coating of hexadecane on the walls of the vessel nor occluding it on to a molecular sieve increased the rate of mineralization over that observed when a "single lump" was added. Further, Thomas, *et al.* (1986) cite reports by Wodzinski and co-workers (1972, 1974) that growth rates of bacteria on naphthalene, biphenyl or phenanthrene were independent of the surface area of the solid substrate.

Thomas, *et al.* (1986) examined four other chemicals. The mineralization rates of two chemicals-palmitic acid and Sevin (1-naphthyl N-methylcarbamate) - were lower than their dissolution rates, while for octadecane and di-ethylhexylphthalate the reverse was true. For example, palmitic acid was mineralized at 29 ng/ml.h^{-1} and dissolved at 70 ng/ml.h^{-1}, while for octadecane the values are 3,800 pg/ml.h^{-1} and 48pg/ml.h^{-1}, respectively. The authors concluded that micro-organisms may use an insoluble chemical as it dissolves spontaneously in water, or they may metabolize it after a microbial-mediated solubilization or by mechanisms involving physical contact with the solid phase of the chemical. They stress that additional studies are required to assess the significance of these and other possible mechanisms.

A novel use of liposomes (phosphatidylcholine, from egg yolk) was reported by Miller and Bartha (1989) to encapsulate insoluble alkanes so that they would easily pass through the gram-negative cell walls. Thus, if recalcitrance of the hydrocarbon was due to its inability to enter the cell rather than the absence of a necessary enzyme, this technique should be able to differentiate between the two possibilities. Hexatriacontane (C_{36}) was shown not to cause growth of a *Pseudomonas sp.* isolated from soil when added alone but when added in the encapsulated form, growth occurred at a rate comparable with that on succinate. Growth on octadecane was similarly increased; no growth occurred on the "empty" liposome. Thus the recalcitrance of the C_{36} hydrocarbon was due to its inability to pass into the cell.

(b)   Volatile chemicals

In early work (e.g. Sato and Akiyama, 1972) volatile acids were tested in Warburg or similar respirometers as their sodium, or other, salts. For volatile chemicals generally Hantsveit and de Kreuk (1977) indicated a die-away method in which a glass stopper of special design was fitted to the normal conical flask. The stopper was provided with a side tube carrying a tap and a rubber injection cap so that sampling could be carried out by means of syringes with minimal loss of volatile material. Presumably either DOC and/or specific chemical was determined, but generally speaking it seems that the method of determining DOC of volatile chemicals has not been substantiated.

Another method, put forward by Shell Research Ltd (ISO Paper N40 1984), used a conical "iodine" flask with a side tube fused into the lower side fitted with a mininert valve allowing direct injection of a test liquid into the bottle via micro-syringe. The port is also used to withdraw samples. The flask is filled with the inoculated medium for the Closed Bottle test, then stoppered and the sample injected. The contents were well stirred by means of a magnetic stirring bar previously added to the flask. The course of degradation could be followed by sacrificing flasks at timed intervals for dissolved oxygen determination (e.g. by electrode) and/or by withdrawing samples via the mininert valve for GC analysis to assess primary biodegradation. It was found that recovery of added toluene was about 90% (low because of some of the hydrocarbon remaining as discrete globules) while recovery of acetone was 95-100% (Shell Research Ltd). Using a culture, known to degrade toluene, as

inoculum and making DO measurements, toluene at 2 and 4 mg/l were found to degrade by 60-62% ThOD in 5 to 7d, with good reproducibility. However, using the same method but with an inoculum of 0.5ml sewage effluent/l, difficulties with high blank values and with nitrification were encountered even with test chemicals not containing N(Battersby and Mallett, 1988). The problems could not be solved by adding inhibitors of nitrification but were minimized by reducing the N content of the medium and by carrying out the tedious analyses of ammonium, nitrite and nitrate, as is described in the latest version of the OECD methods (OECD, 1992). The change in the concentration of ammonium N has reduced the "worst case" value (that is, full nitrification in the test bottle, none in the control) for a nominal 60% ThOD (C oxidation only) from 93% ThOD to 67% ThOD. Ethyl acetate was degraded by >90% (GC) and 55% ThOD but although sec-butylamine could not be detected after 28d only 25% ThOD loss was recorded; t-butanol was not degraded and recovery was >90%. The method was also applied to cinnamylalcohol (45% ThOD, 5d; 85%, 28d), cinnamylaldehyde (41,72%), di-isopropylamine (1,4%) di-isopropyl ether (2,7%) allyl bromide (1,62%) and methyl isopropyl ketone (1,80%). Although effluent from a nitrifying activated sludge unit was taken for the inoculum, very little or no nitrification occurred.

Other studies have included that of Tabak, *et al.* (1981) who used the Bunch-Chambers version of the shake flask die-away test and sealed the flasks with glass stoppers. In most cases primary degradation was assessed. Benzene and derivatives, halomethanes and haloethanes were among the chemicals tested and control flasks were set up to determine loss by volatilization. Also, since the Bunch-Chambers medium contains 50 mg yeast extract/l (though Tabak, *et al.* seem to have used only 5 mg/l), cometabolism and other processes may have been operating. Some results obtained were expected, for example, phenol degraded by 96% in the original culture (7d), but others were unexpected; for example, benzene removed by 49% in 7d rising to 100% in 14d (first subculture); chloroform - 49% in 7d and 100% in 28d, with 6-24% lost by volatilization.

Sims and Sommers (1986) determined total loss of pyridine and some derivatives by UV spectroscopy in the presence of ethanol as solvent and co-substrate in shake flasks with soil suspension as the inoculum. The flasks were plugged with 2.5cm thick polyurethane foam stoppers to trap volatile pyridine compounds; separate tests in the absence of soil showed 89-100% of the pyridine lost from solution was trapped in the plug, as determined by extracting the plugs with a KCl solution. By determining the amount of pyridine derivative adsorbed on the soil and that remaining in solution, it was found that one-half of the 28 chemicals tested were degraded (primarily) by 50% or more.

To overcome the inability of the Zahn-Wellens and SCAS tests to assess the inherent biodegradability of volatile compounds over 28d, Struijs and Stoltenkamp (1986a) exposed sludge to the volatile chemical as in the Pitter (1976) method for three weeks. The resulting sludge was used as inocula in DOC die-away tests in closed flasks, in which the ratio of headspace: liquid was about 1 : 1. The methods were applicable only to chemicals which are not so volatile that they are completely lost between the daily additions in the Pitter "SCAS" system.

By headspace gas analysis, the primary degradation of 400 μg/l of trichloroethylene (TCE) by a methane-oxidizing culture was followed in 250 ml septum bottles containing 100ml medium by GC analysis (Little, *et al.* 1988). Each bottle contained 12 ml filter-sterilized methane. When ^{14}C-TCE was tested, ultimate degradation (72% Th^{14}CO$_2$) by a mixed ground water culture was observed. A similar system, but about one-tenth the size, was used by Henson, *et al.* (1989) who derived their mixed culture from soil which was able to remove

halogenated aliphatic hydrocarbons. Dichloromethane, chloroform and even carbon tetrachloride - though this more slowly - were removed by 90, >80 and 30% respectively, in 30d. Trichloroethylene, 1,2-dichloroethylene and 1,2-dichloroethane were also removed by about 90% in the test. These removals were not caused by direct metabolism but some form of cometabolism since the presence of methane was essential.

A.5.2 Effect of test chemical concentration

Fortmann and Rosenberg (1984) tested e-caprolactam at 100, 1000 and 2000 mg/l and found the highest % $ThCO_2$ with 100 mg/l - 80% in 21d and least with 2000 mg/l. However, the highest amount of chemical was degraded at 1000 mg/l, indicating that inhibition began above this concentration.

In the Zahn-Wellens test (Nyholm, *et al.* 1984) the lag period for the degradation of 4-NP increased from 1 to 3d at initial concentrations of 40 to 800 mg/l. In the modified OECD method, the lag period was roughly proportional to the initial concentration of the nitrophenol, increasing from 14d at 1 mg/l to 70d at 35 mg/l. Counts of bacteria indicated that optimum growth occurred at 4 mg 4NP/l; above that the chemical was inhibitory. The % DOC removal was about the same in all cases. Similarly, with a series of eleven surfactants, Urano and Saito (1985) found inhibition at higher initial concentrations for a number of test chemicals. They tested 3, 10, 30 and 100 mg/l and determined the oxygen uptake in the MITI test and calculated the ratio BOD_{14} to ThOD. Only for dodecyl sulphate was the ratio about unity at all four concentrations; with all the other surfactants at least 100 mg/l gave values significantly lower than 1. LAS gave a high value only at 3 mg/l with 0.37 at 10 mg/l falling to 0.06 at 100 mg/l. Even naturally occurring stearate and oleate gave a ratio of only 0.6 at 100 mg/l.

There are other examples showing that chemicals at 20 to 100 mg/l are inhibitory but degrade at lower concentrations.

Some chemicals degrade easily over a wide range of concentration; dibutylphthalate degraded by 64% after 7d at 80 μg/l and by 73% after 14d at 8 mg/l (Johnson and Heitkamp 1984). Boethling and Alexander (1979a) found that dimethylamine, diethylamine and diethanolamine degraded at rates proportional to their initial concentrations over the range of several ng/l to several mg/l. The rate of glucose removal at 18 μg/l, however, was lower than that predicted by Michaelis-Menten kinetics.

Using concentrations normally employed in the MITI test, Urano and Kato (1986a) reported only small differences in lag time, degradation time, rate constant, % ThOD and % DOC removal in 10d with initial concentrations of 10, 30 or 100 mg ethanol/l. Phenol and aniline gave similar results at each concentration, but benzene sulphonic acid at 100 mg/l took up less oxygen after 10d (26% ThOD) than at 10 and 30 mg/l (45 and 42% ThOD, respectively). 3-Amino-phenol did not degrade at any of the three initial concentrations.

Whereas the t_{50} for 2-chlorobiphenyl (primary degradation) in river water increased only slightly from 2.5 to 3.5d at initial concentrations of 1, 10 and 100 μg/l, the times required to achieve 50% $Th^{14}CO_2$ were longer, being 7d at 1 μg/l, 10d at 10 μg/l and >50d at 100 μg/l (Bailey, *et al.*, 1983). An intermediate metabolite, peaking at 5d, degraded at the two lower concentrations but not so readily at 100 μg/l. At the highest concentration, the 3- and 4-isomers degraded more readily than the 2-isomer.

Whereas isopropyl-N-phenylcarbamate degraded at 0.4 μg/l and 1 mg/l in sewage (Wang, *et al.* 1984), it was mineralized only at the lower concentration in lake water; at 1 mg/l organic intermediates accumulated. Mineralization of 0.4 μg/l in lake water was enhanced by adding inorganic nutrients or a mixture of non-chlorinated water pollutants but not by yeast extract, aromatics or excretions from primary producers. Similarly, the degradation of 2,4-D at 0.2 μg/l in lake water was not affected by the addition of yeast extract or compounds excreted by primary producers but was enhanced by low concentrations of mixtures of water pollutants.

The lag occurring before the onset of degradation of 4-NP in sewage decreased as the concentration of the chemical increased; at 2 μg/l the lag was 11d while at 10 mg/l it was reduced to 2d. On the other hand, the lag increased from 1 to 2d at 100 mg phenol/l to 12d at 1400 mg/l; with both chemicals the lag times were reproducible (Wiggins and Alexander, 1988a).

The rate of biodegradation in stream water of 4-chlorobenzoate and chloroacetate decreased at lower concentrations of the chemicals from 47 mg/l down to 47 μg/l; even so >50% ThCO$_2$ was evolved within 8d at the lower concentration (Boethling and Alexander 1979b). In contrast, little degradation of 2,4-D and 1-naphthyl-N-methylcarbamate occurred at 2 to 3 μg/l or less, though 60% ThCO$_2$ was produced at higher concentrations.

Rubin, *et al.* (1982) showed that phenol, benzoate, benzylamine, 4-NP and di-ethylhexylphthalate added to lake water were mineralized at rates proportional to their concentration from a few ng/l to μg/l. 2,4-D degraded at rates less than predicted at 1.5 ng/l while at 200 μg/l it was not degraded, although it was mineralized in sewage. The slope of plots of rates of phenol mineralization in lake waters against initial concentration was lower at lower levels of 1 to 100 mg/l than at higher concentrations. Values of % ThCO$_2$ in sewage and in some lake waters approached 100% for benzoate, phenylacetate and 2,4-D, so that little or none of the chemical was assimilated into cells. From these and other data, it was concluded that mineralization of some chemicals at <1 mg/l is carried out by organisms (oligotrophic) different from those functioning at higher concentrations or of organisms that metabolize the chemicals at low concentrations but assimilate little or none of the substrate.

The same group (Subba-Rao, *et al.* 1982b) showed that 93-98% of benzoate, benzylamine, aniline, phenol and 2,4-D was mineralized in lake water at one or more concentrations below 300 μg/l, again indicating little or no assimilation. Aniline, from 50 to 500 μg/l, and phthalate, from 200 ng to 2 μg/l, were degraded at rates which increased with time. Although phthalate and 2,4-D were mineralized in eutrophic lake water, they were not degraded in water from oligotrophic lakes.

When lake water was inoculated with Corynebacterium sp., 4-NP was rapidly mineralized at 100 μg/l to 1 mg/l but not at 26 μg/l; the rate at this last concentration varied from sample to sample of the lake water. The addition of phosphate increased the rate (Zaidi, *et al.* 1988).

Seto and Alexander (1985) investigated the effect of concentration of ^{14}C-glucose on the yield of cells in a synthetic medium inoculated with bacteria derived from sewage, doubly filtered to remove protozoa. The yield coefficient in the early stationary phase when glucose had gone was approximately 0.48 on a C basis and did not vary with glucose concentration from 43 ng C/l to 100 mg C/l nor with initial bacterial densities of 0.4 μg to 40 mg cell carbon/l. About 44% C was converted to CO$_2$ and 7.4% excreted as organic products.

To sum up, in conventional tests the concentration of test chemical should be chosen to be as low as possible consistent with the detection limits of the analytical methods (DOC, CO_2 oxygen uptake). A guide for inhibiting chemicals is that the initial concentration should be at about 10% of the EC_{50} value (to activated sludges) for values over about 300 mg/l. For more inhibitory compounds a range of initial concentrations should be tested. The complex situation in natural waters regarding the effect of concentration of the test chemical cannot be stated simply or easily taken into account in tests simulating rivers, lakes, etc. Conditions would have to be applied in any simulation test to suit the purposes in hand (Sections A.9, C.5.2) and the phenomena outlined here should be considered when interpreting the results obtained.

A.6   Duration of tests: 10-day window

Before the OECD methods had been agreed, the durations of the tests were 14d (MITI), 19d (Mod. OECD), 28d plus 14d acclimation (Sturm), 30d (Closed Bottle) and 42d (AFNOR); there were no "window" limitations. The OECD Group standardised the duration at 28d as a result of discussions on the two ring tests. This decision was partly based on experience and partly arbitrary, just as were decisions on other aspects such as density of inoculum. But it was clear that these tests were not to be taken as simulating any part of the aquatic environment contrary to the views of Gerike and Fischer (1979). The conditions were deliberately chosen to be stringent – more so than in most parts of the environment and it was decided that, although there are differences in the degree of stringency between the tests, chemicals reaching agreed levels of removal could be assumed to be rapidly and completely biodegraded in a wide variety of natural aerobic environments.

No differences were to be made in conclusions to be drawn from "passes" between the five screening tests – all chemicals passing any of the tests were readily biodegradable. The tests are not simulation tests, but tests for potential to biodegrade, so that using data from them to assess the performance of a substance within a particular route to the environment would be a misuse of the data and an unwarranted extrapolation. For example, because a test used activated sludge as an inoculum it was not to be taken as a simulation of waste water treatment. The data are currently used to state that chemicals passing the tests do not offer a serious challenge to the metabolic capability of aerobic environments (given the presence of bacteria, nutrients, etc.) and that they would be readily degraded in the real environment. Then, based on the quantities produced, routes of entry to the environment and its ecotoxicity, they would require only limited or no further study as far as biodegradability is concerned. The object of the test harmonization programmes has been to bring together the environmental parameters of the test system (media, inocula, incubation conditions) to avoid as far as possible anomalies between the test systems.

Similarly, the 10d window was inserted within the 28d period in an arbitrary manner. The 28d duration was instituted to encourage adaptation to occur within the test period, but no reason was given for 10d. It is true that in the original OECD method for assessing the biodegradability of anionic surfactants (OECD, 1970) the primary degradation of the 'soft" standard normally took 7 to 10d, although 14d was allowed in the test period of 19d; all times were measured from the beginning of the test. It is known that mineralization of LAS or Marlon A takes longer than its primary degradation and there is a very large body of evidence to show that such surfactants are efficiently mineralized in the environment.

The AFNOR T73-260 (1971) test for assessing primary biodegradability of surfactants requires 10d, but the cell density was very much higher (10^5-10^6 cells/mm^3 or 10^8-10^9/ml) than in the OECD test (10^2-10^3/ml).

In their updated review of biodegradability tests, Howard, *et al.* (1981) make no reference to a 10d window, although they refer to the "1980" OECD report which is probably the same as that marked "Draft" published in December 1979 (OECD, 1979). Also, in a recent book on biodegradability, Pitter and Chudoba (1990) make no reference to the 10d window, although all the OECD tests are discussed.

Few, if any, references have been seen in published work in the open literature on the 10d window, though much data have been accumulated in testing laboratories and by competent authorities, which have not been published even in anonymous form. At a workshop in Munich (Rohleder, *et al.* 1983) no consensus was found on the topic. One view was that the 10d window would exclude results from degradation curves which do not reflect "normal" kinetics (de Kreuk). Indeed, this was probably the reason that the 10d window was originally introduced (Hanstveit, pers. comm.) De Kreuk has suggested that if removal was non-biological it would take place at a more or less constant rate throughout the test. Thus a slow abiotic removal over 28d could reach the pass level and the chemical would be classified as readily biodegradable. By using the 10d-window, he suggested that such false results would be eliminated. An opposite view was that the degradation curve supplied sufficient information for a complete and sensible assessment; in any case, abiotic controls would have shown that the removal was not biological. Another opinion expressed, not incompatible with the latter view, was that the window concept rendered very stringent tests even more and unnecessarily stringent and should therefore be deleted (Gerike). In their review, ECETOC (Blok, *et al.* 1985) did not appear to consider the 10d window and the draft revision of the tests by the OECD (1991) omitted the 10d rule.

When these revised test methods were circulated for comments, a number of objections were made to the deletion of the 10d window on the grounds that it made the tests insufficiently stringent; this view highlighted differences in the understanding of the objectives behind the scheme of testing (see earlier in this section). However, at a meeting called by the OECD in Copenhagen (Dambourg and Lindegaard-Jorgensen 1990) data collected on the effect of the 10d window on biodegradation results suggested that only 47% of 302 test results on 72 chemicals were above the pass level in the shorter period, while 52% required the longer period of 28d. The meeting decided, on this evidence, to agree to reinstate the 10d time frame, but for technical reasons to lengthen it to 14d in the Closed Bottle method.

Unfortunately, the two largest contributors to the data suspected that there were errors in their data. Also, it was thought that the use of the number of test results rather than number of chemicals biased the proportion in favour of chemicals which did not pass in 10d since there was a tendency for them to be tested more frequently. One contributor, Water Research Centre, UK, (Painter, in letter to Lindegaard-Jorgensen, 1990) had indeed presented incorrect data by virtue of a series of misunderstandings. It was found that of 42 chemicals which achieved more than 70% DOC removal two failed the 10d window that is, 4.8%. If the total number of test results on the 42 chemicals are used, 146 passed at 28d of which 17 did not meet the window limit, that is 11.6%. On the other hand, the other contributor, Shell Research, confirmed their original data (Battersby, pers. comm.). In the Closed Bottle test four out of twelve chemicals (33%) failed the 10d window limit while in the modified Sturm test as many as 50% of 22 chemicals failed. Many of the eleven chemicals "failing" the 10d window had achieved >50% ThCO$_2$ and all eleven had reached >40% by ten days. Also, doubt has been

thrown on the rate of collection of CO_2 (as opposed to the rate of production); at pH 7.4 CO_2 tends to stay in solution, thus giving falsely low values of % $ThCO_2$ (see section A.7.4).

A random choice of 25 chemicals from the large MITI data-base showed five (20%) which did not reach the 60% ThOD level within the 10d window (Takatsaki pers. comm.). A similar proportion (24%) was reported by Douglas (Dambourg and Lindgaard Jorgensen, 1990) of 21 chemicals (out of 106) which reached >60% ThOD in the Closed Bottle test in 28d but not in the 10d window.

In the second ring test of the EU Respirometer method (CEC, 1985), the % ThOD values for both (t_L+10)d and 28d were given for 14 chemicals tested by between nine and 25 laboratories $(t_L=$lag period). It is difficult to express the differences between passes under these two conditions, since though all chemicals were 'passed" by some laboratories not all chemicals were 'passed" by the same number of laboratories; this confounds any difference between results at (t_L+10)d and 28d. Additionally, there is evidence that 60% ThOD is an unnecessarily high value; 50% or even 40% ThOD has been shown sometimes to be equivalent to 80-100% DOC removal. The chosen method was to express the increase in the number of laboratories passing each chemical (>60% ThOD) at 28d over the number passing at (t_L+10)d as a percentage of the number passing at 28d. The values for the 14 chemicals ranged from 0 to 50% with a mean of 13% (median 12%).

Nyholm (1991) repeated his earlier suggestion that the 10d window should be substituted by the t_{50}, which he defined as the time taken to reach 50% removal of DOC from the time 10% removal was reached. A pass limit, if needed, for ready biodegradability may be on t_{50}, such as 10d. In respirometric tests t_{50} would be 50% of the final % degradation of ThOD or $ThCO_2$. However, the value of t_{50} would be dependant to some extent on the initial concentration of the test chemical used; higher concentrations would require a longer time to achieve 50% removal, so that the initial concentration would have to be fixed for comparative purposes.

Two studies of the kinetics of bacterial growth in ready biodegradation tests (Painter and King 1983; Blok and Booy 1984) showed that after making 'fail-safe" assumptions, all five methods would pass in 28d, chemicals degraded by bacteria with specific growth rates (μ) greater than $0.6d^{-1}$ and all would fail those chemicals for which $\mu<0.15d^{-1}$. Chemicals degraded by species with μ between 0.15 and $0.6d^{-1}$ would be degraded only in tests with higher cell densities. If the 10d rule applied, the models predicted only a slight effect in the low cell density methods and a raising of the threshold in other tests from about 0.15 to $0.25d^{-1}$.

A view which has to be considered is that it is not only the scientific aspect of the duration and 10-d window which is important. Equally vital are the application of the tests, their use in test strategies and the interpretation of results in the various systems of chemical hazard assessment (discussed further in section E). Much data and consequent decisions, made from those data, on many chemicals have been accumulated over the past decade and obviously changes will not be accepted unless the evidence is strong.

To summarize, present knowledge in not adequate to enable a firm decision to be made on this problem though the reviewer thinks that the 10d-window is unnecessary. The data required are results from tests which simulate rivers and lakes and comparing them with those from ready tests within the 10d window and also with those after 28d. Ultimately, monitoring of the environment for the presence/concentration of chemicals is the only way to prove whether the assessment has been correct.

A.6.1 A problem

Since 100% removal is possible for DOC but not for CO_2 production, because some carbon is diverted to cell growth, the biodegradation curve for DOC would be expected to be steeper than that for CO_2 production. Thus, for this and other reasons the time taken to reach 70% DOC removal could be different from the time needed to reach 60% $ThCO_2$. Birch (Unilever, pers. comm.) has found this to be so for benzoate and 1,6-hexanediol (and probably others). When DOC removal and CO_2 production were measured simultaneously for benzoate, the time window for DOC was 0.9d and for $ThCO_2$ it was 1.2d. Taking his detailed data for 1,6-hexanediol from the 1988 OECD ring test, he found DOC removal took 3.0d while CO_2 removal required as long as 11.7d when fresh sludge inoculum was used; with pre-conditioned inoculum the times were 2.2 and 7.2d respectively. (It is possible that these observations are connected with the phenomenon reported by Weytjens (pers. comm.) discussed in section A.7.4). Thus, the situation arises in which one method passes a chemical and another method does not; this anomaly must be resolved.

A.7   Analysis

A.7.1 Sampling

Just sufficient volumes of the reaction mixtures should be taken as samples for analysis (DOC, COD, test chemical) from DOC die-away test flasks. Care should be taken to remove any "rim" formed at the liquid/air interface before sampling. The samples are filtered or, less usually, centrifuged before analysis or storage. To obtain a good representation of the biodegradation curve, frequent samples should be taken and preserved (A.7.2) and judicious choice of preserved samples for analysis can lead to the identification of the 10d-window with a minimum of analytical effort. By appropriate modification of the apparatus, samples for DOC analysis may be withdrawn from Sturm-test vessels (Ruffo, *et al.*, 1984; Weytjens pers. comm) and respirometers.

The favoured method of determining CO_2 in the Sturm test is to collect the gas in NaOH and to take small samples of the absorbent solution for direct injection into a carbon analyzer.

A.7.2 Sterilization of controls; preservation of samples

Abiotic degradation can be followed by sterilizing a solution of the test chemical in the uninoculated mineral medium. Traditionally, mercury (II) chloride has been used but substitutes are being sought because of its effect on the environment. The use of mercury (II) chloride can give misleading results; for example, Pagga (1982) found a "loss" of about 50% DOC, tested at time zero, from three amines-p-anisidine, p-phenetidine and o-toluidine. The loss could have been due to precipitation of the amines perhaps with too high a concentration of mercury (II) chloride. The same author also reported that 0.01 and 0.1ml/100 ml medium of a "concentrated" solution of sodium hypochlorite added to an uninoculated medium prevented the removal of 4-nitrophenol over 28d; in the absence of the inhibitor 95% DOC was removed.

Alternatively, sterilization of the media components (and flasks) could be achieved by autoclaving or by sterile filtration.

Ogawa, *et al.* (1981) compared a number of methods for preserving ground water samples containing hydrocarbons at about 100 μg/l. Simple chilling to 4°C was inefficient, all the carbon being removed within 12d. The addition of 1ml N sodium hypochlorite per litre at ambient temperature was not much better since the carbon was completely removed in 18d. Surprisingly, as much as 30% of the carbon was lost in one week and 70% in three weeks in the presence of mercury (II) chloride (of unstated concentration) at ambient temperature. The corresponding losses in the presence of unstated concentrations of silver sulfate were as low as 5 and 20%. However, preservation was highest in samples filtered through 0.45μm silver membranes with only 5% lost after 36d.

In order to study the survival of added bacteria, filtration through 0.2μm nylon membranes was used to prepare sterile lake water (Klein and Alexander, 1986). Filtration through Nuclepore membranes was also used by Servais, *et al.* (1989) to assess biodegradable DOC in environmental samples. Sub-samples were sterilized prior to analysis by the addition of sodium azide to give a final concentration of 0.05% (500 mg/l).

A.7.3 Dissolved Organic Carbon (DOC)

It is obviously important that in the determination of DOC any inorganic carbon present is removed by acidification and purging; this is done either outside or within the analyser. In the latter case the concentration of DIC (Dissolved Inorganic Carbon) is sometimes recorded. Removal of DIC is particularly important in analysing environmental samples, which in hard water areas can contain as much as 40 mg DIC/l.

Another practical point of importance is the method of preparation of samples for DOC analysis. Any method using filtration should be tested for gain of DOC by leaching of organic matter from the membrane and loss by adsorption. Poly-carbonate membranes of 0.2μm pore size have been found to be acceptable.

The reproducibility of the modified OECD screening test was examined by Gerike and Richterich (1983), using three chemicals and two carbon analysers - one a UV type, the other a furnace type. The chemicals were tested in six replicates on six occasions and they obtained similar values with the two analysers. LAS was removed by 71.2 and 71.1% at 5 mg C/l and by 63.6 and 60% at 10 mg/l after 21d. The relative standard deviations (RSD) were in the range 5 to 10%. Similar results were obtained with the readily biodegradable hydroquinone but the third chemical, tetrapropylene benzene sulfonate, was poorly degraded with much higher RSDs.

A note of caution was sounded by Lockwood (1985), who reported on three carbon analysers; two were of the furnace type made by one manufacturer and the third a UV-persulfate instrument made by a second manufacturer. The furnace instruments behaved erratically while the UV analyser was stable and reliable. The furnace analysers gave variable results from day to day, even though they were calibrated daily against potassium hydrogen phthalate; for example a sterile solution of N-methylaniline (NMA) of nominal concentration of 10 mg C/l gave values between 10.7 and 20.3 mg C/l over a 15-day period. Neither did these analysers give accurate values; for example a solution of NMA of 27 mg C/l gave 39.7 mg/l and 35.4 mg/l by the furnace analysers and 26.8 mg/l by the UV analyser. Also, some volatile

chemicals were not fully recovered by the furnace instruments, only 38% being recorded for N-methylpyrrole. After adjustments were made to the two analysers by the manufacturer three chemicals (aniline, t-butanol, sec-butylamine) were analysed on four successive days by all three analysers. Good recoveries - 90 to 95% were obtained by the UV instrument, but the recoveries obtained with the furnace analysers were 71-78% (aniline) and 30-52% for the other chemicals. The RSD was as low as 1% for the former analyser but as high as 10-43% for the others. The furnace analysers always gave higher values for distilled-deionised water than did the UV analyser (1.5-3.3 mg/l compared with 0.3-0.6 mg/l) and also for environmental samples.

To what extent these furnace instruments were representative of their type is not known, although the experience of Gerike and Richterich (1983) suggests that they were not representative.

It is the practice to subtract the DOC value of controls, containing inoculum but no test chemical, taken at each sampling time from the DOC value for the flasks containing the added test chemical. This is done on the assumption that events in the bacterial cells would be the same in the control as when substrate was present. However, this is known not to be true and in the control cells lyse in the absence of external substrate thus increasing the concentration of DOC during incubation. In the presence of substrate less lysis occurs so that the subtraction of control DOC from the test-flask DOC does not give a true reflection of the amount of substrate remaining. Indeed, towards the end of the incubation, negative net values for DOC are sometimes obtained.

A.7.4 Carbon dioxide

Improvements to the Sturm method have been made by Oudot (1979) and by Ruffo, *et al.* (1984). Oudot added a U-tube containing CO_2 absorbent at the end of the CO_2 absorption train to prevent CO_2 from the atmosphere getting into the solution of barium hydroxide. Ruffo, *et al.* (1984) circulated the gas in the system using a pump to pass exit gas from the single absorber back to the aeration vessel. This reduced the amount of extraneous CO_2 picked up from traces of CO_2 still present in the "CO_2-free" air normally used. They also introduced a side-port on to the aeration vessel to facilitate the taking of samples of the medium, but unfortunately did not report any results of DOC and DIC analysis, which might have thrown light on the problem of CO_2 retention in the medium (see later in this section).

Blum, *et al.* (1983) compared the rate of collection of CO_2 into the absorbers in six types of flasks, including 250ml modified soil biometer flasks and 1l- and 2l-Gledhill flasks. Using $Na_2^{14}CO_3$ acidified when the flasks were sealed, they showed that in the short term (6-24h), CO_2 in the small flasks reached the absorbers more quickly than in the Gledhill flasks; for example, in the soil biometer flasks 88% $ThCO_2$ was collected in 6h and 92% in 24h compared with 58% and 79% in the Gledhill flasks. But in biodegradation tests the Gledhill flasks recorded higher proportions of $ThCO_2$, in 21d, for glucose (88%) and LAS (69%) than in the soil biometer flasks - 66% and 18% respectively. However, this may have been due to the use of 100 mg/l of test chemical in the smaller flasks compared with only 10 mg/l in the larger vessels. This was necessary in order to have sufficient CO_2 in the absorbers to be measured by the titration method.

Using sodium benzoate and hexadecane (insoluble), Fogel, *et al.* (1985) also found higher proportions collected in Gledhill flasks than in the 250ml biometer vessels. Again, the

same weight of test chemical (10 mg) was added to each flask, giving concentrations of 20 mg/l in the large flasks and 200 mg/l in the smaller flasks. The yields of CO_2 after 21d were 82+/-2% $ThCO_2$ and 67+/-2% $ThCO_2$, respectively, although after 7d 55% $ThCO_2$ was collected in both types of flask. The authors thought that the difference was due to greater agitation in the larger flasks and recommended the use of flasks with baffles.

In section A.6.1 it was reported that Birch (1990) had evidence for a higher rate of removal of DOC than of collection of CO_2 in the conventional Sturm test. For some chemicals this could mean that they "pass" the 10d window on DOC removal results but not on CO_2 collection; for example, 1,6-hexanediol needed only 3d for removal of 70% DOC but as long as 11.7d for collection of 60% $ThCO_2$. Other evidence (Weytjens, pers. comm.) suggests a possible explanation for this phenomenon, but further data need to be gathered before it can be fully accepted. Weytjens determined the DOC removal of a number of chemicals in shake flasks under normal conditions with 30 mg activated sludge/l as inoculum, but he also determined the concentration of DIC at the same time and on the same samples. The results (**Table A.7.1**) show that in each case DIC was absent initially but increased as DOC was removed reaching a peak value (6 to 8 mg/l) when DOC removal attained the plateau value (>90%). After the peak DIC value had been reached it took several days for the value to reach zero. For aniline, 1,6-hexanediol and monoethanolamine the average rate of removal of DIC was 0.7 to 1.0 mg/l per d; for pentaerythritol it was lower at about 0.3 mg/l per d.

Further tests were made with the conventional Sturm test apparatus modified so that samples of medium could be taken for DOC/DIC analysis without loss or gain of CO_2 to or from the atmosphere (Weytjens, pers. comm). Air free from CO_2 was passed through the vessel at 50-100 ml/min, measured by means of a rotameter and the vessel was stirred, using a magnetic stirrer. The exit gas was passed through 0.05N NaOH and the concentration of DIC was determined by both titration and by carbon analyser (**Figure A.7.1**). DOC and DIC were determined in samples of medium taken from the stirred vessel (**Figure A.7.2**). These latter samples (aniline and an un-named degradable chemical) showed the same results as given in Table A.7.1, namely that when the DOC removal plateau was reached (at 3d), the DIC content had reached a peak of about 8 mg/l. But at this time only 10-15% of the total C added (20 mg/l) was detected in the absorbers determined by both titration and carbon analyser. The "pass" level of 60% $ThCO_2$ was not reached until about 8d in the case of aniline. However, if the DIC (8 mg/l) still present in solution at 3d is added to the DIC collected in the absorber after 3d, the % $ThCO_2$ would increase from 10-15 to 50-55. The plateau of DOC removal of the un-named chemical was reached after 10d, having begun at 4d. DIC peaked at 7 mg C/l on day 8, while the percentage $ThCO_2$ did not attain the pass level until day 18, having started on day 7.

In the history of the Sturm test (Sturm, 1973; Larson, 1979), nothing seems to have been said about CO_2 in solution and the rate at which it escaped to the absorbent, except that on the last day of incubation acid was added, to pH3, and aeration was continued for a further 24h. Little, if any, CO_2 resulted from this acidification. The rate of bubbling CO_2- free air was not quantified, except that it was regulated so that the "scrubbing capacity of the NaOH and barium hydroxide scrubbers was not exceeded". Sturm (1973) used static aeration vessels but Larson (1979) continuously agitated his flasks at 120 rpm on a rotary shaker but passed the CO_2- free air through the headspace; both the OECD and EU versions of the test stipulate static vessels.

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**Table A.7.1   DIC concentrations during DOC die-away tests
(initial DOC concentration = 20 mg/l)**

Chemical	Concentration of DIC (mg/l) in medium after (d)								
	0	3	4	7	10	11	14	21	28
1,6-hexanediol	0	1.5 (3)	-	7.8*	5.5	-	2.0	-	-
monoethanolamine	0	&*	-	5	0	-	-	-	-
pentaerythritol	0	-	-	0.5 (10)	-	2 (30)	6*	4	2
aniline	0	-	8*	6	-	2	1	-	-

* DOC removal reached plateau (>90%) Values in brackets are % DOC removal.

The problem is not confined to the Sturm test. Larson and Games (1981) reported that less than 10% of total $^{14}CO_2$ production was recovered in external traps when ^{14}C-labelled chemicals were tested in water from the River Ohio in closed shake flasks. The carbonate-buffering capacity of the river water was given as the cause. Similar results were reported by Larson and Payne (1981) and Jensen k.*et al.* (1988). In other studies the collection and estimation of $^{14}CO_2$ is either not clearly explained or is suspect.

There is little doubt that the effect is due to a physical process, namely a slow release of CO_2 from solution at pH 7.2-7.4. There seems to be no physiological process which delays the production of CO_2 in the period of growth when DOC is being assimilated. In any event the delay is not in the production of CO_2 but in its release from solution - the concentration of DIC peaks at 6-8 mg/l. After the production of 40-60% $ThCO_2$, resulting from the need to produce energy from the substrate to synthesize new cells, over a period in which DOC is completely removed, there is a slower production of CO_2 caused by endogenous metabolism of the cell contents. This metabolism continues until internal polymers, carbohydrates, proteins have been transformed resulting ultimately in cell death.

It is urgent that this matter be investigated and clarified not only because it affects the 10-day window problem, but also since it may influence the determination of kinetic constants of ultimate degradation reactions, such as those reported by Larson (1979), Gledhill (1975b) and Carson, *et al.* (1990).

Figure A.7.1 Aniline — Sturm Test
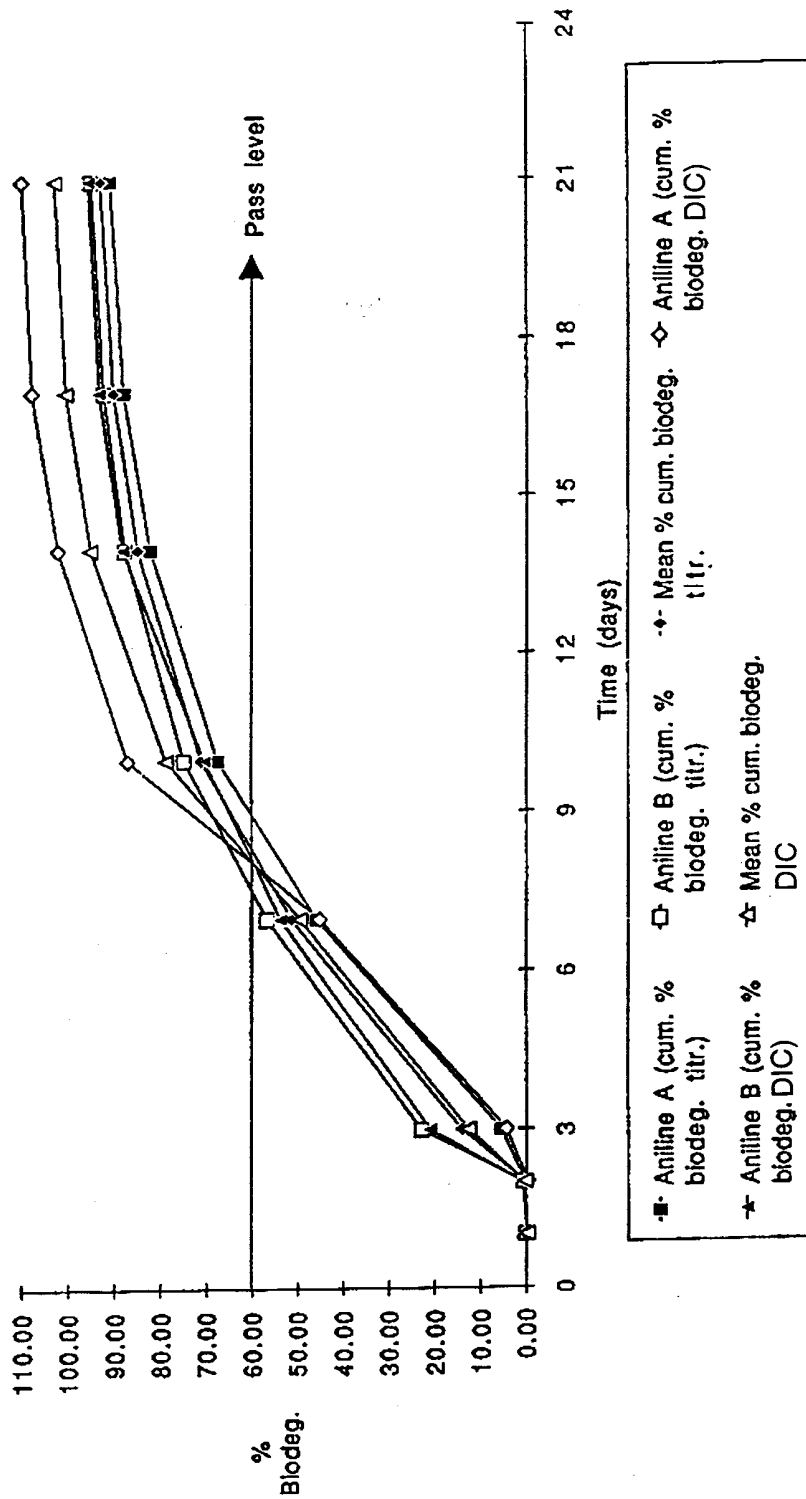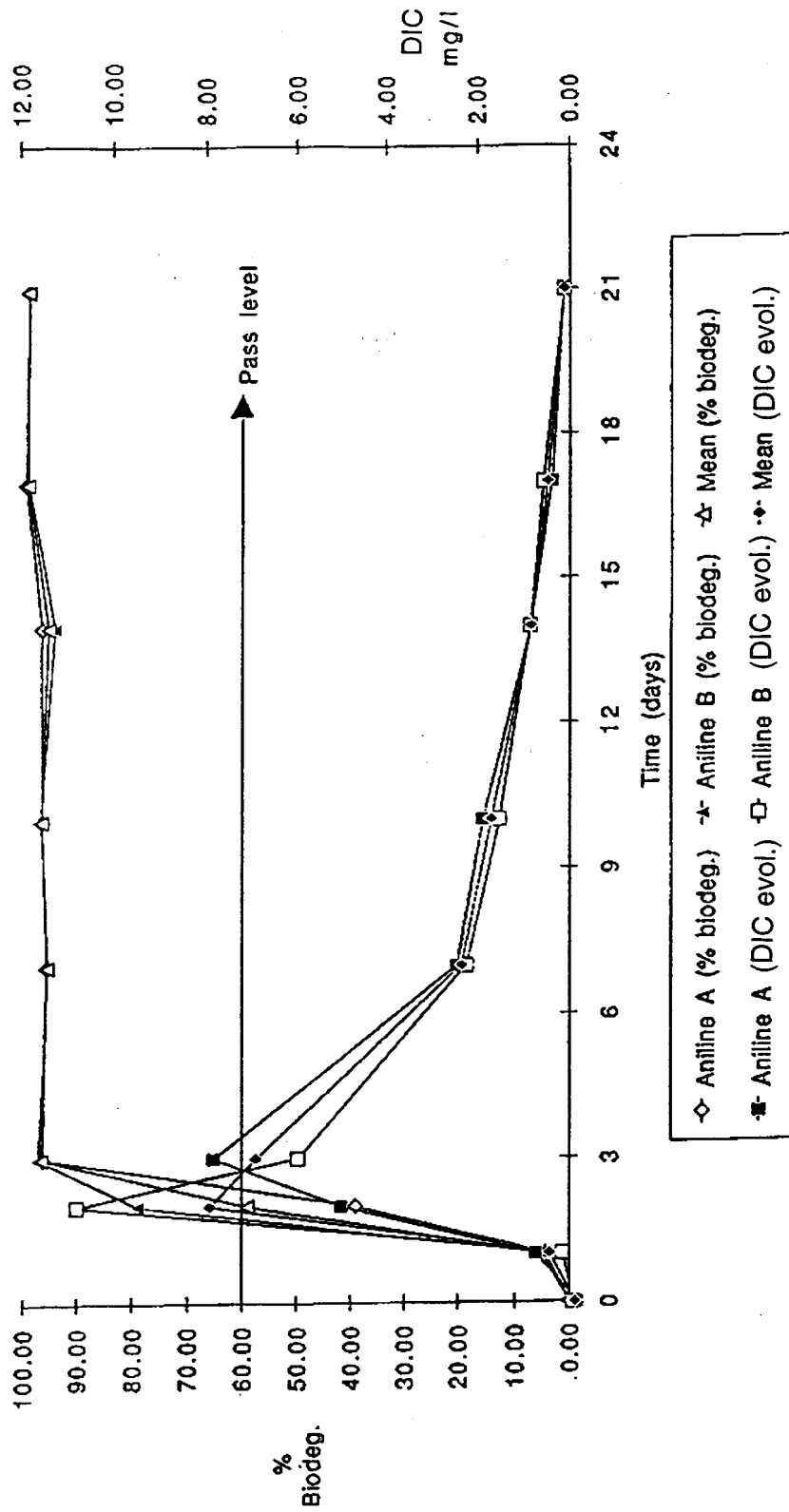Biodegradation (results from titration and DIC analysis of absorbers)

Figure A.7.2 Aniline — Sturm test
Biodegradation and inorganic carbon evolution

Apart from this newly raised doubt as to whether the rate of CO_2 collection in the absorbent is a true reflection of the microbial rate of formation, the Sturm test has been criticised on other counts, including its cumbersomeness and inability to assess volatile chemicals. Further, Struijs (1991) has pointed out that since loss by volatilization is largely determined by Henry's law constant, estimated from the vapour pressure/solubility ratio, loss by volatilization cannot be completely ruled out for sparingly soluble chemicals of low vapour pressure. For these reasons other methods have been considered. Boatman, *et al.* (1986) rejected the Gledhill test because it requires specialized glassware and the removal of CO_2 and titration of the absorbent is time-consuming. They devised a GC procedure to analyze the CO_2 produced in 20ml vials, designed for the automatic headspace chromatograph and containing 10ml of inoculated mineral medium plus test chemical. At intervals vessels are acidified and equilibrated for 30 min before analysis for CO_2. There are two disadvantages of the system relating to its size (10 ml medium); the introduction of insoluble chemicals would be difficult and such a small volume could result in low values especially for chemicals which degrade only after a lag period, presumably due to the low absolute number of potentially competent micro-organisms. This point was illustrated by Struijs (1991) who assessed the biodegradability of pentaerythritol (PE) in die-away tests using a single batch of inoculated medium (11.5 mg sludge solids/l) containing 20 mg PE/l. Different sized volumes of the inoculated medium – from 900 ml down to 20 ml – were incubated and it was found that this reduction in volume increased the lag period from ten to 22 days.

Two groups have independently improved the technique of Boatman, *et al.* (1986) by applying the technique used in assessments of anaerobic biodegradability. Struijs and Stoltenkamp (1990) introduced normal serum bottles (120 ml) using conditions similar to those in other ready biodegradability tests and concentration of test chemical of around 10 mg C/l. At timed intervals the contents of triplicate bottles were acidified to pH < 3 and equilibrated by shaking for 1h. Gas samples were taken from the headspace by syringe and analysed for CO_2 in a carbon analyser. The authors demonstrated that the procedure is a successful alternative to the Sturm method, since it is suitable for testing insoluble chemicals and most volatile chemicals.

Birch and Fletcher (1991) used a similar, but slightly larger, apparatus and other conditions as used by Struijs and Stoltenkamp (1990). However, the bottles sacrificed at timed intervals were not acidified; instead, they measured CO_2 in headspace samples by carbon analysers and in addition they determined DIC in the medium immediately after breaking the bottle's seal. The carbon produced as CO_2 was thus taken as the sum of the gaseous CO_2-C and DIC. They also showed that insoluble and volatile chemicals could be assessed with good precision and reproducibility. As inocula, these authors used sewage effluent at 0.5-10% (V/V) of final medium, recommending 5-10%. Struijs and Stoltenkamp (1990) favoured the use of activated sludge at 4-30 mg/l, although later they recommended a maximum of about 16 mg/l (see A.7.5).

In the above three modifications the vessels were enclosed and shaken throughout the test so that the contents were more agitated than in the conventional Sturm test. Nevertheless, it would be instructive to know how the CO_2 was distributed between the two phases; unfortunately, the paper by Birch and Fletcher does not reveal this.

A.7.5 Oxygen uptake

It is worth remembering that some reactions can occur in which results of oxygen uptake can be misleading. For example, the oxidation of ethanol to acetic acid uses up oxygen but no carbon is removed, while decarboxylation reactions cause the loss of carbon without the uptake of oxygen.

The variability of the temperature in BOD/Closed Bottle test incubators has been investigated by Gray (1989). He found that only three out of 13 incubators (made by five manufacturers) met reasonable standards. Variations in temperature in the incubators over time ranged from 0.3 to 3°C at 20°C. The temperature in BOD bottles in an incubator remained reasonably constant (0.2 to 0.6°C) over 24h but the mean bottle temperature varied from 18.7 to 20.9°C depending on the position of the bottle within the incubator. Door-opening and bottle density were also important factors in temperature variation. BOD values varied by +/-10%.

Struijs (1991) has calculated the maximum concentrations of activated sludge which can be safely used in the Closed Bottle, 2-phase Closed Bottle and the Sturm tests. Among the criteria used were that the oxygen uptake by the control (endogenous) should not exceed 10% of the oxygen available and that the endogenous respiration of the activated sludge used was 0.85 +/- 0.3 mg oxygen/mg dry weight. This was the value for sludge used in his laboratory over a number of years. For the Closed Bottle test the maximum inoculum was calculated to be 1.1 mg solids/l and for the 2-phase test with a liquid to gaseous phase ratio of two to one the maximum was 16 mg/l. For the headspace CO_2 test the value was also 16 mg/l. The prescribed maximum in the 2-phase Closed Bottle test is 30 mg/l so it is not surprising that this concentration has often caused too high a blank oxygen uptake and an adverse effect on the accuracy and precision of the test. Struijs (1991) suggests that it is better to use a maximum of 15 mg/l rather than preconditioning 30 mg/l inocula to the mineral medium. He rejected pre-conditioning because it is more complicated, lengthens the test and nitrification is more likely to occur.

A technical point in the draft ISO 2-phase Closed Bottle method (1990a) for insoluble chemicals concerns the oxygen electrode; it is important that emulsions and oily suspensions are shown not to affect the calibration of the electrode and that only insignificant amounts of test chemical are removed from the bottle by adhering to the electrode.

A.7.6 Chemical Oxygen Demand (COD)

When a test chemical/material, e.g. oils, is not pure enough for its ThOD to be calculated from its formula, resort has to be made to determination of its COD in order to assess biodegradability. A number of chemicals, such as hydrocarbons (benzene, toluene), methylamine, pyridine, are known not to be completely oxidized by acid dichromate in the standard 2h test. Difficulty has also been encountered with insoluble compounds. Because experimental COD values may be lower than the corresponding ThOD, it must be borne in mind that estimates of biodegradability based on % COD are likely to be falsely high.

Gerike (1984) called attention to the enclosed, modified Kelkenberg method with higher (0.25N) concentration of dichromate and stronger sulfuric acid than in standard methods. This method gave higher recoveries than standard methods, for example, diphenyl 100%, 62% ThOD; octadecane 94%, 6% ThOD. Results of pyridine and methylamine by this

method was not reported. The Kelkenberg modification is referred to in the updated OECD guidelines(1991).

Chudoba and Dalesicky (1973), using a standard method, showed that of many heterocyclic N compounds only pyridine and its derivatives were not completely oxidized. Later, Chudoba and Zeis (1975) determined the first order rate constant (k_1) for the dichromate oxidation of a number of chemicals. Compounds with k_1 values greater than 5×10^{-1} gave >85% ThOD in 2h. Ethylamine ($k_1 = 2.5 \times 10^{-1}$) gave only 36% in 2h but after 7h almost 100% had been achieved. Similarly, other chemicals were oxidized satisfactorily after longer periods than 2h. Perhaps a combination of a longer period and the Kelkenberg modification should be applied in doubtful cases.

A.8   Miscellaneous aspects

A.8.1 Adsorption

Adsorption has not been widely studied except in simulations of the activated sludge process. In tests for ready biodegradability adsorption can be a problem in DOC die-away tests and would be indicated if less than the expected DOC concentration was present in samples taken within a few hours after the beginning of the test. If the concentration stayed at a more or less constant value throughout, this would indicate that adsorption was the only mechanism. If the test chemical is suspected of being significantly adsorbed onto glass, sludge, etc. it is advisable to make a separate, preliminary assessment to determine the likely extent of adsorption and then to ascertain the suitability of the DOC die-away test for that chemical. If the DOC die-away test is applied, an extra flask should be set up containing the test chemical, inoculum and a sterilizing agent which should not change the adsorptive properties of the suspended solids.

Mackrell and Walker (1978) grew bacterial isolates on octanoic acid and cetyltrimethylammonium bromide (CTAB) and the washed cells were suspended in 2.8mM CTAB. Analysis showed a reduction to 1.1mM, a 60% reduction. Lyons, *et al.* (1984), studying the mechanism of elimination of aniline in pond water, reported only small amounts (about 1%) the chemical being bound to activated sludge added to the water. The amount adsorbed did not change over 7d; a distillation method showed that the aniline was bound strongly to the sludge.

In the activated sludge process, Dohanyos, *et al.* (1978) showed that 20 dyes were removed by adsorption processes which could be characterised by the Freundlich isotherm equation. Wierich and Gerike (1981) formulated a mathematical model to describe the fate of soluble chemicals which were non-degradable and strongly adsorbing onto sludge. Wierich (1985) later refined the model to make it easier to apply. The model calculates the retention time of the chemical in the system and indicates that a compound having a high adsorption constant, determined in a batch test, is not eliminated to the same extent in the activated sludge process at equilibrium. For example, under the conditions of the Husmann test a chemical with an adsorption constant of 98% is eliminated to the extent of 40%. Mass balances are carried out in simulation tests to determine the fate of chemicals. For example, Janicke and Hilge (1980) showed that for 3,4- and 3,5-dichloroaniline only 15-20% was eliminated; 10-15% was due to abiotic processes mostly by adsorption onto the plastic material of the apparatus. Similarly, Gerike, *et al.* (1978) found only very small proportions

(2-3%) adsorbed on sludge in one test but none in three other tests with dimethyldistearylammonium chloride.

Topping (1987) reported that although about one third of the 1,4-dichlorobenzene added to raw sewage was present in settled sludge, very little, if any, was found in the waste activated sludge from a porous pot unit treating the settled sewage.

A.8.2 Inhibition

If a chemical fails to be degraded in a screening test, it may be because the chemical inhibits the micro-organisms; there is the facility in most tests to set up extra vessels containing both the test chemical and the reference chemical to assess inhibition. Sometimes inhibition can be demonstrated by using a number of concentrations of the test chemical; for example, Urano and Saito (1985) reported that in the MITI test degradation of LAS took place at 3 and 10 mg/l but not at 30 mg/l; at 100 mg/l the oxygen uptake was less than that of the control.

There have been no major developments on assessing inhibition within the biodegradability tests. However, Lindgaard-Jorgensen and Riemann (1989) and Struijs (1991) have warned that only the MITI and other automatic respirometric methods, in which continuous records are made, are able to demonstrate inhibitory effects which may occur in the initial stages of incubation. Recovery of an inhibited population able to degrade reference chemicals, such as acetate and benzoate, can take place fairly rapidly, so that very frequent analyses are needed to observe this and weekly measurements may be insufficient.

Guidance on how to assess the inhibitory properties of a chemical has been given by Reynolds, *et al.* (1987). They examined nine tests and six chemicals and rejected MICROTOX and nitrification inhibition tests as being too sensitive in some cases. The other tests included BOD_5 and Closed Bottle, growth, respiration and repetitive die-away; results of these were compared with those obtained in the five ready biodegradability tests at anticipated non-toxic and toxic concentrations. No test method evaluated consistently forecast toxicity due to the chemicals tested. They concluded that to avoid toxicity, biodegradability tests should be made at 10% of the EC_{50} value so that compounds with EC_{50} value greater than 300 mg/l are unlikely to be toxic in the tests. Compounds with an EC_{50} value less than 20 mg/l may pose problems, so that the Closed Bottle test or the use of ^{14}C-labelling is necessary. Compounds having intermediate EC_{50} values (20 to 300 mg/l) should be evaluated over a range of concentrations.

Several methods to be used independently of the biodegradability assessment have been employed besides the OECD (1984) activated sludge respiration method. This method was examined by Yoshioka, *et al.* (1986) who tested 32 chemicals not only by this method but also against red killifish and the protozoan *Tetrahymena pyriformis*. They concluded that the test was easy to carry out and that reproducibility was good, but thought that its sensitivity was too low compared with the fish and protozoan tests for assessing chemical toxicity in the environment. The author also found that moderate deviations from stated test conditions - duration, aeration rate, temperature, sludge suspended solids, acclimatization to synthetic sewage - did not significantly affect EC_{50} values. A method similar to the OECD test was reported by Pagga (1981), who later used a "continuous" version ("BASF toximeter") linked to an activated sludge unit (Pagga and Gunther, 1981).

Alsop, *et al.* (1980) proposed a growth method with an inoculum of coarsely filtered settled sewage; growth was measured by optical density. The method has been adopted by the UK Department of the Environment (HMSO, 1988). A similar method using pure cultures of *Pseudomonas putida* was reported by Bringmann and Kuhn (1975) and is expected to be adopted soon by ISO (1991).

Among others, Lindgaard-Jorgensen and Riemann (1989) have applied the incorporation into bacterial cells of micro-organisms of 3H-thymidine, using organisms from freshwater, sea water and activated sludge. The test was cheap, simple, easy to perform and gave good reproducibility; it could be performed under conditions which more truly reflect environmental conditions than do the usual biodegradability tests. Eutrophic bacteria were less sensitive than oligotrophic bacteria towards the two chemicals tested, 3,5-dichlorophenol and potassium dichromate.   This could explain, the authors say, the degradation of dichlorophenols at 10-20 mg/l by activated sludge and their non-biodegradation in sea water.

Struijs (1991) favour the use of glucose-glutamic acid mixtures as in the BOD test using homogenized activated sludge inoculum, as described by Larson (1979) and suggest the use of ATP as the parameter to be measured.

Broecker and Zahn (1977) applied a number of batch toxicity tests to 3,5-dichlorophenol and compared the results with those from a continuous activated sludge unit. The tests included oxygen uptake in the Sapromat and oxygen-electrode respirometers, BOD_5 and growth of *P. putida* as well as a few enzyme tests. Most tests gave a toxicity limit (no effect) of 3-5 mg/l, which agreed with the value obtained from the simulation tests; for river water (BOD_5) the toxicity limit was 2 mg/l. EC_{50} values were in the range 12 to 50 mg/l. Pagga (1982) similarly applied some batch tests to 4-nitrophenol and compared the results using the BASF-toximeter in conjunction with a model activated sludge plant. The batch tests gave an EC_{50} value of 160 mg/l compared with 110 mg/l from the toximeter.

King (1984) compared the OECD method with the growth (Alsop, *et al.* 1980) and BOD_5 methods. The author used eight chemicals singly and in various mixtures and found reasonable agreement with one exception; growth and BOD tests were more sensitive than respiration for methylene bis-thiocyanate, namely 1.2 and 0.3 mg/l compared with 13 mg/l respectively. The 15-min MICROTOX, based on the inhibition of light production from a reconstituted culture of a marine bacterium *Photobacterium phosphoreum*, was much more sensitive for all eight chemicals. For three chemicals the MICROTOX values were about two orders of magnitude lower than the OECD values, namely, lauryl sulfate, 1.5 and >500 mg/l; p-benzoquinone, 0.02 and 7 mg/l;2,2[1]-dihydroxy-5,5[1]-dichlorodiphenylmethane, 0.06 and 17 mg/l. There was no evidence of significant synergism or antagonism in any of the tests. King and Painter (1985) added six chemicals, separately, to activated sludge units for three months, and in batch tests, found no evidence of lessening of toxicity, except with pentachlorophenol for which the OECD test value rose from 24 to 134 mg PCP/l.

Rothkopf and Bartha (1984) tested the biodegradability of 60 chemicals (at 500 mg/l) all containing the NH2 group, by measuring the protein content of the culture. Additional flasks, containing 1% glucose as well as the amine, were set up to test the inhibition of the chemicals. About 75% showed at least 10% inhibition, two were 50-60% inhibitory, a half were 25-50% inhibitory and one compound was 90% inhibitory. No consistent correlation was found between these moderate inhibitory effects and the ability of the amines to serve as growth substrate.

The inhibition of pentachlorophenol degradation was assessed in continuous enrichment cultures with the phenol as the sole source of carbon (Klecka and Maier 1985) and was expressed as k_i - the highest concentration at which the specific growth rate is half of the maximum - from the Haldane modification of the Monod equation. The saturation constant (k_s) was 60 μg/l and k_i was 1375 μg/l. The ks value for phenol (Jones, *et al.* 1973) was 1 mg/l and k_i was 110 mg/l.

A.8.3 Nitrification

The oxidation of ammonium to nitrate via nitrite -nitrification - can cause interference in some biodegradability assessments by reducing the pH value, thus inhibiting bacterial action, and more importantly, by taking up oxygen unpredictably in the Closed Bottle and Respirometric methods (e.g. Painter, 1987). It would appear that at least in some countries, the incidence of nitrification in biodegradability tests has increased over the past two or three decades because of improvements in sewage and waste-water treatment. Nitrification in standard BOD_5 tests has long been inhibited by the addition of specific inhibitors, for example, 2-chloro-6-(trichloromethyl)pyridine (TCMP) in the USA and allylthiourea (ATU) in Germany (Wunderlich, 1984) and the UK. While these inhibitors acted efficiently over periods of up to 8 - 10d, they were found not always to be effective over a 28-d period for various reasons - degradation of ATU, inhibition of C oxidation (Richterich and Steber 1989; Painter, 1986; Battersby, *et al.*, 1987). Also, there are technical problems with TCMP which is extremely poorly soluble in water and there are difficulties with ATU in the determination of dissolved oxygen by iodine titration. Other inhibitors also had drawbacks (Richterich and Steber, 1989).

Richterich and Steber (1989) investigated the problem by reducing the concentration of ammonium in the medium or completely removing it and replacing it with nitrate. Reduction from 0.45 to 0.13 mg NH_4-N/l reduced the incidence of significant concentrations of nitrate (>0.05 mg N/l) from 16 out of 16 replicates to 2/15 for sodium benzoate, 4/8 to 0/7 for dodecanol 8EO and 14/14 to 0/15 for dodecyl sulfate. With N-containing chemicals the reduction of incidence of nitrification in the bottles was not so great - from 14/16 to 12/16 for 4-aminobenzoic acid and 10/10 to 4/16 for 4-nitrobenzoic acid. This sporadic behaviour is typical of a reaction brought about by micro-organisms which are relatively slow-growing and which are present in the population in a small minority. Replacement of ammonium by nitrate was also unsuccessful as had been indicated by Blok, *et al.* (1985) although de Waart and van den Most (1986) have reported using nitrate instead of ammonium in the Closed bottle test, but gave no evidence of the effectiveness of the substitution. Nitrification still occurred in 20% of replicates of NH2-compounds (Richterich and Steber, 1989) and nitrite was formed from 4-nitrobenzoic acid in both cases leading to false results. Thus, while interferences due to nitrification (and denitrification) can be reduced by lowering the concentration of ammonium in the medium or by substituting it completely by nitrate, interferences cannot be completely eliminated. The authors showed that by making allowances for the oxygen uptake due to the formation of nitrite and nitrate, determined by analysis, values for % ThOD were obtained which appeared to represent true values.

This correction procedure, which was adopted by Dias and Alexander (1971) and Hammond and Alexander (1972), has now been incorporated into the relevant OECD Guidelines (1991).

A.8.4 "Pass" levels

The "pass" levels for classifying a chemical as readily biodegradable are 80% for specific analysis, 70% DOC and 60% ThOD or $ThCO_2$. These values were established in the original OECD (1979) report on the test guidelines based on practical experience. One factor influencing the decision was that it is well known that in batch cultures events do not mimic accurately events in the environment and as a result less removal takes place in laboratory tests than would occur in sewage treatment, rivers, etc. For example, it was found that 1-10% of the initial DOC of even very readily degradable chemicals remained at the end of incubation in various forms (Pitter and Chudoba, 1990). The values for oxygen uptake and carbon dioxide production are lower than those for specific analysis and DOC because some DOC is utilized for synthesis of new cells and is not oxidized in the process.

However, there is evidence that the value of 60% is unrealistically high and there is a strong case for its reduction. For example, in the two ring tests of the Respirometer method (CEC, 1982, 1985) simultaneous determination of % ThOD and % DOC at 28d showed that when >90% DOC was removed, the % ThOD obtained by a majority of participants for many chemicals was <60%, often 45 to 50%. Similarly, Kuenemann, *et al.* (1990), in examining their data from the Tokyo ring test (OECD, 1988), found that >40% DOC was converted to bacterial cells so that <60% $ThCO_2$ was produced.

It is interesting to note that other workers preferred to classify chemicals in different ways, for example, Pitter (1976) used four classes based on their biodegradability and toxicity and Urano and Kato (1986b) proposed ten classes based on BOD/ThOD obtained from the MITI test over ten days, involving both acclimatised and unacclimatised inocula. Reynolds, *et al.* (1987) classified chemicals into five groups according to the degree of delay before degradation begins and on the extent of degradation. Struijs and Stoltenkamp (1986b) similarly used five classes, introducing the terms "highly biodegradable" for chemicals degrading within 10d and "intermediate in biodegradability" for borderline cases and those giving inconclusive results in ring tests.

Gerike, *et al.* (1984) challenged the value of 70% DOC removal and argued that to obtain clear evidence for "complete" degradation, in simulation as well as in screening tests, consideration should be given to the number of carbon atoms in the molecule under test. Briefly, if the molecule contains four C atoms, more than 75% DOC must be removed to ensure that a recalcitrant compound containing one C atom did not remain. For C_5 the limit would be 80%, and for C_{20} more than 95% DOC would have to be removed. For the formation of recalcitrant compounds containing more than one C atom, the limit if DOC removal would, of course, be lower, for example, for a C_{20} compounds and a C_4 product the limiting value would be 80%. They illustrated the argument with 3-nitrophenol, postulating that a nitro-C_1 compound might be formed. As a result they devised a test for detecting quantitatively, but not the identity of, recalcitrant metabolites based on the activated sludge confirmatory test (coupled-units version). The use of ^{14}C-labelling was considered to be superior but was rejected as being too costly. Briefly, the test chemical was added daily at 5 mg C/l to one vessel and the organic/inorganic nutrients were added, in concentrated form, to both vessels. The effluents were filtered and recycled back to their respective aeration vessels and trans-inoculation was effected by transferring centrifuged sludge, the supernatant being returned to the original vessel. All sampling was carried out sparingly, any excess being returned to the original vessel. Analyses for DOC was made on effluents and % removal was calculated, making any necessary corrections for material transfer. It was emphasized that great care should be taken in carrying out all operations.

Results for three chemicals exemplify the test (Gerike, *et al.* 1984). 3-Nitrophenol was removed by 83+/-11% in a normal simulation test, leaving some doubt about the formation of a recalcitrant metabolite (C_1/C_6 = 16.7%, limit for removal = 83.3%). But in the new test, during the last 22d of a total period of 45d, 101+/-2% DOC was removed; this leaves no doubt as to any recalcitrant residue. Similarly, with benzyldimethyldodecyl-ammonium chloride (C_{21}) 83+/-7% was removed in the normal test, while the "limit" is 20/21x100 = 95.2%; in this test 101+/-2% was removed at 5 mg C/l and 99 +/-5% at 1.2 mg C/l. At the other end of the scale, metanilic acid was not biodegradable (minus 8% in the normal test) and was removed by only 18+/-8%, showing that a false positive did not occur.

Later, Gerike and Jasiak (1984) applied the test to eight surfactants and found values ranging from 88 to 104%. Six of the values, including that for LAS, indicated that recalcitrant metabolites were not formed. However, for nonylphenol ethoxylate (10EO) and a C_{12-14} alcohol ethoxylate (EO_{30}) with values of 94+/-3 and 88+/-2%, respectively, recalcitrant metabolites were probably present.

A.8.5 Metabolites and intermediates

As indicated earlier (A.8.4) pass levels have been agreed for all parameters which are lower than 100% and Gerike, *et al.* (1984) have devised a test for determining whether recalcitrant metabolites were formed. The method does not indicate their identity, but presumably any residual organic carbon could be extracted from the coupled units effluent and be identified by usual chemical techniques.

Of the tests for ready biodegradability only the MITI test requires an investigation into the identity of possible intermediates if the removal of the parent compound is relatively high and the % ThOD is relatively low. As mentioned earlier (A.8.4), the limit of 60% ThOD for the pass level is too high so that it would be advisable to check the % DOC removal before embarking on a chemical investigation of the identity of metabolites. The philosophy behind the other methods is that they are screening tests which should be uncomplicated, cheap and straightforward and that a search for metabolites should be undertaken as a special investigation only if other factors, such as extreme toxicity, justify it. In the OECD Scheme of testing, intermediates are to be sought at the second tier of testing, inherent biodegradability (see Section E). For a strict consideration, the argument of Gerike, *et al.* (1984) concerning the possible, but improbable, formation of C_1 metabolites should be applied; otherwise an arbitrary limit of, say, >90% DOC could be used. In the MITI test, the chemical analysis would presumably be done by the powerful tools of T_LC, HPLC, GC-MS, etc.

Many studies of intermediate metabolites are to be found in the literature but most relate to pure cultures and the metabolites are usually transient forming a metabolic pathway to CO_2 and H_2O. Few studies were seen in which mixed cultures were used and fewer still under the conditions of the screening tests after 28d. Indications of the sort of chemicals to look for as intermediates can be gleaned from such texts as that of Dagley (1978), Gibson (1984) and Pitter and Chudoba (1990).

During die-away tests with LAS, especially when low cell densities are used, intermediates identified as various sulfophenylalkanoic acids formed by w-oxidation followed by b-oxidation of the side chain were detected (Swisher, 1987; Painter and Zabel, 1988). Eight of these acids were present at 10-100 μg/l in the initial stages of a simulation test (20 mg LAS/l) but could not be detected after about three months.

Incubation of ^{14}C-methylcellulose with activated sludge resulted in the formation of at least two intermediates after 12d and were still present at 20d when 55-73% ThCO$_2$ had been collected. The metabolites were probably methyl glucose and methyl cellobiose (Blanchard, *et al.* 1976). Three unstable intermediates were formed during the degradation, at pH7 or below, of hydroquinone by bacterial isolates (from activated sludge); T$_L$C and colorimetric analyses were applied (Harbison and Belly, 1982). They were identified as 1,4-benzo-quinone, 2-hydroxy-1,4-benzoquinone and B-keto-adipic acid. After 5d they represented about 2% of DOC left in solution. Above pH7, humic acids could be formed which resist biodegradation. No intermediates were detected in effluents from activated sludge units receiving hydroquinone.

In die-away tests with activated sludge inocula, Chow and Ng (1982) found 95% primary removal of N-methyl-2-pyrrolidone after two weeks but only 45% removal of COD; similar results were obtained in SCAS tests (inherent biodegradability). Metabolites were sought by GC and IR without success but it was ascertained that a carbonyl compound was present. Krzeminski, *et al.* (1975) were able to identify a number of intermediates during the degradation of two 3-isothiazolones, one containing a chlorine atom, using T$_L$C, GC-MS and high-voltage electrophoresis. Effluent from an enclosed SCAS unit at equilibrium, in which only about 20% ThCO$_2$ was evolved, was extracted, concentrated and analyzed. The principal pathway was ring-opening, loss of Cl and S to form N-methylmalonamic acid and thence malonamic acid, malonic acid, acetic and formic acids. Other products were tentatively identified as 5-chloro-2-methyl-4-isothiazolin-1-oxide, N-methylglyoxylamide, ethylene glycol and urea.

Lyons, *et al.* (1984) followed the degradation of aniline at 25 and 250 mg/l in pond water alone and with the addition of sewage, and identified various intermediates by the use of T$_L$C and GC-MS before and after derivatization. Differences in the pathways were observed; in the absence of sewage catechol was formed which then degraded to B-ketoadipic acid and succinate and finally to CO$_2$. In the presence of sewage two additional, minor pathways were detected; in one acetanilide and formanilide were formed while in the other N-oxidation occurred to form aromatic hydroxylamine derivatives and oligomeric condensation products. Acetanilide was present at about 14% of the initial concentration of aniline (on a molar basis) at day 1, falling to about 2% by day 7. The corresponding values for the total of other products were about 8% on day 1 and 5% on day 7. About 3% of the initial aniline content was present throughout the seven days bound directly to humus material in sewage and by day 7 about 5% had been evaporated or auto-oxidized.

Unidentified intermediates in the degradation in river water of pyridine and quinoline were detected by the use of HPLC. Two peaks were observed during degradation of 30 mg quinoline/l; one appeared on day 1 at 7 mg/l falling to about 1 mg/l after 3d, while the other also appeared on day 1 and rose to about 1 mg/l after 3d (Cassidy, *et al.* 1988). Working with water-soluble fractions of gas oil in ground water, Kappeler and Wuhrmann (1978) identified by GC some of the intermediates formed during the degradation of 47 hydrocarbons over a 10d-period. Some thirteen pure aromatic hydrocarbons were tested at 10 mg/l with bacterial isolates over 3d and one or more intermediates from eleven of the hydrocarbons were identified. These intermediates were mostly aromatic alcohols, aldehydes and ketones which are biodegradable.

Heitkamp, *et al.* (1986, 1987) have characterized the metabolites formed from t-butylphenyldiphenyl phosphate (BPDP) and naphthalene in freshwater and estuarine microcosms. Normal and ^{14}C-labelled chemicals were used. Mineralization was low, ranging

from 1.7 to 37% of $Th^{14}CO_2$ in eight weeks, being highest with sediments from eutrophic systems. Extracts of the microcosms were found by GC-MS to contain unchanged BPDP plus phenol, t-butylphenol, diphenyl phosphate and triphenyl phosphate. Metabolites of naphthalene and other hydrocarbons were identified (Heitkamp, *et al.,* 1988) by extraction, T_LC, HPLC and GS-MS as 1-naphthol, salicylic acid and catechol as well as cis-1,2-dihydroxy-1, 2-dihydronaphthalene; this was the first occasion on which this metabolite had been identified in freshwater and estuarine sediments. After one week intermediates accounted for 1-3% of total naphthalene and most of this small proportion was subsequently degraded.

Bacterial isolates derived from activated sludge and grown on biphenyl converted 2,4-dichlorobiphenyl (2-20 mg/l) in overnight cultures to monochloro-benzoic acids, plus monohydroxydichloro-biphenyls and a yellow chloro-hexadienoic acid (by GC-MS) (Baxter and Sutherland, 1984). The latter compound could be converted to monochloro-acetophenones by UV-radiation and to chlorobenzoic acids by bacteria. Masse, *et al.* (1984), also using pure cultures isolated from activated sludge, reported that 4-chloro-benzoic acid was the major intermediate in the degradation of 4-chlorobiphenyl. Liu, *et al.* (1983) investigated the enhanced biodegradation by the addition of fulvic acid (FA), known to enhance photochemical degradation and also to be present in freshwater as a large proportion of the DOC. Using inocula of activated sludge-derived bacteria, they found that 100% of 2-(methylthio)benzothiazole (MMBT) was removed in 140h and this was reduced to 70h in the presence of FA. As MMBT degraded, a yellow colour appeared which peaked at 144h without FA and at 69h with FA, after which it began to disappear. The yellow compound was not amenable to GC analysis and was not identified. A transient yellow intermediate was also formed by bacteria, isolated from marine sediments, from pure biphenyl, 3- and 4-methylbiphenyl; the intermediate had the spectrophotometric characteristics of an α-hydroxy-muconic semi-aldehyde (Fedorak and Westlake 1983). Growth on 3- and 4-methylbiphenyl produced different methylbenzoic acids (TLC) which were further degraded.

A.9   Simulation tests

Standardized simulation tests have been established for only the activated sludge system, but there are many papers in the literature describing simulations of a wide variety of environments. Some of these are discussed here and recommendations are made in section C5.

A.9.1 Sewage treatment

(a)   Summary

The prediction of the fate of chemicals in each environment and the estimation of their exposure to human beings are important to assess the safety of chemicals to the environment and human beings. In the prediction process for the concentration of chemicals in the environment it is very important to know the amount of the chemicals released into environment.

Recently, the direct release of chemicals into the environment has been restricted in many countries, thus, chemicals are released into environment largely through sewage treatment plant or waste water treatment plant. Therefore, the estimation of the removal rate

of chemicals in the treatment plant is important to predict the concentration in the environment and the exposure to human beings.

The coupled unit method was adopted in the OECD Test Guidelines as a simulation method for sewage treatment. It was concluded that results obtained from the coupled unit test were sufficient to estimate the removal waste of all chemicals in treatment plants. Furthermore, the necessity for new simulation methods was also discussed.

(b)   Comparison between sewage treatment plant and coupled unit method

Systems of sewage treatment plants in Switzerland were reported by Giger, *et al.* (1987) as listed in **Table A9 1.1**, which shows the different systems in use. The flow scheme for general sewage treatment system is shown in **Figure A9.1.1**.

Table A9.1.1   Treatment process of sewage treatment plants in Switzerland

Treatment Plant	PEC	Operational Types of Waste and Sludge Treatment
Basserdorf	22,500	primary clarifier / aeration tank / secondary clarifier; digestion
Bülach	34,000	primary clarifier / aeration tank / secondary clarifier; digestion
Dietikon	67,000	primary clarifier / aeration tank / secondary clarifier; digestion
Dübendorf	41,000	primary clarifier with chemical flocculation / aeration tank / secondary clarifier; centrifugation
Fällanden	50,000	primary clarifier / aeration tank / secondary clarifier; digestion
Flaach	4,000	primary clarifier / aeration tank / secondary clarifier; digestion
Nänikon	12,000	primary clarifier / aeration tank / secondary clarifier; external sludge treatment
Niederglatt	37,750	first aeration tank / first clarifier / second aeration tank / secondary clarifier; digestion
Opfikon	34,000	primary clarifier with chemical flocculation / aeration tank / secondary clarifier; digestion
Pfungen	33,000	primary clarifier / aeration tank / secondary clarifier; digestion
Regensdorf	15,000	primary clarifier with chemical flocculation / aeration tank / secondary clarifier; digestion
Uster	6,000	primary clarifier / aeration tank / secondary clarifier / filtration; digestion
Zürich-Glatt	240,000	primary clarifier / aeration tank / secondary clarifier; digestion

PEC: population equivalent capacity

The schematic diagram of one Husmann unit of the coupled unit system is shown in **Figure A9.1.2**; sludge is intercharged daily between one unit receiving the test chemical and the second which does not. In the coupled unit test, the biodegradability of a chemical substance is calculated from the removed DOC from test solution under a residence time of three or six hours. The test method simulates the general sewage treatment plant. However, the coupled unit test is normally applicable only to those organic substances which, at the concentration used in the test:

- are soluble in water to the extent necessary for the preparation of the test solution;
- have negligible vapour pressure;
- are not inhibitory to bacteria; and
- do not significantly adsorb on glass surface.

Figure A9.1.1 Flow scheme of general sewage treatment plant
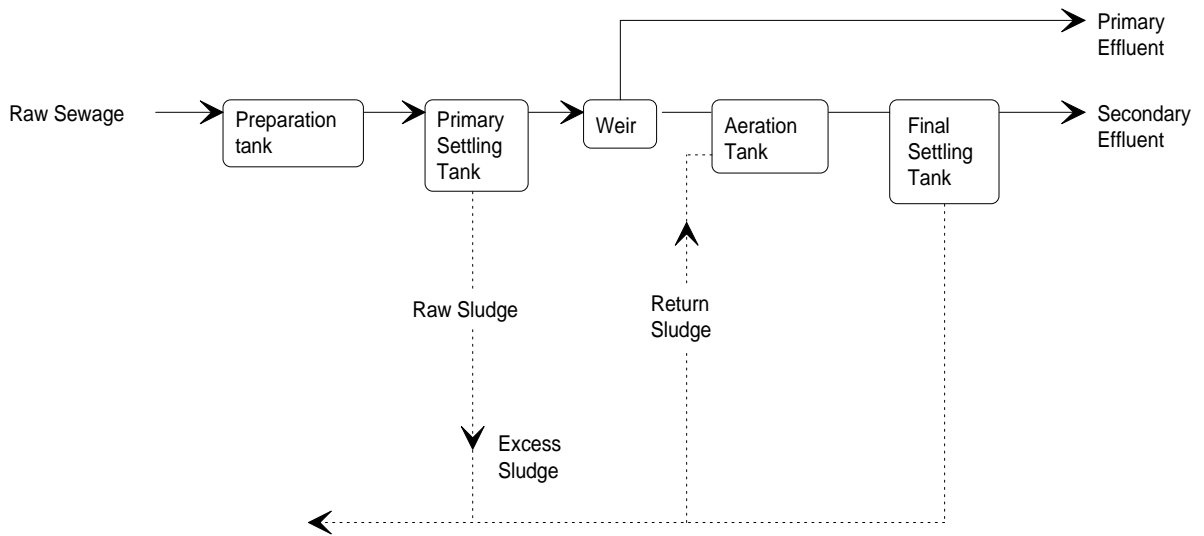
Figure A9.1.2 Husmann unit
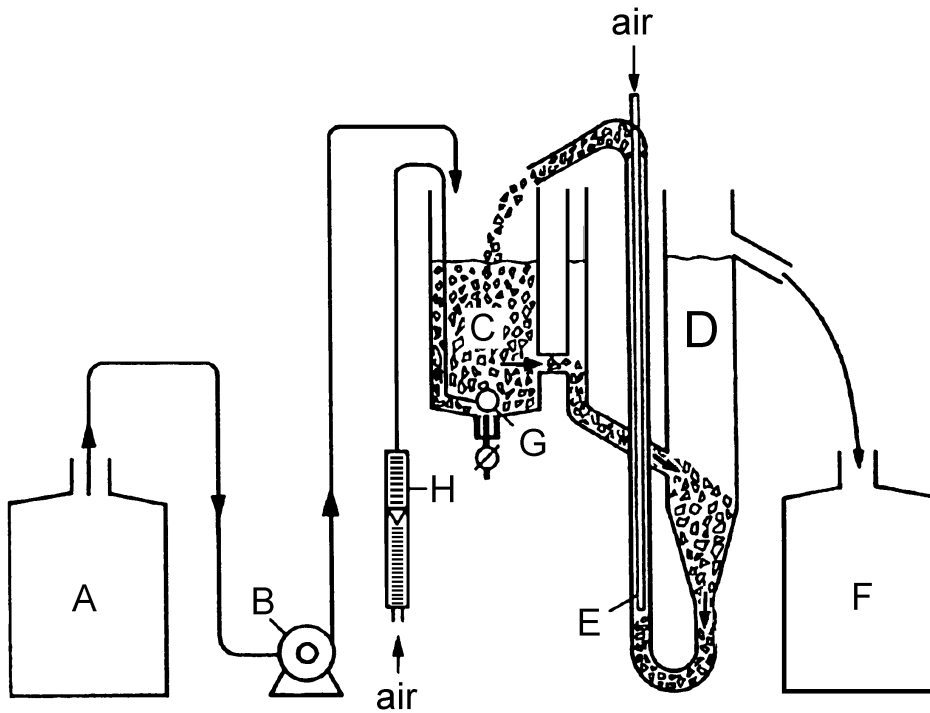
A   = storage vessel
B   = dosing device
C   = aeration chamber (3 l capacity)
D   = settling vessel
E   = air lift
F   = collector
G   = aerator
H   = air flow meter

On the other hand, in sewage treatment plants and waste water treatment plants insoluble substances and volatile substances are also treated. Therefore, the present test should be modified or a new simulation test method which is applicable to those substances should be developed (see sections C.1 and C.2).

(c) Studies on sewage treatment plant

Yogo, *et al.* (1982) studied the biodegradability of oil components in sewage and showed that the oil components were degraded by micro-organisms after adsorption onto activated sludge. This study suggests that the simulation test for insoluble substances will be possible by analysing chemicals by HPLC and GC, etc. after suitable extraction.

Pagga (1985) estimated the toxicity of chemicals to biodegradation process by measuring the respiration rate in the "Toximeter" which is a simple model of waste water treatment plant. The Toximeter is useful to test the acute inhibition to the metabolic system, the long term toxicity, and the biodegradability of chemicals.

De Henau, *et al.* (1989) studied the biodegradation process of LAS in sewage treatment plant and applied a mathematical model to the process. Giger, *et al.* (1987) reported that both biotransformations and physico-chemical processes determined the behaviour and fate of nonylphenolic substances in sewage treatment. Furthermore, they established that the various treatment plants showed different removal efficiencies for nitrilotriacetate depending on the operating conditions. Steber, *et al.* (1985), Matthijs, *et al.* (1987), and Giger, *et al.* (1986) studied the degradation process in sewage treatment of fatty alcohol ethoxylates, monoalkylquaternaries, and alkylphenol polyethoxylate surfactants, respectively. Buisson, *et al.* (1988) studied the behaviour of chloro-compounds in pilot plant. Park, *et al.* (1989) studied the biodegradability of sodium polyglyoxylate in laboratory simulation of a sewage treatment system. Brauer, *et al.* (1986) reported that ammonia in sewage was effectively decreased in pilot plant which consisted of two aerobic bioreactors maintained at different pH values. Reference to these studies may suggest a new or modified test method for simulating sewage treatment plant.

Rozich, *et al.* (1987), Maria, *et al.* (1989), Van Haandel, *et al.* (1981), and Namkung, *et al.* (1987) proposed mathematical models for degradation process in treatment plant. These studies can also be referred to in developing a new or modified simulation test method.

Seiber, *et al.* (1982) analysed the variation of micro-organisms in activated sludge of sewage treatment plant by using cluster analysis; the results should be of use in studying inocula and populations in the simulation method.

Han, *et al.* (1985) reported the importance of anaerobic biodegradation of chemicals by using a model system which includes an anaerobic attached film expanded bed (AAFEB). Pfeiffer, *et al.* (1986) also reported the efficiency of anaerobic treatment in sewage plant. These and other studies suggest the necessity for a method to simulate the anaerobic treatment.

(d)   Other tests with simulation methods

Painter and Bealing (1989) showed that no advantages were gained by using coupled units over operation as single, non-coupled units with no sludge interchanged. Also, the OECD confirmation test gave virtually the same results as the porous pot system; domestic sewage gave more consistent and often higher % DOC values. Birch (1984,1991) operated porous pot units at a range of sludge retention times and temperatures to show more precisely the operating condition under which various surfactants were successfully biodegraded.

Nyholm, *et al.* (1992) operated continuous flow activated sludge units in enclosed vessels so that volatile compounds in the exit gas could be examined. They used OECD synthetic sewage but had to modify it to overcome poor settling. The test chemicals were added at 5-1000 μg/l (unlike the 15-20 mg/l added in the OECD test) but problems were experienced with spiking the sewage. The optimal method was to add a methanolic solution of test chemical to 1l water stirred for 30 min and then transferring the mixture to 10l of synthetic sewage, which was strongly stirred throughout the test.

Shimp (submitted) added ^{14}C-labelled surfactants to domestic sewage and treated the mixtures in 6-d (sludge retention time) activated sludge units. The concentrations added were less than 20 fg/l, though some of the surfactants were already present in the sewage. $^{14}CO_2$ was not collected but the percentage "probable" biodegradation was calculated from mass balances.

Percolating, or trickling, biofilters have been simulated by rotating tubes (HMSO, 1983) using both OECD synthetic sewage or domestic sewage, but no results were reported. Gerike, *et al.* (1980) developed trickling filter units and, operating them in the coupled mode (Gerike and Fischer, 1981), found higher rates of removal of 3-chloro-benzoic acid, diethylene glycol, sulfanilic acid and 2-chloraniline than in the OECD confirmatory simulation test in the coupled mode. The authors explained these differences by reference to the loading gradient in filters which does not exist in the activated sludge system.

Baumann, *et al.* (1990) simulated trickling filters by inserting polyester "fleece" strips into 1m tubes (12 mm int.diam.) after the strips had been immersed in concentrated activated sludge for 30 min. The test chemical was fed to the vertical tube as a solution of the test chemical (100 mg C/l) as the sole C source, in a salts solution. Biodegradability was assessed by determining DOC in the effluent and CO_2 in the exiting gas.

A.9.2   Environmental waters

(a)   Summary

Information on the fate of a chemical in the environment, especially its biodegradability in natural waters, is important to assess the effect of the chemical on the environment.   Although some test methods have been proposed to evaluate the biodegradability of chemicals in environmental water (e.g. Means, *et al.* 1981), no Test Guidelines of simulation of biodegradation in environmental water have been established.

Studies related to each factor which should be considered to establish the simulation test method for the biodegradation in environmental water are summarized, and a selection of conditions proposed for some simulation tests are given in Table A.9.2.1, and are further considered in section C.5.2.

(b)   Source of micro-organisms

Micro-organisms inoculated into a test system should be selected considering the environment where a target substance is degraded. Micro-organisms are usually collected from river or lake water (Wylie, *et al.* 1982), epilithic microbial communities (Anderson, *et al.* 1990, Gantzer, *et al.* 1988) or sediment (Larson and Payne, 1981). Collection from water is the most convenient; indeed, uninoculated water from the appropriate environmental compartment is often used. However, the epilithic communities are preferable to avoid data scattering which may depend on sampling time and sampling point (Anderson, *et al.* 1990, Gantzer, *et al.* 1988).

The adaptation of micro-organism to a chemical affects lag-time and degradation rate, etc. in the biodegradation process (Shimp, *et al.* 1989). Therefore, in the evaluation of generated data, the effect of adaptation should be considered, since it can be assumed that some micro-organisms are adapted to those chemicals which are released constantly into environment.

(c)   Composition of test media

Test media should represent the environmental water into which chemicals are released. However, the composition of environmental water is variable. Therefore, artificial media are sometimes used for a test in an attempt to generate reproducible data. Most artificial test media are composed of inorganic salts necessary for the growth of micro-organisms. Organic materials, such as meat extract or yeast extract, are sometimes added to test media. Although the addition of organic materials is preferable for the growth of some micro-organisms, they may induce some difficulties in the evaluation of test data (especially in analysis of chemicals). However, for a true simulation natural waters, taken from the environment of interest must be used; any deficiency in nutrients or bacteria is part of that environment.

(d)   Concentration of test substance

It was reported that the concentration of test chemical affected the biodegradation process (for example, Larson and Payne, 1981). Therefore, it is necessary that a test is conducted at a test chemical concentration predicted to be present in real environments. However, the predicted concentrations of chemicals in the environment are usually very low, and this, together with the analytical limit of detection, must be taken into account when choosing the concentration to be tested. Ruling out the use of ^{14}C-labelling, reasonably low concentrations of test chemicals can be used if they can be determined by HPLC, GC, etc., but only primary biodegradation will then be assessed.

Table A.9.2.1   Representative simulation test methods (1)

System	Stream Model		
Reference	Oba, *et al.* (1977)	Shimp, *et al.* (1989)	Engelmann (1978)
Test solution	Dechlorinated tap water	Mixture of water and river water (CaCO$_3$: 125 mg/l)	Nutrient medium (DOC 300 ppm, PO$_4$-P 4.6 ppm, organic N 51 ppm)
Source of micro-organism	Supernatant of activated sludge	River water, sediment	
Test conditions	Stream with L. 1080 cm x W. 15 cm x D. 5 cm, residence time 50h, 20°C, DO 5-8 ppm	L. 20 m x W. 30 cm x D. 27 cm, sediment D. 1-2 cm, light 10 h/day 20-25°C, DO 8 ppm, pH 7.5-8.0	50 ml vessel x 500 (overflow system) residence time 5h, pH 7.2
Concentration of test substance	10 ppm, flow rate 1.5l/h (test sample LAS)	0-2.1 ppm	Flow rate 34 ml/min
Analysis	MBAS and TOC, etc. at each point	$^{14}CO_2$	
Characteristics	Quantitative and qualitative changes of test substance are continuously measured. Stabilization is needed after the addition of micro-organism	The importance of adaptation is emphasized. Model system in lab.	It takes four weeks to stabilize the conditions. Samples are available from each test vessel. River simulation model in lab.

Table A.9.2.1　Representative simulation test methods (1) (Continued)

System	Lagoon	Microcosm	Model ecosystem
Reference	**Walker and Leclerc (1973)**	**Porcella, *et al.* (1982)**	**Metcalf (1974)**
Test solution	River water	Nutrient medium	Inorganic salts ($MgSO_4$, K_2SO_4, $CaCl_2$, $NaHCO_3$, NH_4NO_3, K_2HPO_4, $CaCO_3$, $NaSiO_3$, $FeCl_3$)
Source of micro-organism	River water	Sediment (or natural water)	Plankton from old aquarium water
Test conditions	Scale: 16m x 14 m x 1.95 m with twelve streams (L. 14 m x W. 1 m x D. 1.5 m) residence time 6h, 6-18°C, pH 7-8	Cylinder of φ 15 cm x H. 75 cm with 15 cm depth of sediment and 2 cm of gas phase. Media are inletted from bottom and outletted from top. Semi-continuous flow system. Light, temperature, and stirring water phase are controlled.	10 x 12 x 18 inch glass aquarium which contains a miniature farm and lake, a food crop, and food chains of at least seven elements. Plants are treated with radio labelled test substances. pH 7.9. Test period 33d.
Concentration of test substance	Pollutant (river water) is injected with 2.6 l/min.		1-5 mg (1-10m Ci/mmol) was exposed to plant
Analysis	Water quality, amount of organic substance, and number of micro-organisms, etc. are measured at each point.	Gas analysis. Mass balance of materials	Liquid scintillation, TLC
Characteristics	Lagoon simulation (like river model) large scale (field) with long residence time.	Simulation of three phases (air/water/sediment). Cheap.	Degradative pathway through various organisms can be examined. Reference: Metcalf, *et al.* (1971), Kanazawa, *et al.* (1975)

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Table A.9.2.1 Representative simulation test methods (1) (continued)

System	Die-away	
Reference	Wylie, *et al.* (1982)	Anderson, *et al.* (1990)
Test solution	River water	River water
Source of micro-organisms	River water	River water and epilithic micro-organisms attached to stone in river.
Test conditions	2.5 l, dark, test period one month	500 ml of water sample in 1-litre Erlenmeyer flask. Incubation at 5 or 10°C in agitatory incubator at 100 rpm.
Concentration of test substance	0.1 mg/l	25 mg/l
Analysis	Degradability: $^{14}CO_2$, Water quality: DO, $CaCO_3$, SS, NH_3-N, NO_3-N, PO_4, P, pH, conductivity, temp, MPN, ATP	MBAS
Characteristics	Rapid. Economical. Scattering of data is relatively large to evaluate the biodegradability	Experimental data were fitted to a variety of possible kinetic models.

(e)   Analytical method

The degree of biodegradation is usually estimated from direct analysis, such as HPLC, GC, and colorimetry, etc., or from indirect analysis, such as Biochemical Oxygen Demand (BOD), CO_2 evolution, and Dissolved Organic Carbon (Larson and Payne, 1981). In general, the direct analysis generates more sensitive data than the indirect analysis. On the other hand, in order to evaluate ultimate biodegradability, the indirect analysis is necessary, although HPLC, GC and the like can be used to identify and quantify intermediates.

In the measurement of CO_2 evolution, the use of test chemical labelled with ^{14}C will produce more sensitive data (Larson and Payne, 1981; Shimp, *et al.*, 1989). However, it seems that the application of radio-active substances to biodegradation tests generally is too expensive, unless a *de novo* HPLC method is needed.

For the measurement of BOD and CO_2 evolution, test vessels should be closed systems, and the volume of the test vessel should be optimised considering the sensitivity of measurement.

(f)   Test system

Most proposed test systems for the simulation of biodegradation in environmental water are open systems, such as model streams (Oba, *et al.*, 1977; Walker and Leclerc, 1973; Shimp, *et al.* 1989; Engelmann, *et al.*, 1978) and microcosms (Porcella, *et al.*, 1982). In order to simulate the real environment, systematic models will be of interest, but it is preferable to avoid huge model systems which may represent the real environment precisely. Although a long stream system will produce many valuable data to evaluate the fate of chemicals in the environment, it needs large areas and expensive running costs and takes a long time to stabilize test conditions (Walker and Leclerc, 1973). Because of this, the possibility of using simple, small closed or open systems, batch or continuous, as surrogates for simulation tests.

(g)   Applicability and evaluation of results

Although any model systems can be applied to water-soluble chemicals without any trouble, they should be modified to apply to insoluble and, if possible, to volatile substances using restricted conditions.

In the simulation test, kinetic data expressed as half-life time or biodegradation rate (mass per unit volume and time) are more informative than the degree of biodegradation. The adoption of standard (reference) chemicals should be examined to evaluate the validity of the test results.

A.9.3 Sediments

(a)   Summary

Chemicals produced on land ultimately flow directly or via rivers into lakes or coastal zones, where they are often deposited in sediments, in which some chemicals are degraded by micro-organisms under aerobic and/or anaerobic conditions.

Although standarized methods to simulate the biodegradation in sediments have not been established, biodegradation processes of chemicals have been studied in sediments under various conditions.

(b)   Studies on biodegradation in sediment

Matsumoto and Hanaki (1990) studied the biodegradation of acetate and glucose in coastal sediments and in sediments acclimated with artificial sea water under anaerobic conditions. They showed that both methane production and sulfate reduction could contribute to the mineralization of organic matters in coastal sediment.

Kuhn, *et al.* (1987) studied the biodegradation of nitrilo-triacetate under aerobic conditions by using saturated-upflow sediment columns (Figure A.9.3.1).

Figure A.9.3.1 Experimental set-up of saturated-upflow sediment columns
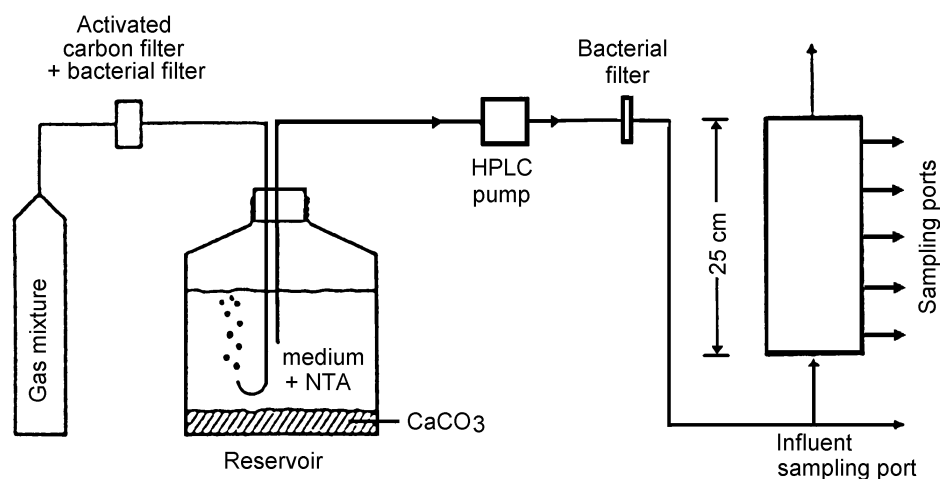
Fleischer (1987) studied glucose transformation rates during one year in sediments from six lakes, and showed that the glucose transformation rates fluctuated seasonally within a single lake.

M.J. Jensen, *et al.* (1988) examined the effect of simulated acid precipitation on the breakdown of organic matter in streams under laboratory condition using two different sediment types from streams. The authors showed that sediment with higher organic matter was more sensitive to pH under conditions of suspension, but less sensitive than the sediment with less organic matter under settled conditions.

The characteristics of sediments will be affected by various factors, such as fresh water or sea water origin, aerobic or anaerobic conditions, and high or low organic matter, etc. Therefore, the establishment of a consolidated simulation method for the biodegradability of chemicals in sediments seems to be difficult. In order to simulate the biodegradability in sediments, methods whose test conditions are suitable for each particular environment will have to be established. Before this can be done more fundamental studies are required.

A.9.4 Sea water

(a) Summary

Relatively few of studies for the simulation of the biodegradation of chemical substances in marine and estuarine environments have been published. These studies were classified into three categories: 1) Single-stage continuous culture system; 2) Multi-stage continuous culture system; and 3) a type of die-away method for radiolabelled compounds.

(b) Studies on biodegradation in sea water

A single-stage continuous culture system was described by (Kirk, *et al.*, 1983). Approximately 500 ml of surface coastal sea water in 1 litre wide-neck reaction vessel were acclimated to develop the bacterial population by supplying synthetic sea water and synthetic sewage at a dilution rate of 0.0167 h^{-1} for twelve days under aerobic or anaerobic condition. The filtered sea-water contained 1.8×10^5 bacterial cells/ml. The test chemical (NTA) was added to aerobic and anaerobic systems at a concentration of 0.5 ppm or 7.5 ppm for a period of 80 days at a dilution rate of 0.05h^{-1}. All systems were reinoculated with fresh sea water (100 ml) after 40 days. The entire apparatus was maintained at $15 \pm 1^{\circ}$C in a darkened room. The biodegradability was estimated from the measurement of the concentration of test chemical in the vessel at appropriate intervals.

A schematic diagram of the multi-stage continuous culture system (Hunter, *et al.*, 1986, Lovitt and Wimpenny, 1981) which is called a Gradostat is shown in Figure A.9.4.1. Five 1-litre reaction vessels with 500 ml sidearm overflow outlets were arranged in a stepped sequence. Freshwater was delivered to the top vessel at a rate of 30 ml/h. A saline solution was delivered to the bottom vessel at a rate of 30 ml/h and a passive downward overflow of less saline water between adjacent vessels at a rate of 60 ml/h.

Figure A.9.4.1   Schematic diagram of the estuarine independent microcosm layout (Hunter, *et al.*, 1986)

GRADOSTAT
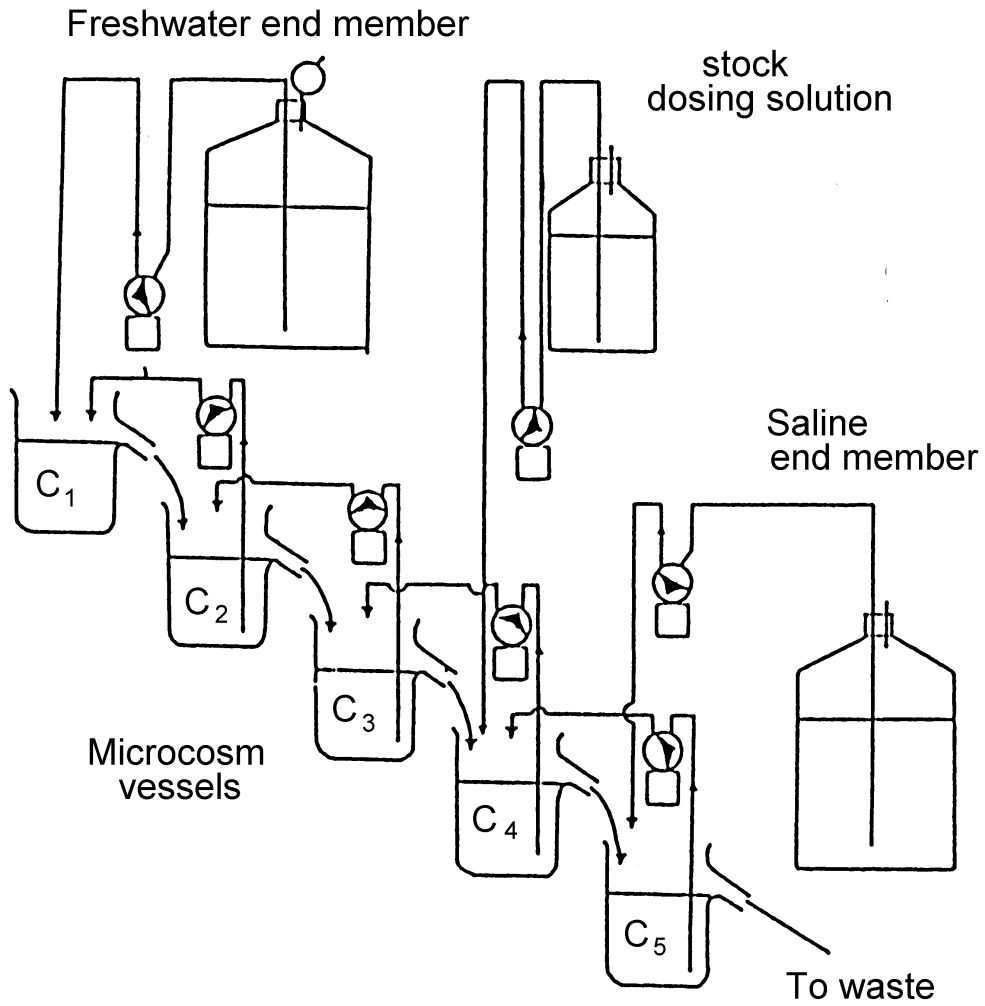
Freshwater was pumped to the top vessel and surface coastal water was pumped to the bottom vessel for a period of seven days to seed the microcosms. Subsequently, the bacterial populations were maintained with a weak synthetic sewage solution. The chemical was added, via a stock solution to the freshwater end to obtain the nominated concentration in the top vessel. Salinity, temperature and dissolved oxygen concentration were measured every two days as was the concentration of test chemical in appropriate vessels.

Biodegradability test methods in which radiolabelled compounds were used (Palumbo, *et al.* 1988; Pfaender and Bartholomew, 1982) have been conducted to simulate not only marine environment but also other environments.

Shimp and Young (1987) showed that the use of radioisotopes in biodegradation studies could significantly increase the sensitivity of biodegradation measurements.

(c)   *Recommendation*

Simulation methods for the biodegradability of chemicals in marine or estuarine environments have not been studied enough to be established as standardized methods.

Although the use of radiolabelled compounds may be suitable to simulate the biodegradability of chemical substance in the low concentration as found in the environment, special facilities are required and expensive running costs are incurred. Therefore, the use of radiolabelled compounds is unlikely to be adopted as a standardized test guideline.

Continuous culture systems may be suitable simulation methods. However, these methods have not yet been studied sufficiently to be adopted as a standardized test guideline. In order to standardize the continuous culture system, further work on apparatus, source of micro-organisms, and test conditions, is yet required.

A.9.5 Soil

(a)   *Summary*

Generally, chemicals are rarely released directly into the soil environment, except for special uses, such as pesticides. The fate of chemicals in soil will be affected not only by biodegradation but also by adsorption and desorption, photodegradation, hydrolysis, evaporation, etc. Thus simulation methods for biodegradability of chemicals in soil were far less frequently studied than those in the aquatic environment.

Data on biodegradation of pesticides in the soil are required in some countries for notification. The following test guidelines may be referred to in order to establish a simulation method for biodegradation of chemical substance in soil.

1. FIFRA (USA) Pesticide assessment guidelines, Subdivision N, Oct. 1982.

   • Soil Column Leaching Studies

   • Dissipation Studies
      · Field Dissipation Studies for Terrestrial Uses
      · Field Dissipation Studies for Aquatic Uses and Aquatic Impact Uses
      · Dissipation Studies for Forestry Uses
      · Long Term Soil Dissipation Studies

2. BBA (German) (Federal Biological Research Centre for Agriculture and Forestry) Guidelines for the Official Testing of Plant Protection Agents

    - BBA Guideline, Part IV, 4-3, 1986
      · Lysimeter Studies

    - BBA Guideline, Part IV, 4-1, 1986
      · Fate in soil. Degradation, Transformation and Metabolism

    - BBA Guideline, Part , 5-1, 1990
      · Degradation and Fate in a Water Sediment System

3. Pesticides Control Law (Japan)

    - Soil Dissipation Studies
      · Vessel Studies
      · Field Studies

4. Sweden

In Sweden, a research programme on many aspects of soil was launched in 1988 (Bengtsson and Torstensson, 1988; Rundgren, 1991) and finished in 1993 (Torstensson, 1993). One facet deals with pesticide movement in soils and another with test methods for the decomposition of organic chemicals in soil. The aim of the second aspect is to develop a test system for the quantitative description of the degradation of organic chemicals and to be able to predict their persistence from equations, taking account of such factors as initial concentration, temperature, pH, water content, adsorption/desorption. Among early papers Stenstrom (1989) has measured the removal of pesticides in 300 g soil in plastic containers, both by HPLC and $^{14}CO_2$ evolution, and found that degradation did not follow first order kinetics. A test for inherent biodegradability in soil was incorporated in the OECD Guidelines (1981) but it required the use of ^{14}C-labelled test chemicals. There have been a number of papers describing similar methods for example, Thomas, *et al.*, 1987, Ward and Larson, 1989, Focht and Brunner, 1985; the latter two papers describe a flow-through method, using CO_2-free air. Other workers have used soil-water slurries, some assessing biodegradation by $^{14}CO_2$ production (Swindoll, *et al.*, 1988) and others by HPLC or GC, without resorting to ^{14}C-labelling (Morris and Novak 1989, Hickman and Novak 1989). The kinetics of degradation in soils were modelled by Verstraete, *et al.* (1975) and Alexander and Scow (1989).

(b)   Recommendation

Standardized methods by which the fate of chemicals in soil can be simulated have not been established. On the other hand, computer simulation methods, such as SESOIL (Seasonal Soil Compartment Model) and PRZM (Pesticide Root Zone Model), etc., have been developed to predict the concentration of pesticide in environmental compartments from physicochemical data, information on usage, and weather conditions. Furthermore, monitoring studies for existing chemicals have been carried out, from which more realistic data have been obtained. However, the simulation test method should be established to predict the fate of new chemical substances in soil and to complement the computer simulation model.

For this purpose, test conditions of simulation test methods which include transportation in soil, metabolism, and effect of rain fall and ground water, etc., are indicated in **Table A.9.5.1**. For the preparation of Test Guideline of simulation test of biodegradation of chemical substances in soil, some additional studies may be needed, though much experience in this field has been obtained. It is known that (Vonk, pers. comm.) the ISO Soil Technical Committee (TC140/SC4/WG1) is working actively in this area and a draft method for assessing biodegradation of organic chemicals in soil under aerobic conditions is available.

Table A.9.5.1   An example of consolidated test condition

	Test I (Leaching studies)	Test II (Lysimeter studies)	Test III (Dissipation studies)
Test scale	Lab.	Semi field	Field
Number of soils	More than two	More than one	More than one
Soil type	Alfisol, Spodsol or Entisol	Alfisol, Spodsol or Entisol	Alfisol, Spodsol or Entisol
Test substance	Radio labelled	Radio labelled or unlabelled	Radio labelled or unlabelled
Application method of test substance	Surface area	Surface area	Surface area
Water-soluble Water-insoluble	Aqueous solution Suspended slurry or CH_3CN solution	Aqueous solution Suspended slurry	Aqueous solution Suspended slurry
Application rate to soil for ageing	Not specified	Not specified	Not specified
Amount of aged soil applied	Depth: more than 30 cm column	Depth: more than 1m. Surface area: more than 0.5m	Not specified
Replication	More than two	More than two	Not specified
Temperature	20~25°C	Depending on the weather	Depending on weather
Elution/desorption medium	Deionised water	Rain water	Rain water
Elution volume	Equivalent 20 ins of rain	Depending on the weather	Depending on weather
Flow rate	Elution completed in two days	Depending on the weather	Depending on weather
Ageing period	Two half-lives or 30 days	Two half-lives or more than six months	Two half-lives or more than two years
Number of sampling times (minimum)	6 (20~80% residue)	12	24
Sampling site	6 even parts in each sampling time	Upper 10 cm layer	Upper 10 cm layer

Table A.9.5.1 An example of consolidated test condition (continued)

	Test I (Leaching studies)	Test II (Lysimeter studies)	Test III (Dissipation studies)
Mass balance	required	not required	not required
Analysis of test substance in soil	required	required	required
Analysis of test substance in the leachate	required	required	not required
Analysis of metabolites in soil	required	not specified	not required
Analysis of test metabolites in the leachate	required	required	not required
Reporting and evaluation of data:			
1. Decline curve of residue in soil	required	required	required
2. Amount of metabolites in soil and leachate	required	not specified	not required
3. Amount of test substance in the leachate	required	required	not specified
4. Application time and method	required	required	required
5. Amount of elution/desorption medium	required	required (rainfall)	required (rainfall)
6. Water table	required	required	required
7. Soil and air temperature data	required	required	required
8. Sampling times and techniques	required	required	required
9. Test period	required	required	required
10. Depth, weight or volume of each sample taken analysis	required	required	required
11. Analysis method	required	required	required

B. Biodegradation in the Environment

It is out of the question to test the behaviour of all chemicals in the field, because of the enormous cost and time it would entail. Some means of prediction are therefore required. (Structural activity relationships are discussed in section F.) Whereas the present tests for ready biodegradability are not designed to predict in detail the behaviour of a chemical in specific sectors of the aquatic environment, laboratory test systems provide the most practical means of obtaining data that, under some circumstances, could be used to predict the biodegradation and fate of organic pollutants. If a relatively small number of chemicals of various types are tested in field tests (waste water treatment, rivers, lakes, etc.) and if the results are compared with those from various types of laboratory test, the relevance and usefulness of the laboratory data may be assessed. Such laboratory tests could range from simple tests, such as activated sludge units, biofilm reactors, slurries, eco-cores and microcosms. Results of such tests are given in section B.1.

OECD tests so far established are carried out at 2-100 mg test chemical/l, whereas environmental concentrations are much lower than this. Section B.2 indicates the concentrations of some organic pollutants. In section B.3, the kinetics of degradation of chemicals are discussed with special reference to the concentration of the chemical. This subject has recently been extensively reviewed (ECETOC, 1991; Battersby, 1990).

Many other factors appear to influence the biodegradation of xenobiotic chemicals, both the rate and whether it occurs at all. These are discussed in section B.4, some having already been discussed in section A.4. Finally, the phenomena which are collectively described as "cometabolism", and how they impinge on biodegradability testing, are addressed in section B.5.

B.1 Prediction

Tests for ready and inherent biodegradability were designed to determine whether or not a chemical has the potential to biodegrade; readily biodegradable chemicals were assumed to degrade in the environment. The tests were not intended to predict the behaviour of chemicals in the various compartments of the aquatic environment; this was to be the function of the simulation tests. Nevertheless, the question arises as to whether results of tests for ready biodegradability can be used for predictive purposes.

In the environment there are a number of factors which have to be fulfilled for a potentially degradable chemical to be mineralized. First, physical conditions have to be acceptable – certain ranges of pH value, temperature, dissolved oxygen, salinity and possibly redox potential, surface tension and sorption. Then inorganic nutrients, especially N and P, have to be present in adequate concentration for complete and consistent metabolism. The concentrations of the test chemical in the environment should be above a threshold value to allow degradation unless the phenomena of cometabolism or secondary oxidation are able to operate, and the speciation of some test chemicals, for example chelating agents (Madsen and Alexander, 1985), has been shown to be important. The presence of other organics can influence the fate of a given chemical and inhibitors, both natural and synthetic, can also play a part. Finally, the most important factor is the absolute necessity for species which are competent to degrade the chemical or which can be adapted to do so. There may be positive

and negative interaction between species, and there could be competition for inorganic nutrients. Predation by protozoa may also play a part.

B.1.1 Aerobic sewage treatment

Most of these conditions are fulfilled in aerobic waste water treatment, especially if the waste contains domestic sewage, but in many other compartments – some rivers, lakes – some conditions are not or are not fully fulfilled.

In their study of 44 chemicals, Gerike and Fischer (1979, 1981) found that nearly all the chemicals which passed the screening tests were degraded in their simulation test (coupled units activated sludge units). The exceptions were 3-chloro-benzoic acid, sulfanilic acid and 3-amino-benzoic acid. The former two chemicals were found to be readily biodegradable by other workers, and these chemicals are discussed in Appendix III. They also found that some chemicals which failed most or all of the screening tests were degraded in the simulation test. Similarly most of the 14 chemicals which had been studied in a ring test of OECD Respirometric method (CEC, 1985) were found to behave as expected in the Husmann and porous pot activated sludge simulation tests. Higher degrees of removal were recorded for some chemicals when domestic sewage was used instead of the OECD synthetic sewage (Painter and Bealing, 1989). The exceptions were hexamethylene-tetramine and sodium benzene sulphinate; the former passed the screening test, probably because of its hydrolysis, but not the simulation test (see Appendix III), while the latter passed the simulation test on four out of six occasions.

These examples show that screening tests can predict that readily degradable chemicals are degraded in sewage treatment, but the chemicals had been added at 5-20 mg C/l for analytical reasons. Few chemicals are present at these concentrations in sewage or waste waters (except in certain factory discharges or accidental spillages). Most chemicals will be present at the μg/l level, and the removal of these concentrations is problematic. However, such concentrations have been shown to be removed (primary biodegradation) by secondary substrate oxidation, for example by Nyholm, *et al.* (1992). They applied five chemicals separately at 5-1000 μg/l to activated sludge units operated at sludge ages of 1 to 32d. The three readily biodegradable chemicals, 4-nitrophenol, 2,4,6-tri-chlorophenol and 2,4-dichlorophenoxyacetic acid (2, 4-D), were all biodegraded after periods of adaptation in the units, volatility and adsorption being allowed for. Lindane and pentachlorophenol, not readily biodegradable, were degraded but only under some conditions. The authors proposed that on the basis of their evidence, the OECD and EC activated sludge simulation test be evaluated with regard to its predictive value by performing tests at both high and low concentrations.

Mathematical models representing events in the screening tests (Blok and Booy, 1984; Painter and King, 1983) established that chemicals passing even the less stringent tests would be removed in normal sewage treatment; the mathematical models do not, however, take account of processes such as inhibition, synergism, antagonism, etc. Making the very conservative assumption that all bacteria in the inoculum would degrade all chemicals tested, they further showed that for chemicals with "decay rates" of <0.7/d inconsistent results would begin to appear, although chemicals with "decay rates" of down to 0.2 to 0.3/d would still be degraded in normal sewage treatment.

B.1.2 Rivers, lakes, estuaries

Although the relationship between results of tests for ready biodegradability and those from sewage treatment simulated tests is well established (but many failed chemicals degrade in the simulation test), it is by no means so with other compartments of the environment. For example, in pristine river systems there may be few if any competent bacteria present, or there may be a lack of N or P. Some practical examples illustrate the point. Evans and David (1974) found that the extent and rate of degradation of various glycols in river die-away tests depended on the particular river and the time the sample was taken. Similar variations in the biodegradation of 2, 4-D in river waters was reported by Watson (1977).

Wylie, *et al.* (1982) reported a wide variation, 11 to 78% $Th^{14}CO_2$, in the results for the biodegradation of 100 μg ^{14}C-di 2-ethylhexylphthalate/l in various samples of water taken from the Missouri River. The reference chemical (phthalic acid) showed much less variation, 66-92% $Th^{14}CO_2$. The results did not correlate with determined values of N, P, etc. in the river water and the relationship with suspended solids was complex. MPN and ATP concentrations fluctuated throughout the tests, showing no trends. The authors attributed this to change in the temperature and perhaps other factors, between the environment in the river to the laboratory; unfortunately no control values of MPN or ATP were given, so that no judgement can be made. They concluded that river die-away tests had their uses, but for accurate predictions replication within samples and over time should be taken for assessment.

Vaishnav and Korthals (1988) determined the pseudo-first order constant (k_1) for the biodegradation of nine chemicals in BOD dilution water, using inocula acclimated to the respective chemicals. They found that the values of k_1 in the dilution water compared with values reported in the literature (Paris, *et al.* 1983, 1984), thus justifying the method; they warned that more diverse chemical structures and environments should be investigated.

Kuhn, *et al.* (1985) found that the chemicals they tested in upward flow sediment columns in the laboratory under aerobic conditions behaved qualitatively in the same manner as at a field site. But the transfer of rate constants determined in the laboratory at elevated substrated concentrations to the field was difficult. For 1,4-dichlorobenzene the rate constant at the field site during the summer was $0.4d^{-1}$, which compares well with 0.5 to $1.0d^{-1}$ calculated for the first few days in the laboratory column. However, by day 10 the rate was as high as $20d^{-1}$ and by day 39 it was reported to be as high as $110d^{-1}$, caused by the increase in biomass and/or activity in the column.

Since it had been observed that in some experiments a threshold concentration of the test chemical had been found below which little or no degradation occurred, Howard and Banerjee (1984) suggested that this could be evidence for the presence of eutrophs rather than oligotrophs. It was thus important to carry out tests using water taken, for example, from both eutrophic and oligotrophic lakes. In the case of ground water, in which the concentration and activity of bacteria are generally lower than in surface waters, low concentrations of C are likely to be present in pristine water and hence the population will be oligotrophic. But in situations in which the ground water is near a waste disposal site or has been contaminated by a spill, the bacteria will be eutrophic.

The problem of prediction was approached in a different way by Struijs and Stoltenkamp (1986b) and by Carson, *et al.* (1990). The former authors classified chemicals by ranking them in increasing order of persistence into five groups:

1	highly biodegradable	complete mineralization within 10d; time window <4d
2	readily biodegradable	high level of mineralization (>70%) within 28d
3	intermediate in biodegradability	borderline cases of ready biodegradability; inconclusive results in ring tests
4	inherently biodegradable	not readily biodegradable but shown to be biodegradable with other test methods
5	non-biodegradable	after unsuccessful attempts to demonstrate inherent biodegradability

They compared class 1 chemicals (e.g. aniline, sodium benzoate, 1-naphthol, 2-phenylphenol, 4-nitrophenol) with results in natural waters taken from the literature; all were rapidly degraded in the environment. They tested nine other chemicals, for which half-lives ($t_{1/2}$) in natural waters had been reported in the literature, by the ISO 7827 (ISO 1985) method with 5 mg SS/l. All nine chemicals were completely degraded after 9d and this was in good agreement with $t_{1/2}$ values of <1d (m-cresol) up to <14d (4-methoxyphenol), illustrating good predictive qualities of the screening test. This led the authors to propose that efforts should be concentrated on class 2, 3 and 4 compounds and that tests should be used for potential biodegradability in particular compartments (as "surrogate" tests?), thus avoiding the use of the costly simulation tests.

Carson, *et al.* (1990) have demonstrated, using phthalate esters, phosphate esters, biphenyl and LAS, that complex simulation tests, such as complex large-scale micro- or mesocosms like outdoor ponds, would not be required if the behaviour of several (say five) varied standard chemicals was first established in simpler, laboratory microcosm tests. Their solution for prediction of environmental behaviour was to test the chemical in one or more screening tests similar to the Sturm test, the Gledhill CO_2- shake flask test, river die-away test (primary and ultimate biodegradation) and the SCAS test. The chemicals were also tested in simple microcosms containing river water and stabilized sediments; some chemicals were also tested in more complex microcosms containing undisturbed sediments and in outdoor simulated ponds. They found that results from the simpler ("surrogate") tests were sufficient to be able to obtain accurate data concerning the degradation of a chemical; so that the need for using more complex and expensive microcosm tests should be questioned. The biodegradation properties of standard chemicals (of known behaviour in the environment) established along with those of the chemical would allow comparison or ranking of the comparative persistence of the chemical. In this way there would only rarely be a need to conduct more costly studies. Obviously, more chemicals would have to be put through this system, which makes assumptions about the distribution of microbial abilities, before a conclusion on it can be made.

B.1.3 Sea water

Difficulties in assessing biodegradation in sea water are that the concentration of nutrients, especially of N and P, is low so that the concentration of test chemical which can be tested is too low for analytical determination by DOC, and, to a lesser extent, that the density of micro-organisms is relatively low and variable. However, the addition of N and P to sea water does not consistently increase the BOD of the chemicals tested (Street, 1984; Lindgaard-Jorgensen, 1989); the BOD in sea water of a number of chemicals was 10-20% lower than that in BOD dilution water (Street, 1984).

Nyholm, *et al.* (1992) compared the OECD sea water "screening" test, that is, using uninoculated nutrient-supplemented natural sea water containing 20 mg test chemical-C/l with a proposed "simulation" test. The latter test was similar to the shake flask test, but was conducted with ^{14}C-labelled chemicals at 1 to 30 μg/l and with no nutrient addition. Biodegradation was measured as disappearance of ^{14}C in solution; the samples for analysis were prepared by bubbling acidified samples with nitrogen gas to remove $^{14}CO_2$ and filtering to remove biomass. Prediction was good in so far as all those chemicals which were degraded in the "screening" test were also degraded in the "simulation" test; some chemicals degraded in the latter test even when screening tests were negative. However, more chemicals should be tested, as only seven were investigated in this study. Shimp and Young (1987) also compared the OECD sea water DOC die-away test using 20 mg benzoic acid/l with 50 μg/l of ^{14}C-labelled benzoic acid in nutrient supplemented water taken from two estuaries. One estuary was more polluted than the other. The unpolluted water gave variable results (coeff. of variation 12 to 85%) for DOC removal, which reached only about 60% after 8d, but the more polluted water gave less variation (about 10%) and reached 90% DOC removal in 4d. However, using only 50 μg/l, good replication was obtained with both estuarine waters for removal of ^{14}C in solution with 80-85% attained in about 6d. Thus, the OECD test at 20 mg/l gave a wrong impression of benzoic acid in the more pristine estuarine water. The authors suggest that either 20 mg/l was partially inhibitory to the microbial population in the less polluted water, and/or the population in the polluted water was more acclimated to the compound by virtue of the polluting discharges it had received. Other evidence of inhibition to marine micro-organisms was given by Lindgaard-Jorgensen (1989), who found that the rate of primary biodegradation of three chloro-phenols in sea water was lowered appreciably by the presence of a waste water. At 10-18 μg/l, the chlorophenols were degraded at about 1.3-2.8 μg/l.d^{-1} in sea water, but in the presence of a waste water containing organic compounds including chlorinated phenoxyalkanoic acids, the three chlorophenols present at 2-4 μg/l were degraded at a rate about 60 times lower than previously.

Kuiper and Hanstveit (1987) made a different comparison; they compared results of laboratory tests with those from model plankton ecosystems systems – 1.5 m^3 of sea water in plastic bags – and used HPLC to determine primary biodegradation of nine chemicals. Generally, there was agreement between results from the two types of test; two PCBs, 4,6-dinitro-orthocresol and 3,4-dichloroaniline were not degraded in either test. Phenol and the chlorinated phenols tested were degraded in both systems, but differences were found in rates of degradation. At times, the degradation rate of phenol and 4-chloro-phenol was much lower in the model plankton ecosystem than in the laboratory die-away tests with the same batch of sea water. The difference was even larger with 4-nitrophenol and TPBS; sometimes no degradation of 4-NP took place in the ecosystem. The authors attributed the differences to a number of factors, the main one appearing to be that laboratory tests were conducted in the dark. In the plastic bags suspended in the sea, exposed to natural light and temperature, competition for inorganic nutrients could occur between bacteria and algae, as well as there

being other competitive reactions and possibly predation, all of which would lower the rate of degradation of the added chemical.

B.1.4 Other approaches

Larson (1983) compared the pseudo-first order rates of degradation (k_1) in screening tests (Sturm) and in tests using natural water ($^{14}CO_2$) of four compounds associated with detergents. He reported that two – NTA and LAS – showed reasonable agreement using both acclimated and unacclimated natural waters.   However, two quaternary ammonium compounds exhibited far less good agreement with the screening test, showing virtually no degradation due to the toxicity of the cationic compounds at the high concentration (10 mg/l) needed in the Sturm test. Thus, good predictive results were obtained for NTA and LAS, but "fail-safe" poor predictions were obtained for the other two compounds.

Shimp, *et al.* (1990), concerned that the large array of biodegradation data collected over the years for the protection of the environment have not been used very effectively, have proposed a new system. The system relies on a "practical biodegradation" model which relates laboratory-derived or estimated rates of biodegradation to estimates of the time a chemical may spend in a particular environmental compartment (residence time). Thus at least a semi-quantitative estimate may be made of the removal of a chemical due to biodegradation. The authors assume first order degradation so that:

$$\text{half-life} = HL = \ln 2 / k_1$$

and if C/Co is the removal ratio for a chemical at the end of a given retention time, RT,

$$C/Co = e^{-0.693\, RT/HL}$$

Graphs can be constructed relating HL:RT to percentage chemical remaining. They stress that it is not HL alone which controls removal, accumulation or persistence in a particular environmental setting, but the ratio of HL to RT. For example, in a river HL must be short (<1d) for the biodegradation process to be important in the river, but, by contrast, HL of months may be acceptable in some estuaries and in ground water; in other words HL must be less than RT for biodegradation to be practically significant in the compartment in question.

B.2   Concentrations in the environment

Rough estimates of the concentrations of xenobiotic in the environment may be made from the annual amounts of chemical used, the daily volume of water usage per person, and the total population and dilution factors related to discharge of sewage or sewage effluents to rivers or sea. (The degree of biodegradability at each stage, if known, should also be taken into account.) For example, the annual amount of anionic surfactants sold in the UK was about 70,000 tonnes in 1978, the population was 55×10^6 with an average daily usage of water of 240 l, so that the estimated "average" concentration in sewage is:

$$\frac{70{,}000 \times 10^9}{365 \times 55 \times 10^6 \times 240} = 14.5 \text{ mg/l}$$

The recorded concentrations in sewage were in the range 12 to 20 mg/l, and in sewage effluents the concentration was around 0.3 mg/l.

Thus, for every tonne used per year the concentration in sewage is predicted to be about 0.2 µg/l; assuming a dilution of 1 in 10 with no degradation the concentration in river water would be 0.02 µg/l. Similar estimates may be made for discharges direct to rivers from factories synthesizing a chemical. These estimates are made on the assumption that the distribution of the chemical is reasonably uniform. While this is probably true for chemicals such as surfactants which are used by most households, it is not so for "specialty" chemicals, so that the estimate must be made on different patterns of usage if these are known. For example, dyestuffs are usually made in relatively small tonnages/year but concentrated in three or four batches per year. The effluents from the factories where the dyes are made will contain concentrations of the dye which can fairly easily be calculated. The only other significant source will be effluents from textile factories where the dyes are used and, again, the concentration can be estimated from data on dyeing efficiency, adsorption onto sewage solids, etc.

Some examples of reported measured concentrations taken from CEC (1987) are given in the table below:

Table B.2.1   Environmental concentrations of some chemicals by chemical analysis

Chemical	Sample	Location	Concentration (µg/l)
glucose	lake	New Zealand	5
glucose	treated sewage	UK	34
aniline	river	Japan	28
cresols	rivers	USA and UK	0.01-2
phenol	river	Germany	0.2-1.6
benzoic acid	well water	USA	10-100
chloroform	sea water	inshore ocean	0.1 0.015
carbon tetrachloride	sea water	inshore ocean	0.15 0.005

B.3   Kinetics of biodegradation in aqueous systems

B.3.1 Introduction

In order to be able to predict concentrations of chemicals in the environment more precisely and accurately than can be obtained by the approximate method described in Section B.2, it is necessary to know the kinetic constants of degradation of chemicals in the various environmental compartments. By ascertaining by experimentation the kinetic equation which best fits degradation in the compartment of interest, the constants associated with the equation may be calculated. These constants are then used in the chosen "fate" model of the many which are available (for example, EXAMS, QWASI, EXWAT, MEXWA; see ECETOC, 1991) for predicting the exposure level as a function of time, distance along a river, etc. Models are also used for predicting the concentration of chemicals in effluents from waste water treatment plants under a wide variety of operating conditions.

The present OECD tests, even in improved form, provide only the percentage removal of a test chemical after a given period. Although the tests were not designed for obtaining values of kinetic constants, the sigmoidal degradation curves they produce can be used to provide such constants (for example, for anionic surfactant removal, Downing and Knowles, 1967; Painter and Durrant, 1976) and, by a process of re-iteration, "best-fit" values are obtained. Conversely, sets of theoretical decay curves (but not allowing for lag periods or the need for adaptation of one sort or another) can be calculated to demonstrate limiting values of μ (specific growth rate of bacteria) for different initial bacterial cell densities (Painter and King, 1983; Blok and Booy, 1984). These studies were based on the Monod equations (see later), but values for the saturation constant (K_s) had to be assumed. From the data obtained from these combined studies, reasonably accurate predictions could be made of the behaviour of chemicals in waste water treatment plants, but extrapolation to events in rivers, lakes, etc. could not be made safely because of the differences between the eutrophic and oligotrophic modes of life.

Two thoroughly comprehensive reviews of the kinetics of biodegradation and their application to calculations of environmental concentrations have recently been published by Battersby (1990) and ECETOC (1991). These reviewers have discussed the many proposed equations/models which have been put forward. However, both concluded that the Monod and the Michaelis-Menten models are the most satisfactory and that the approach by Simkins and Alexander (1984) yielding a series of six simplified equations is suitable for the purposes of biodegradability testing. These derived equations can be used for primary biodegradation or mineralization, but different values for the kinetic constants will result.

It must be emphasized that in tests for degradation in soil, consideration must be given to other important environmental fate processes such as sorption/desorption and diffusivity.

B.3.2 Additional literature

It has been found that in practice, especially in waste water treatment, the Monod and Michaelis-Menten equations are not always obeyed and the two reviewers have given some examples. A few further examples of limits and modifications are cited here.

Jones (1973, 1976) suggested from his experiments that only a minority of cells (viable) present in activated sludge divided within the system and that the majority (non-viable) metabolized substrates without dividing. He added a Michaelis-Menten term to his Monod

equation to allow for this metabolism without growth, and obtained better agreement between predictions from his model and experimental data from both die-away and continuous activated sludge tests. Other authors, for example Schmidt, *et al.* (1985), have added similar terms and also further terms to allow for cell maintenance.

Grady and Williams (1975) reported that the Monod equation was obeyed by pure continuous culture with single substrate, but not when mixed cultures and mixed substrates were used. In the latter case the equilibrium (effluent) concentration, expressed as COD, was affected by the influent concentration, whereas theory predicts that effluent concentration should be independent of the influent concentration. The authors modelled their system by a linear approximation of the Monod equation:

$$S = k^1 S_0 D + k^{11} S_0 \text{-------------------(1)}$$

where S = COD in effluent
        S_0 = COD in influent
        D = dilution rate
        k_1 = proportionately constant relating k and S_0
        k_{11} = $k_1 b$
        b = bacterial specific decay rate constant.

Costa, *et al.* (1985) in a study of ethylene glycol reported that degradation in batch and continuous activated sludge tests was best represented by:

$$dS/dt = - KB(S/S_0) \text{ or } - KB(S/S_0)^n$$

where K = constant
        B = bacterial density,

rather than by other models, including Monod and Michaelis-Menten equations. Using Monod expressions, Cech, *et al.* (1985) found that values of K_s and μ_{max} were lower for sludge grown in completely mixed reactors (filamentous organisms) than with those cultivated in selector type reactors (non-filamentous organisms).

The conventional equations were rejected by Sissons, *et al.* (1986) who adopted a phenomenological approach in attempting to represent the lag, acceleration, deceleration and endogenous phases of growth. Monod and Michaelis-Menten deal only with the logarithmic phase. They proposed three states of bacteria as being important:

- viable and reproducing cells;
- viable without a full complement of enzymes; and
- non-viable, dying or dead cells.

They found reasonable agreement in batch die-away tests at 100-500 mg substrate/l but not in continuous activated sludge; the authors thought this was due to the flocculated nature of the sludge.

A review of methods for determining the saturation constant, K_s, of the Monod equation (Williamson and McCarty, 1975) showed that all methods considered had serious disadvantages, whether batch or continuous systems were used. The authors proposed and validated a new technique which they called the "infinite-dilution" method, which used the Lineweaver-Burke procedure for the calculation.

Other factors, such as inhibition and dissolved oxygen concentration, affect the rate of removal. Larson, *et al.* (1981) reported that the half-life (= ln2/1st order reaction constant) for the degradation of nitrilotri-acetic acid increased from 1.3d at oxygen saturation to 5.8d at 0.3 mg DO/l. This effect was quantified by Shaler and Klecka (1986) by adapting the Monod equation so that it described the relationship between specific growth rate, μ, and the concentrations of both substrate and dissolved oxygen.

B.3.3 The Monod equation

As mentioned earlier the Monod equation is widely accepted, although it is realized that there are certain limitations in applying it to biodegradation in the aquatic environment. More complex equations, twelve in all, have been produced to take account of such factors as inhibition and secondary substrates by Schmidt, *et al.* (1985). But for the purposes of biodegradability testing these additional equations do not yield sufficient extra information to justify the effort required.

Simkins and Alexander (1984) expressed the Monod equation as

$1/B.dB/dt = \mu = \mu_{max}.S/K_s + S)$ ---------(2)

where　B 　=　concentration of bacteria degrading the substrate
　　　　μ 　=　specific growth rate of these bacteria
　　　　μ_{max} 　=　maximum value of specific growth rate
　　　　S 　=　concentration of substrate
　　　　K_s 　=　saturation constant, equal to the concentration of substrate giving growth rate of $\mu_{max/2}$,

and proposed that the variety of substrate removal curves experienced in biodegradability testing is the result of interaction between S and B.

B.3.4 Batch systems

They expressed a mass balance in batch systems by

$S_0 + qB_0 = S + qB$ ---------------(3)

to accommodate changes in population density,

where　S_0 　=　initial substrate concentration
　　　　B_0 　=　initial bacterial density
　　　　q 　=　a cell quota, the reciprocal of cell yield, Y.

The value of q is said to be reasonably constant when carbon is the limiting nutrient, but changes markedly with concentrations of P and N; thus they took q to be a constant. The actual value of q is not needed when substrate disappearance is of interest and qB was replaced by X which corresponds to the amount of substrate required to produce a population density equal to B. Similarly, X_0 is defined as qB_0, so that equation (3) becomes

$S_0 + X_0 = S + X$ ------------(4)

and (2) may be written as

$$dX/dt. \; 1/X = \mu_{max} \; S/(K_s + S) \; ------------(5)$$

By solving for X and dX/dt from equation (4) and substituting in equation (5) a differential equation is obtained:

$$-ds/dt = \mu_{max}S(S_0 + X_0 - S)/(K_s + S) \; ------------(6)$$

The authors called this the "integrated Monod equation", which may be approximated by six simplified forms representing extreme ratios of inoculum density to initial concentration of substrate, or of substrate concentration to K_s. These forms are given in **Table B.3.1**, which also contains the conditions yielding them and the derived rate constants. The six equations and the conditions under which they operate are best understood by reference to **Figure B.3.1** (Simkins and Alexander, 1984). As an example, K_s is taken as 1 mg/l and is represented by the full vertical line, and q is taken as 1 pg substrate/cell. Points along the diagonal line represent inoculum sizes which yield one division of the active cells at various initial substrate concentrations. Initial cell densities above the line at a given substrate concentration indicate approximately constant cell densities, since there would be insufficient substrate to produce a significant increase in cells. The broken vertical line is placed at a concentration at approximately 1.5 orders of magnitude greater than K_s and separates conditions under which the uptake systems of the active organisms are efficiently saturated until near exhaustion of substrate (right of the line), from those under which the biodegradation rate per cell varies appreciably with concentration of substrate. From the two vertical lines and the diagonal line, the approximate ranges of initial concentrations of substrate and cell densities which should show mineralization kinetics corresponding to the six models can be predicted. Other substrates and populations would be represented by similar diagrams, but the vertical lines and the vertical intercept of the diagonal line would be in different positions. Also, the position of the lines is somewhat arbitrary and may vary with the precision of the methods used.

At concentrations of substrate below K_s, which is the normal situation for nearly all chemicals in the aquatic environment, degradation can be described by first order or logistic kinetics. In zone A (**Figure B.3.1**) no significant increase in cells occurs, so that first order kinetics apply and in zone B the small population grows on the test chemical at ever decreasing rates, typical of logistic kinetics, but the precision of the data may not be good enough to distinguish between the two types (Alexander, 1985). Zone D represents the addition of a small number of cells to a system containing substrate at a concentration greater than K_s and degradation is described by classical Monod kinetics, growth occurring as the substrate disappears. On the other hand, when the substrate concentration is much greater than K_s, the bulk of the substrate will be degraded while the uptake systems are saturated. These conditions, in zone F, allow $(K_s + S)$ to be approximated to S and kinetics follow the logarithmic model until the substrate is nearly exhausted. When the initial concentration of substrate is insufficient to support a significant increase in population and when the uptake systems are saturated, as in zone E, substrate removal will be linear and zero-order kinetics apply. Lastly, the Monod (no growth) kinetic equation can be used in situations in which the uptake systems of the active bacteria are not saturated at the start of incubation, although the initial concentration of substrate is insufficient to support a significant increase in biomass (zone C). This "no growth" equation is analogous with Michalis-Menten kinetics for enzyme catalysed reactions (see later).

Table B.3.1 Models for biodegradation kinetics, using only the variables of substrate concentration and bacterial cell density (Simkins and Alexander, 1984)

Model	Necessary conditions	Equation $-ds/dt=$	Rate constants (units)
A First-order	$X_0 \gg S_0$ $S_0 \ll K_s$	$k_1 S$	$k_1 = \mu_{max} X_0 / K_s$ (h^{-1})
B Logistic	$S_0 \ll K_s$	$k3 S(S_0 + X_0 - S)$	$k3 = \mu_{max}/K_s$ $(1h^{-1}mg^{-1})$
C Monod (no growth)	$X_0 \gg S_0$	$k0 S/(K_s + S)$	$k_0 = \mu_{max} X_0$ $(mgl^{-1}h^{-1})$
D Monod (growth)	None	Equation (6)	μ_{max} (h^{-1})
E Zero-order	$X_0 \gg S_0$ $S_0 \gg K_s$	k_0	$k_0 = \mu_{max} X_0$ $(mg\ l^{-1}h^{-1})$
F Logarithmic	$S_0 \gg K_s$	$\mu_{max}(S_0 + X_0 - S)$	u_{max} (h^{-1})

Biodegradation curves representing the various kinetic models are shown in **Figure B.3.2** (Alexander, 1985). It should be borne in mind that in the Monod equation used it is assumed that the cell yield is constant with time. Also, the model does not apply to organisms requiring a lag period before biodegradation occurs and that other factors affect degradation in the environment, such as the present of other substrates, predation by protozoa and phage, toxic chemicals produced by other organisms and the availability of inorganic nutrients, discussed elsewhere (in A.2.2 and B.4).

Briefly, the method for obtaining kinetic constants for the environmental conditions of interest is to determine the biodegradation curve, starting with the relevant initial concentration of the chemical, in river water, river water plus sediment, estuarine water, etc. The disappearance is followed by one of a number of methods, depending on the conditions and on the purpose. HPLC or GC may be used for primary biodegradation if a suitably sensitive analytical method is available. Mineralization can be followed by following CO_2 production, usually $^{14}CO_2$ at low concentrations of test chemical or by determining residual ^{14}C in solution, obtained after membrane filtration or centrifuging and removal of CO_2. The plotted curves may be compared with curves A to F in **Figure B.3.2**, but to obtain values for the appropriate rate constant the data are fitted to each model (or to two or three, if some can be eliminated) by non-linear regression analysis (using MARQFIT computer program, Simkins and Alexander, 1984). The program fits the data by minimizing the least-squares differences between the data and the model curve. The model giving the lowest residual sum of squares for a particular data-set was taken to be the model of best fit, but only if the difference between it and models with fewer parameters was significant at the 90% confidence level or higher.

Figure B.3.1 Kinetic models as a function of initial substrate concentration and bacterial cell density (Simkins and Alexander, 1984)
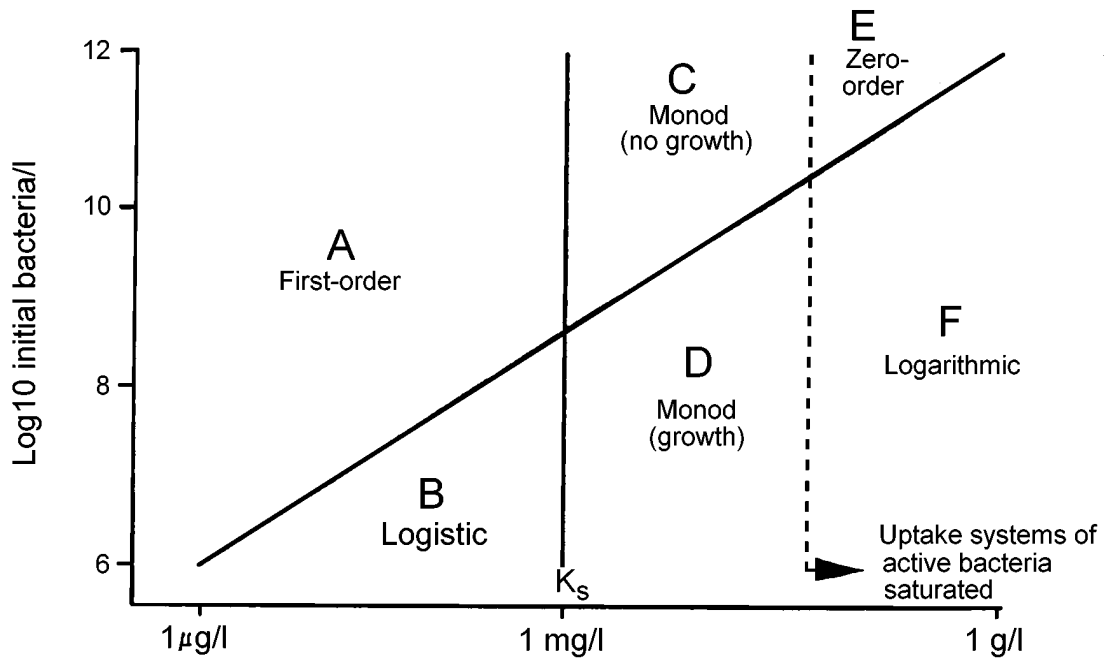
Figure B.3.2 Disappearance curves for a chemical degraded according to the kinetic models shown above (Alexander, 1985)
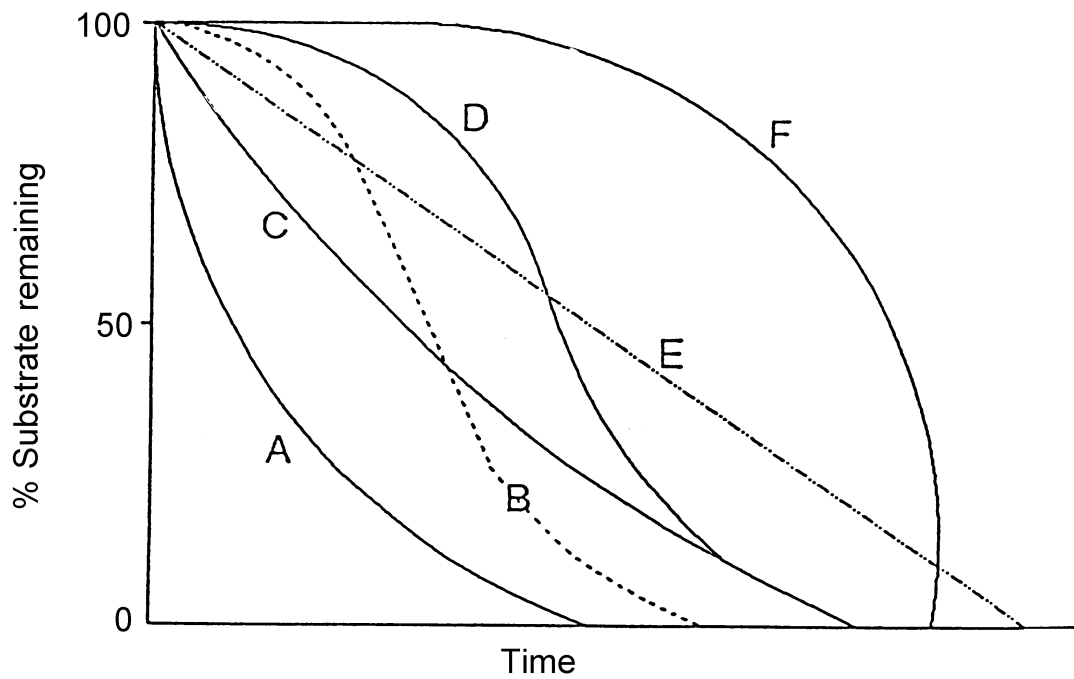
B.3.5 Second-order kinetics

The Monod equation can be approximated to

$$-dS/dt = \mu_{max}/(YK_s). S.B \text{ -----------(7)}$$

when S is much lower than K_s, as it is in most aquatic environments. Paris, *et al.* (1981, 1982) have reduced it to

$$-dS/dt = k_2. S.B \text{ -----------(8)}$$

where k_2 is termed the second-order rate constant (= μ_{max}/YK_s). First, the pseudo-first order reaction rate constant is determined by adding the test chemical, at a concentration expected to be present, to the relevant environmental sample (river water, etc.). The disappearance of the chemical is monitored by specific analysis or by organic-^{14}C in solution and a plot of log S against time should yield a straight line. The pseudo-first order constant, k_2B, is calculated from the slope by linear or non-linear regression of the data (e.g. Larson, 1980). Samples of the culture taken for chemical analysis are also examined for bacterial density, by one of a variety of methods. The second order constant, k_2, is then calculated by dividing the pseudo-first order constant by the bacterial density, that is

$$k_2 = (k_2B)/B.$$

This "normalises" the observed pseudo first-order constant for biomass (Paris, *et al.*, 1981; Larson, 1984). The cell density, B, has been determined in terms of colony-forming units, by statistical plate counts, acridine orange staining, dry weight, ATP.

This method assumes that:

- plate counts, or other methods used, detect similar percentages of total bacterial population in all samples;

- all, or a constant proportion of, enumerated bacteria can transform the chemical;

- transformation rates per cell (or average rates per microbial assemblage) are approximately the same for all sites examined.

However, Battersby (1990) concludes from data in the literature that, while the three conditions may hold for easily hydrolysable chemicals transformed by free-floating or attached micro-organisms, this is unlikely to be the case for mineralization, or for recalcitrant molecules.

B.3.6 Secondary substrate utilization

Another application of Monod kinetics is to the biodegradation of secondary substrates, at concentrations below their threshold values for degradation, in the presence of other substrates at higher concentrations (Bouwer and McCarty, 1985). The limiting value for secondary substrate concentration, Smin, is given by:

$$Smin = K_sb/(Yk - b) \text{ -----------(9)}$$

where K_s = concentration of substrate giving half maximum rate of removal of substrate
k = maximum specific rate of removal of substrate
b = first order decay constant of the bacteria.

However, the authors demonstrated that in the presence of other (primary) substrates at higher concentrations than their Smin values, the secondary substrate may be degraded. For example, in biofilms the utilization rate of the secondary substrate within the film is given by:

$$- dS_f/dt = k.B_f.S_f/(K_s + S_f) \text{ -----------(10)}$$

where S_f = rate limiting concentration in the biofilm
B_f = active cell density

Since S_f is much lower than K_s,

$$- dS_f/.dt = k.B_f.S_f/K_s \text{ ------------(11)}$$

Thus, for trace substrates, there should be a linear relationship between substrate flux into the biofilm and substrate concentration; at equilibrium the rate of degradation of secondary substrate should also be directly proportional to the density of active organisms and to the ratio k/K_s.

B.3.7 Continuous systems

So far, only batch systems have been considered. In waste water treatment systems (continuous culture) the mass balance equation

$$S + X = S_0 + X_0 \text{ ------------(4)}$$

does not apply, and substrate uptake and change in biomass are considered separately. One treatment is given by Birch (1984, 1991) for primary degradation (of surfactants), and this led to the following equation which incorporates a biomass decay factor (Kd):

$$S_e = \frac{K_s (1 - K_d \cdot SRT)}{SRT (\mu_{max} - K_d) - 1} \text{ ------------(12)}$$

where S_e = equilibrium concentration of substrate in effluent
SRT = sludge retention time (reciprocal of dilution rate).

Birch (1984, 1991) operated a series of porous pots, modified to make control of the sludge wastage rate continuous and more easy, with all parameters identical except for the sludge retention time, SRT, and the equilibrium concentrations (S_e) of the chemical in the effluents were determined. Plotting SRT against S_e enables the K_s and μ_{max} of the system to be estimated, and it is possible to calculate the critical sludge retention time below which the competent species will be washed out. The model also indicates that the effluent concentration is independent of the concentration of chemical in the influent and that the concentration of sludge, and competent micro-organisms, self-adjust at equilibrium.

B.3.8 Michaelis-Menten kinetics

The Michaelis-Menten equation, describing the rate of an enzyme catalysed reaction, is:

$$v = dS/dt = V_{max}S/(K_m + S) \text{ ------------(13)}$$

where v = velocity of reaction (substrate removal)
V_{max} = maximum velocity of reaction for a given concentration of enzyme
S = concentration of substrate (test chemical)
K_m = Michaelis constant, equivalent to a substrate concentration giving v of $V_{max}/2$

Under most environmental concentrations S will be much lower than K_m, so that equation (13) will approximate to:

$$v = V_{max}S/K_m \text{ ------------(14)}$$

If both V_{max} and K_m are constant

$$v = k_1 S \text{ -------------(15)}$$

and substrate removal will follow first order kinetics. However, since V_m is dependent on enzyme concentration, k_1 here is only a pseudo first-order constant.

B.3.9 "Heterotrophic" activity method

Equation (13) has been used to calculate V_{max} and K_m; for example, Pfaender and Bartholomew (1982) modified earlier methods of Wright and Hobbie (1965) and others. They determined both the assimilation of ^{14}C from labelled substrates into microbial cells and the evolution of $^{14}CO_2$ in experiments in which the environmental water samples were incubated for relatively short periods (6-10h) in the presence of low concentrations of the test chemical. Timed samples were membrane-filtered to measure the ^{14}C incorporated into bacterial cells, while replicate vessels were acidified in situ to collect $^{14}CO_2$ on KOH-impregnated filter paper to measure the amount of chemical mineralized. The rate of biodegradation (v) was calculated from

$$v = \frac{s/t \text{ x dpm in cells } + \text{ dpm in } ^{14}CO_2}{\text{dpm originally added}} \text{ ------------(16)}$$

where S = concentration of test chemical added
t = period of incubation
dpm = disintegrations per minute.

By using a range of initial concentrations of test chemical, K_m and V_{max} may be calculated from the slope and intercept of the Lineweaver-Burke plot of $1/v$ against $1/S$. Larson (1984) refined the method to allow for any of the test chemical already present in the environmental samples.

B.3.10 Empirical pseudo first-order relationship

Larson (1980) used equation (15), a pseudo first-order relationship, to determine the half-life of chemicals in the environment. The concentration of the test chemical in the environmental sample (or loss of radioactivity if used) was followed over a much longer period than in the "heterotrophic" activity method. The constant (k_1) was calculated from the slope of the straight line plot of log. concentration versus time. This method relates to primary degradation, but Larson (1980, 1984) also applied first-order kinetics to the mineralization of chemicals, using the expression:

$$P = a(1-\exp[-k_1(t-c)]) \text{ -------------(17)}$$

where
$$
\begin{aligned}
P &= \text{concentration of product formed } (CO_2 \text{ or } {}^{14}CO_2) \\
a &= \text{extent of biodegradation (asymptote)} \\
c &= \text{lag time prior to the onset of biodegradation, and } t > c.
\end{aligned}
$$

It is used to calculate a half-life ($t_{\frac{1}{2}}$) for the chemical from

$$t_{\frac{1}{2}} = \ln2/k_1 \text{ -----------(18)}$$

However, this is a pseudo first-order reaction so that $t_{\frac{1}{2}}$ may not be independent of concentration of the chemical, as it would be for a true first-order reaction. Thus, the value obtained may apply only to the conditions of the test. For example, at very low concentrations the rate of biodegradation may be limited by the rate of entry of the chemical into the cell and at even lower concentrations a threshold value may exist below which the chemical is not degraded.

B.4 Events in the environment

Many factors have to be taken into account when considering events likely to take place in the environment (see **Table B.4.1**); some of these factors have been discussed in section B.1 (Prediction). Broadly, the factors may be divided into those which concern the chemical substrate, the bacterial populations and other organisms, the presence of inorganic compounds and other organic chemicals, and interactions between some or all of these.

Inorganic nutrients have been discussed earlier (section A.2.2); it should be added that if the concentrations of N,P, etc. are insufficient, competition between target chemical-degraders and other bacteria or between bacteria and, for example, protozoa could result in lowering of, or no, biodegradation. The questions of pre-exposure leading to adaptation were considered in section A.4.4, while cometabolism in its narrower sense is discussed in section B.5.

B.4.1 Substrate

First, the concentration of the test chemical is important in determining whether and at what rate the test chemical will be degraded; this has been discussed in section A.5.2 and also in section B.3 on kinetics. At higher concentrations than those defined by Alexander (1985) to be normal for heterotrophic bacteria and fungi (1 to 100 mg/l), a chemical may be inhibitory; some are inhibitory within this range. At very low concentrations, that is, lower than 10 µg/l, there may be a threshold below which the chemical will not support growth and a lower threshold below which the energy requirements for cell maintenance are not met. In

some cases, a chemical can be degraded or transformed at these very low concentrations (below threshold values) by large populations of cells which are not growing, while other chemicals must support growth to achieve significant degradation. The values of the threshold concentration are determined largely by whether the bacteria are eutrophic, e.g. those found in polluted river, or oligotrophic, e.g. those in a largely unpolluted river (see later).

Table B.4.1 Factors affecting biodegradation in the environment

Factor		Effect
A Test chemical	concentration	determines rate inhibition thresholds - growth - maintenance
	availability - sorption - speciation - conjugation - emulsion formulation	)) increases or decreases removal rate) could increase rate
	duration of contact with micro-organisms	opportunity for adaptation
B Mixed substances	concentrations	simultaneous removal
	nature	sequential removal
	temperature	stimulation/inhibition
	"secondary substrate utilization" - primary - higher conc - secondary - lower conc	secondary substrate degrades below threshold
C Microbial populations	consortia, communities - interactions: mutualism, syntrophy, etc.	degradation where none or higher rates than with single species
	other micro-organisms - protozoa - exudates - products	predation/inhibition inhibition inhibition
	eutrophic bacteria	active at higher concentrations higher thresholds
	oligotrophic bacteria	active at lower concentrations lower thresholds
D Abiotic processes, e.g. hydrolysis, photo-oxidation		can augment biodegradation

B.4.2 Adsorption

The general availability of the test chemical can affect its degradation rate; the chemical may be adsorbed, conjugated or bound in some other way so that biodegradability is hindered or enhanced. For example, Subba-Rao and Alexander (1982) reported that the presence of clay may have significant effects on the microbial degradation of low concentrations of certain chemicals. By adsorbing on to the surfaces, chemicals would be less available and their rate of degradation would decrease. But if bacteria were also adsorbed on to the surface, thus increasing the bacterial density the rate of degradation would increase. At 20 ng/l to 200 ng/l in Lake Beebe water, benzylamine (BA) degradation was inhibited by all four concentrations of clay tested; but at 20 mg BA/l 63% was mineralized without clay in 4.5d, while >90% was removed in the presence of the four concentrations of clay tested (11 to 5400 mg/l) in the same time. At 200 μg BA/l there was virtually no effect. Later, using different conditions, Miller and Alexander (1991) reported different effects due to the presence of clay. A *Pseudomonas sp.*, isolated from soil and pre-grown on glucose-benzylamine, was added to a clay suspension of 1 g/l of a mineral salts solution. The suspensions were inoculated to give high cell densities, whereas Subba-Rao and Alexander (1982) had used the cells present in Lake Beebe water. At 0.95 mg BA/l and 10^7 cells/ml, slight inhibition due to the presence of clay was observed but at 25 mg BA/l and 10^8 cells/ml inhibition was greater, the t_{50} value being increased from about 5h to 20h. The authors made no comments on the difference. The mineralization of benzoate was not usually affected by the presence of clay, Kaolinite or glass-beads.

In settled sediments, Shimp and Young (1988) found that the rate of degradation of dodecyltrimethyl-ammonium chloride (TMAC) was proportional to the unadsorbed TMAC concentration; for phenol the rate was a function of its total concentration. Thus the biodegradation of a chemical in sediments was influenced by the structure of the chemical and its mechanism of adsorption. However, in aerated mixtures of sediments and water (slurries) with up to 10 g solids/l, the rate of biodegradation of TMAC was a function of the total amount of TMAC-adsorbed plus unadsorbed. The authors concluded that though some chemicals may not be directly available in settled sediments, they are available in slurries. Thus, the behaviour of a chemical in sediment cannot be predicted from a single laboratory test system. Settled columns can be used as realistic surrogates for bottom sediments and slurries could be used to represent situations with predominantly suspended particles.

About 50% of toluene, at 10 to 60 mg/l, was adsorbed onto soil in a few days and a small proportion was adsorbed more slowly. Some 90% of the sorbed fraction was extractable and once desorbed it was quickly degraded. The remaining adsorbed fraction (10%) was initially undegraded but was eventually biodegraded at a rate limited by the rate of desorption (Robinson, *et al.*, 1990).

B.4.3 Speciation

The effects of speciation of four chemicals on their biodegradation were examined by Madsen and Alexander (1985). The biodegradation of the complexing agents, oxalate, citrate, nitrilotri-acetate (NTA) and ethylenediaminetetra-acetic acid (EDTA), was determined at 1, 10 and 100 μg C/l in media carefully prepared so that the H-, Ca-, Mg-, Fe- or Al-complex, respectively, was predominant. The pH value and ionic strengths were kept at the same values in all media. Sewage inocula mineralized Ca citrate faster than Fe, Al and H citrates, while the Mg salt was degraded at the lowest rate. There was a different order for the rates of degradation of oxalates: although Mg oxalate degraded at the lowest rate, Ca oxalate degraded only slightly faster and the other three were degraded faster still. Of the

NTA complexes, only Ca-NTA was mineralized during 24d. EDTA was undegraded in any form. Tests with pure cultures of five species indicated that the differences in mineralization for four of the species were not related to toxicity or nutrient limitation. The authors offered possible explanations for the observed differences based on transport into the bacterial cell but indicated that further investigation is needed to evaluate the impact of chemical speciation on biodegradation in natural ecosystems. Larson (1983) reported that a cationic surfactant (octadecyltrimethylammonium chloride, OTAC) did not degrade (and was inhibitory) at 10 mg/l in a CO_2 evolution test inoculated with "acclimated" activated sludge. But when a mixture of 10 mg OTAC/l and 10 mg LAS/l was tested 77% joint $ThCO_2$ was evolved, indicating that the "compound" produced between the anionic and cationic surfactants was degradable though on its own the cationic surfactant was not. At 10 µg/l OTAC was found to be biodegraded. Similarly, Masuda, *et al.* (1976) reported degradation of a mixture of 10 mg/l each of dodecyl sulfate and a dialkyl dimethyl-ammonium chloride, but the cationic surfactant alone did not degrade at this concentration.

B.4.4 Conjugation

A large proportion of the dissolved organic carbon in fresh waters is present as fulvic acids (Liu, *et al.*, 1983) and many chemicals are known to be able to bind to such humic materials (e.g. Anderson, 1986). Liu, *et al.* (1983) observed enhancement by the addition of 50 mg fulvic acid/l of the biodegradation of 2-(methylthio)-benzothiazole (MMBT) at 5 mg/l and 2,4-dichlorophenol (24-DCP) in cyclone fermentors, inoculated with cultures derived from activated sludge. The t_{50} for MMPT fell from 110 to 62h in the presence of fulvic acid and from 235 to 145h for 24-DCP. The authors referred to their earlier work in which fulvic acid enhanced the biodegradation of PCBs and insoluble hydrocarbons, possibly by the formation of micro-emulsions. However, this could not be the explanation in the case of MMBT and 24-DCP since they were completely soluble at 5 mg/l. Liu (1980) also showed that an emulsion of Na ligno-sulfonate and PCBs greatly enhanced the degradation of the halo-compounds, presumably by increasing the interfacial area.

B.4.5 Mixed substrates

In batch tests at 20°C, simulating waste water treatment conditions and using heterogeneous populations previously grown on a simple synthetic medium in such a manner as to maintain the diversity of the population, Stumm-Zollinger (1966) reported various situations in two-substrate utilization. The concentrations of the substrates were in the range 5 to 500 mg/l. No interactions were observed between glucose and benzoate; each substrate was utilized at rates independent of the presence of the other. With other pairs of substrates – glucose/galactose, glucose/L-phenylalanine, benzoate/L-phenylalanine – inhibition of the degradation of the second substrate of each pair or repression of the necessary enzymes occurred, so that sequential removal was observed. However, at 7°C no interaction between glucose and galactose was seen and the two compounds were degraded simultaneously. Under similar conditions, acetate and glucose, glucose and LAS, and sucrose and LAS were each degraded in the two-substrate mixtures at rates independent of the presence of the other substrate (Painter, *et al.*, 1968). However, in glucose/fructose mixtures, sequential oxidation occurred on two occasions out of six glucose being degraded first. The degradation of LAS (10 mg/l) in BOD dilution medium inoculated with 30 mg/l activated sludge was unaffected by the presence of membrane-filtered sewage, 50 mg/l of glucose or sucrose, provided that the pH value was maintained above 6 (Painter and Durrant, 1976).

There are several examples of an inhibitory action by the addition of a second organic chemical. The addition of 0.95 g peptone/l to Delaware river water increased the lag period in die-away tests from 8 to 14d and the time for complete removal of 2,4,6-trichlorophenol (50 μg/l) from 14 to 22d (Blades-Fillmore, *et al.*, 1982). The possibility that pH played a role here is high, since the medium was river water and the degradation of 950 mg/l of peptone could have produced a pH change. The inhibition by 10 g/l of glucose or phenol of the mineralization of 4-NP in water from a mesotrophic lake was dependent on the concentration of 4-NP (Subba-Rao, *et al.*, 1982). At 22 ng/l no inhibition occurred but at 200 μg/l the removal was reduced from 72% (no glucose) to 14% (glucose) and 18% (phenol). Here, pH does not seem to have played a part since 10 g glucose/l did not inhibit the mineralization of low concentrations of phenol.

Glucose and amino acids, added separately, severely inhibited the mineralization of toluene, 4-NP and ethylene dibromide in slurries of aquifer solids (Swindoll, *et al.*, 1988). For example, glucose at 28 ng/g and a mixture of amino acids at 2.2 μg/g reduced the $^{14}CO_2$ evolved from 4-NP in 45d from 50% to <5% $ThCO_2$. By using radio-labelled glucose and amino acids, it was shown that 4-NP had little or no effect on %$^{14}CO_2$ evolved from the natural substrates.

Other examples are the inhibition by 500 mg/l of glucose or peptone of the conversion of 4,41-dichlorobiphenyl (50 mg/l) to 4-chlorobenzoic acid by mixed cultures (Tulp, *et al.*, 1978) and the inhibition by pyrrole of benzene oxidation by biomass from a fixed biofilm system (Arvin, *et al.*, 1989).

Examples of stimulation of the degradation of a chemical at low concentration by the addition of another chemical are given by Alexander and his co-workers. Wang, *et al.* (1984) found that the addition of low concentrations of non-chlorinated water pollutants or inorganic nutrients (but not yeast extract or aromatics) enhanced the degradation of isopropyl N-phenyl-carbamate (IPC) at 0.2-0.4 μg/l in lake water; there was no effect on 1 mg/l at which IPC was converted into organic products. In sewage, IPC was degraded at 0.4 μg/l and 1 mg/l. The authors suggest that the differences were due to an oligotrophic population being active in lake water and a eutrophic population in sewage. Alternatively, there was a single population, not two, which mineralized at one concentration and cometabolized at another. Novick and Alexander (1985) reported stimulation by 200 mg glucose/l, but not aniline, in increasing the products of cometabolism of 2-chloro-N-isopropylacetanilide at 63 ng/l to >100 μg/l. Glucose (100 mg/l added to lake water inoculated with *Corynebacterium sp.* increased the mineralization of 4-NP (26 μg/l) from 25% to 75% after 2d (Zaidi, *et al.*, 1988).

An important phenomenon, described among others by McCarty, *et al.* (1981), concerns the ability of cultures of single bacterial species to degrade xenobiotic compounds, present at concentrations below their threshold concentration for degradation, when the population is supported through energy derived from another substrate present at concentrations usually much higher than its threshold. It is known as secondary substrate utilization, and earlier had been shown to occur in mixed cultures. *Pseudomonas sp.* strain LP, isolated from sewage, was able to degrade acetate and methylene chloride simultaneously (La Pat-Polasko, *et al.*, 1984). When acetate was the primary substrate (1 mg/l), the rate of removal of methylene chloride (10-100 μg/l) was increased, but when the roles were reversed the rate of acetate was reduced and was lower than that of methylene chloride. Schmidt and Alexander (1985) studied the behaviour of pure cultures in removing the uncharacterised DOC in distilled water and in removing secondary substrates under various conditions. *Salmonella* typhimurium could not utilize the uncharacterised DOC; neither was 0.5 μg glucose/l utilized, but the addition of 5 mg arabinose/l caused the glucose to be removed rapidly, thus substantiating secondary substrate utilization. Also, *Pseudomonas acidovorans* grew on the

DOC present (equivalent to about 90 μg phenol/l) in distilled water and was able to mineralize 2 μg phenol/l simultaneously.

Bouwer and McCarty (1984) extended the study to continuous flow models using laboratory biofilm reactors with natural mixed cultures, treating acetate at 1 mg/l as the primary substrate. The second substrates were chlorobenzene, 1,2-dichlorobenzene, 1,4-dichloro-benzene, 1,2,4-trichlorobenzene, ethyl benzene, styrene and naphthalene. All these were added at about 10 μg/l and all were well removed in aerobic systems (but not in anaerobic systems) at a retention time of about 20 minutes. Over 85% of 1.5 μg/l of chlorobenzene was removed in the reactor. Some chemicals were shown to have been mineralized by the use of ^{14}C-labelling. Some chlorinated aliphatics were tested but were not degraded aerobically, although they were removed anaerobically.

Kim and Maier (1986) reported that addition of nutrient broth (100 mg/l) and 2,4D or 3,5-dichlorobenzoic acid to activated sludge previously "acclimated" to the xenobiotic resulted in concurrent utilization. In these cases, however, the lowest concentration of xenobiotic used was 100 μg/l and nutrient broth slightly inhibited removal at this concentration. Nyholm, *et al.* (1991) used concentrations down to 5 μg/l of 2,4,6-TCP, 4-NP, PCP, 2,4-D and lindane and showed that they were removed by 70-95%, largely by biodegradation, after various times of adaptation when fed with a synthetic peptone sewage to laboratory activated sludge units.

The concentration-dependent primary and secondary substrate relationships in the concurrent metabolism of phenol and acetate by a *Pseudomonas sp.* isolated from soil was investigated by Heiman and Cooper (1987), who confirmed, in general, the findings of La Pat-Polasko, *et al.* (1984) and Schmidt and Alexander (1985). With acetate (5 mM) as primary substrate and phenol at 1 mM, growth occurred as in the absence of phenol and phenol was readily removed. In the absence of acetate, phenol was removed at about ten-fold that when acetate was present. With phenol the primary substrate at 100 mg/l its removal was enhanced only when acetate was present at equimolar or lower concentrations. These experiments were repeated with ^{13}C-labelled sodium acetate and the carboxy label was determined semi-quantitatively by solid state ^{13}C NMR spectroscopy. This allowed direct examination of C in all of the sample components of the intact lyophilized cells, taken from the respective cultures when all the phenol had been taken up, without the need for extractions, isolations, or other complex operations on the sample. The analysis indicated by virtue of a significant redistribution of the carboxy label that acetate metabolism was extensive and that the presence of phenol as a secondary substrate did not affect the redistribution. However, when phenol was primary the metabolism of acetate was altered; more carboxy label appeared in the methyl and acyl chain methylene C resonances and less appeared in the carbohydrate spectral region. The authors concluded that under the conditions of the biodegradation tests, acetate is preferentially shuttled into fatty acid synthesis while phenol C is funnelled into the tricarboxylic acid cycle.

A possible example of secondary substrate utilization under anaerobic conditions is given by Gibson and Suflita (1990). They reported the enhancement by primary substrates of the degradation of 2,4,5-tri-chlorophenoxyacetic acid (300-500 μM) by samples from a methanogenic aquifer. The addition of 5-10 mM of methanol, ethanol or single acids from formic to butyric reduced the lag from greater than four months to about one to three months and the extent of degradation was increased.

B.4.6 Micro-organisms

Turning to the role of the microbial population, it is worth remembering that because a given species or collection of species carries out a certain step in a laboratory test, it is not necessarily the only or even the most important way the result is achieved in nature (Dagley, 1987).

There are many interactions between bacteria, as well as between bacteria and other micro-organisms, which are known to occur and probably others which have yet to be revealed. It is also probable that events occurring in biodegradability tests do not always reflect those happening in the environment. Harder (1981) described seven types of interaction (mutualism, commensalism, competition, etc.), but is of the view that in nature microbial interactions are more complex and can be combinations of two or more of the seven types.

Nortemann, *et al.* (1986) described a mutualistic interaction between two *Pseudmonas* strains isolated from River Elbe water. Only one strain grew on 6-amino-naphthalene-2-sulfonic acid in monoculture, but produced black polymers and 5-amino-salicylate (5-AS). In mixed culture the second strain degraded 5-AS and no black colour was formed. An example of what the authors (Kawai and Yamanaka, 1986) described as "obligate mutualism" is the growth of a mixed culture of *Flavobacterium sp.* and *Pseudomonas sp.* on polyethylene glycol (PEG) 600; neither species could grow singly on the glycol. *Flavobacterium sp.* was shown to contain all three of the PEG-oxidizing enzymes, while the *Pseudomonas sp.* contains only the depolymerizing enzyme. In experiments using culture filtrates and dialysis cultures, it was shown that *Pseudomonas sp.* was not contributing growth factors. However, glyoxylate was shown to be the key to the mutualism; it was degraded rapidly in mixed culture, but if the acid was added during culture inhibition occurred. Neither species could utilize glyoxylate as sole source of C, but it was metabolized by resting cells of *Pseudomonas sp.* much faster than by *Flavobacterium sp.*

Another example is the two-membered culture which totally degraded 4-chlorobiphenyl (4-CB) (Sylvestre, *et al.*, 1985). A *Pseudomonas sp.* could grow on 4-chlorobenzoic acid (4-CBA) but not on 4-CB, while the second organism grew on 4-CB but not on 4-CBA. The second organism produced 4-CBA from 4-CB together with minor chloro-organic compounds and yellow and black colorations. In mixed culture the minor metabolites and colours were not formed, and the cells of the 4-CB-degrader remained viable for longer periods after 4-CB removal than in single culture. The oxidation of LAS is thought to be carried out by two types of bacteria (Swisher, 1987). One group could grow on LAS and the early metabolic products, such as sulfophenyl-undecanoic acid, but a second group unable to degrade LAS were needed to complete the oxidation. Jimenez, *et al.* (1991) found that all four species in a consortium isolated from a chemostat inoculated with activated sludge and fed LAS, were necessary for mineralization of the surfactant.

Lewis, *et al.* (1984) reported a different sort of interaction which they considered more likely to occur in sediments, soils and aufwuchs than in the water column. The addition of various cultures, filtrates or exudates of algae or fungi could cause lower or higher transformation rates of methyl parathion, diethyl phthalate and 2,3,4-dichlorophenoxyacetic acid butoxyethylester (at 300 µg/l) by pure bacterial cultures. Inhibitions (up to 80%) which occurred were shown to be due to the lowering of the pH value below about 6, thought to be due to acids such as glycolic acid known to be present in algal exudates. There were two stimulatory effects. One was due to the presence in the culture filtrate, etc. of sugars, amino acids, alcohols, etc., which supported extra growth, and this caused increased removal of the xenobiotic. The other took place within a very short period increasing the transport rates by

about ten-fold and was apparently due to the increase in the efficiency of the relevant enzymes or the induction of additional permease or transforming enzymes.

Inhibitory compounds, produced by one of a pair of strains of *Pseudomonas sp.*, and their destruction were cited by Murakami and Alexander (1989) as a possible way in which bacteria interact during the decomposition of a mixture of chemicals. Strain 1 could mineralize phenol (100 mg/l) but not 4-NP, while strain 2 mineralized at 4-NP (100 mg/l) but not phenol; each was inhibited by the substrate of the other strain. The toxicity of phenol to strain 2 could be reduced by inoculating the mixture with strain 1, but the toxicity of 4-NP to strain 1 was not reduced by inoculating the mixture with strain 2. This was shown to be due to the production of an unidentified compound(s) from 4-NP; nitrite, which can inhibit, was ruled out in this case.

A different form of interdependence was the degradation under denitrifying conditions of p-cresol by two species, isolated from polluted river sediments (Bossert, *et al.*, 1986). One species converted p-cresol to 4-hydroxy-benzoate (4-HB) which was mineralized by the second species with the formation of nitrogen gas. Since no C is released by the first step, the first species presumably obtains C from ring fission of 4-HB by the second species. Thus, it is a syntrophic association in which neither isolate can grow without the other on p-cresol as sole C source.

Naturally occurring toxins were present in lake water at different concentrations depending on the season of the year (Klein and Alexander, 1986). The counts of bacteria added to sterile lake water fell rapidly with time and the cause was traced to chemical(s) which could be destroyed by heat or removed on cation-exchange resins. Zaidi, *et al.* (1989) further found that in lake water an antibacterial compound was present which suppressed the mineralization of 4-NP. They also reported that the grazing by protozoa had an inhibitory effect on the degradation of 4-NP at 26 µg/l by added *Corynebacterium sp.*, but not at higher concentrations at which the predator would have less effect since the bacteria grew faster at the higher concentrations. The role of protozoa in sewage on the lag period before the degradation of xenobiotics begins was described by Wiggins and Alexander (1988b). The "acclimation" periods before the mineralization of 4-NP (2 µg/l), 2,4-D (100 µg/l) and to a much lower extent 2,4-DCP (100 µg/l) were shortened or eliminated when indigenous protozoa were inhibited by the addition the cycloheximide or nystatin, but the extent of degradation was decreased.

B.4.7 Oligotrophs and eutrophs

The most important differences between conditions in the OECD biodegradability tests and those in relatively unpolluted bodies of water are the concentration of test chemical and the nature of the microbial populations. It is often suggested (for example, Alexander, 1985) that results of conventional tests cannot be applied to the environment because of the difference in behaviour of micro-organisms under the eutrophic test conditions and that in unpolluted rivers and lakes. Such tests may lead to erroneous conclusions about the occurrence, rates and products of microbial transformation.

Bacteria thriving on relatively high concentrations of organic chemicals are called, variously and not too accurately, "heterotrophs", "saprophytes", "eutrophs" and more recently (Poindexter, 1981) "copiotrophs", and this type of bacteria is normally used in the OECD tests. Types of bacteria which thrive under low nutrient conditions are called "oligotrophs". The nutrient flux suitable for oligotrophs has been put at from zero to a fraction of a mg C/l per day in contrast to a flux of at least 50-fold higher for eutrophs, and does not fall to zero for prolonged periods (Poindexter, 1981). The difference between eutrophs and oligotrophs is

blurred, just as with psychrophiles and mesophiles, and indeed it is not certain that oligate oligotrophs exist as such, or whether oligotrophic habitats may be transiently enriched with nutrients with successful (facultative) oligotrophs surviving the enrichment even though they may be unable to exploit it. Another possibility is that there is a eutrophic and an oligotrophic "way of life" but no eutrophs or oligotrophs.

Whatever the true explanation, "oligotrophs" are able to mineralize organic compounds at low concentrations, having a greater affinity for the substrate but have lower growth rates and much higher generation times than "eutrophs". Oligotrophs are unable to degrade substrates at above about 1 mg/l, and indeed are often inhibited at higher concentrations. Eutrophs are unable to degrade substrates at concentrations as low as those degraded by the oligotrophs. A threshold concentration below which degradation does not occur may be expected in environments in which indigenous populations are obligate eutrophs that are not able to metabolise the test chemical at low levels regardless of the presence of other nutrients. For oligotrophs, the threshold would be lower. The existence of these thresholds may explain the persistence of some chemicals at very low concentrations in natural waters, although the concentrations found could be equilibrium values resulting from continuous production and degradation of the chemical.

It is difficult to demonstrate the threshold value of a chemical because of the presence of DOC in natural waters at levels higher than the estimated or expected threshold value of the chemical (Alexander, 1985). The same applies to synthetic media because of the DOC present in distilled water and in the atmosphere. Even so, threshold values have been found, for example, for 2,4-D in stream water to be between 2.2 μg/l and 0.22 mg/l (Boethling and Alexander, 1979a).

Biphasic mineralization has also been reported (Subba-Rao, *et al.*, 1982) for aniline and di-ethyl-hexyl phthalate (DEHP). In water from an oligotrophic lake the rate of mineralization of aniline increased linearly from 5 ng/l to 50 μg/l, but at higher concentrations it was less than that predicted from tests at lower concentrations. Thus, the rate may have been reduced because it was carried out by aniline-sensitive oligotrophs. At 50 μg/l and 500 μg/l, mineralization was biphasic; in the first phase of a few days the rate was low, probably as a result of the metabolism of oligotrophs, but after about 7d the rate increased, probably as a result of replication of eutrophs active on the substrate. For DEHP biphasic mineralization occurred in eutrophic lake water at 200 ng/l to 2 μg/l. Rubin and Alexander (1982) reported a similar phenomenon for phenol, but the concentration at which the rate fell was between 1 and 100 mg/l.

In addition to the kinetics being different at very low concentrations, a feature of the degradation of a number of chemicals (aniline, benzoate, phenol, benzylamine and 2,4-D) was the very high degree (94-98%) of mineralization to $^{14}CO_2$, indicating very little incorporation into cells. This mechanism is similar to cometabolism, but differs in that cometabolism is characterized by yielding organic products (Subba-Rao, *et al.*, 1982).

It appears that all chemicals degraded at high concentration (for example, in the OECD tests) would be degraded under most circumstances at the low concentrations, assuming the presence of the necessary organisms and nutrients. The exception would be at concentrations under the threshold value for maintenance of the cells. The converse is not true, since some chemicals which are degraded at low concentrations are inhibitory in the 10 to 100 mg/l region.

Lastly, abiotic processes, especially photo-oxidation, have been shown to augment biodegradation in the environment.

B.5　Cometabolism

Broadly, there are three recognized ways by which a chemical can be biodegraded, namely:

(a)　completely mineralized and, except at very low concentration, producing microbial growth;

(b)　part of the molecule is mineralized, while part remains intact, also associated with microbial growth; and

(c)　by a relatively small transformation of the molecule, with no mineralization and not associated with microbial growth.

This last, (c), is called "cometabolism"; other terms have been "co-oxidation", "analogue metabolism" (because often the substrates and co-substrates are analogues), "fortuitous metabolism" and "bioconversion". It had been thought that all chemicals which are biodegradable also produced growth (a), but because of its nature, it is difficult to assess how many chemicals are initially attacked by cometabolic means in the environment. Examples of partial metabolism are 4-chlorobiphenyl giving growth plus 4-chlorobenzoate, 1-chloronaphthalene yielding growth and 3-chlorosalicylate and LAS giving sulfophenylalkanoic acids.

There are many examples of cometabolism.   Most of these, together with other aspects, can be found in reviews on the topic by Horvath (1972), Perry (1979), Alexander (1981, 1985), Dalton and Stirling (1982), Anderson (1986) and also in texts by Dagley (1984, 1987), Boyle (1989) and Pitter and Chudoba (1990).

There are various definitions of cometabolism, and the term has on occasion been misapplied, which has tended to confuse an already complex situation. Foster (1962) observed the oxidation of co-substrates, such as ethane, by methane-utilizing bacteria in the presence of methane to acetic acid. Since the organisms did not grow on ethane, he termed the phenomenon "co-oxidation".   Later, Jensen (1963) included reactions such as dehalogenation and proposed the term "cometabolism" instead of "co-oxidation", as well as omitting the obligate requirement for the presence of the growth substrate (see below).

The term has been misapplied, for example, to the situation in which two or more species act together on one compound to bring about degradation of a chemical when a pure culture has failed. Another example of misapplication is the case of a pure culture failing to degrade a single chemical until another substrate(s) is added. Further, Horvath (1972), for example, lists examples of cometabolic studies in which the conditions varied quite considerably between different investigations and more often than not a stimulation of respiration was observed rather than true cometabolism.

The essential features of cometabolism are, according to Dalton and Stirling (1982):

(1)　it does not support growth of micro-organisms;

(2)　products accumulate, usually stoichiometrically, from the co-substrate;

(3)　transformation of the co-substrate is associated with increase oxygen uptake; and

(4)　transformation of the co-substrate involves adventitious (fortuitous) utilization of existing enzyme systems.

The whole concept of cometabolism was criticized by Hulbert and Krawiec (1977) on the grounds that the transformations observed did not constitute novel metabolic events and were simply manifestations of existing mechanisms of anabolism and catabolism. Also, few observations of cometabolism, they said, had been studied with sufficient care to establish that the four features (above) taken from Horvath (1972) were fulfilled. For example, the lack of growth or lack of increase in biomass due to the co-substrate was, in most cases, not backed up by experimental data. Dalton and Stirling (1982) conceded that some of the criticisms were valid but, with Alexander (1981), they felt that keeping a separate term was justified on the grounds of the quite clear importance of the environmental consequences of the phenomenon. The term also helps to understand the wealth of literature devoted to this area of metabolism.

Partly to accommodate the criticisms, Dalton and Stirling (1982) proposed the following definition:

"Cometabolism is the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound".

The term "non-growth substrate" should be used to describe compounds which do not support cellular division, as opposed to increase in biomass, since it is possible that such compounds could be incorporated into cellular components although they were not essential for growth. Thus, the definition stays true to Foster's original definition of co-oxidation but extends the range of co-substrates to include non-growth substrates as well as growth substrates. The transformation of non-growth substrates in the absence of another substrate (included in the Jensen and other definitions) should be referred to simply as "fortuitous" metabolism, oxidation, or dehalogenation and not be classed as a novel metabolic event. Dalton and Stirling (1982) give examples to justify the new definitions, based largely on Foster's and their own work with methane oxidizers.

Some examples to illustrate cometabolism, all occurring in pure cultures, are benzoate plus 3-chloro-benzoate which is converted to 3- or 4-chlorocatechol, singly or both depending on the species (Horvath and Alexander, 1970); diphenyl methane plus 4,4[1]-dichloro-diphenylmethane which is converted to p-chlorophenylacetate (Pitter and Chudoba, 1990) and methane plus trichloroethylene which is converted to dichloroacetate and glyoxylic acid (Little, *et al.*, 1988). Other examples go beyond the definitions. For example, Horvath and Flathman (1976) reported that a mixed soil population did not support growth of monofluorobenzoates, though the ring was ruptured. When glucose was added, the rate of ring cleavage increased and the results indicated that after an initial cometabolic attack, glucose provided the necessary energy for cometabolism to proceed to a point where complete metabolism (that is, mineralization and growth) became possible.

The mechanism of cometabolism has not been completely elucidated, but appears to be due to the conversion of the non-growth substrate by enzymes of broad specificity to products similar in structure to the substrate. It is thought that these products accumulate because the organism does not possess the necessary enzymes to degrade the cometabolite, or the enzymes had a tight substrate specificity, or the products inhibit the enzymes. The organisms appear to derive no benefit from cometabolism.

Dagley (1984) suggested that cometabolism is akin to detoxication, although it has no obvious physiological function. Many enzymes function simply to modify structures so that substrates are provided for subsequent ATP-generating processes. When they act in isolation and not co-ordinated in metabolic sequences, these enzymes do not provide ATP necessary for synthesizing cellular constituents. Thus, when they act fortuitously and in isolation on analogues of their natural substrates, they give rise to products which accumulate.

Cometabolism in pure cultures has been proved to occur, though not in all published cases. However, direct evidence for cometabolism occurring in the environment is not easy to obtain because of the variety of species present, but it is thought that a significant proportion of the degradation of xenobiotic chemicals in the environment involves cometabolism (Boyle, 1989). The circumstantial evidence includes the lack of transformation of the non-growth substrate in sterilized environmental samples and the fact that no bacterial isolate could be obtained from that environment which could grow on the chemical. Also, Alexander (1985) reported a lack of incorporation of carbon from several pesticides – non-growth substrates. The reverse is not necessarily true; for example, no incorporation was found for aniline in lake water at very low concentration (ng/l).

With some chemicals there appear to be threshold values of their concentration above which they are cometabolized and below which they are directly metabolized. However, attempts to allocate particular observations at very low concentrations of substrate(s) in natural waters to specific mechanisms are made difficult by factors such as the type of bacteria involved (oligotrophic or eutrophic), the nature and amount of "natural" organic matter in the waters, analytical limitations, microbial maintenance energy, and the associated threshold concentration of substrate allowing growth.

A biochemical indication of when fortuitous metabolism might occur is given by Dalton and Stirling (1982), who reported that loss of the "storage" product poly-B-hydroxybutyric acid from a methane-oxidizing species was correlated with loss of the ability of the organisms to oxidize non-growth substrates. The rate of oxidation of non-growth substrates is usually low and, since no growth occurs, the rate is constant.

The implications for biodegradability testing were considered by Swisher (1987). It would be fruitless to try to promote cometabolism routinely in screening tests by adding a co-substrate chosen by intuition or at random. However, the possibility of cometabolism occurring would make it worthwhile choosing natural, instead of synthetic, media if feasible in testing. The co-substrates present could promote co-metabolism and the results would be closer to those which would occur in the environment.

In the interpretation of results, Dagley (1984) warned that the most serious oversimplification is to assume that, because an organism catalyses a certain reaction in the laboratory, this is the only or even the most important way in which the result is achieved in nature. The same task in nature may in principle be done in several ways by diverse microbial species.

C. Gaps in methods

Many gaps or imperfections in the methods necessary to describe more completely the behaviour of chemicals have been identified in the course of this review, and these are listed in **Table C.1** together with possible solutions. For some of these needs there is more than one method available which have been practised to the point at which they could fairly soon become agreed methods C1 to C3. For other needs, although the requirements are clearly defined, such as a laboratory screening test at environmentally realistic concentrations, the solution is elusive unless radio-labelled versions of the test chemical are available.

C.1   Insoluble and poorly soluble chemicals

The literature review (section A.5.1) has shown that a number of methods of dispersing chemicals of low or no solubility are available, but that no one method can be successfully applied to all such chemicals. This may be due to different mechanisms, all of which have not been fully elucidated, by which bacteria can carry out the initial attack on insoluble chemicals. The ISO paper 10634 (ISO, 1990b) and the ECETOC report (1987) sum up most of the methods of dispersion and much experience has been gained by many laboratories in using these methods. However, the advice of Gerike (1984) should be considered, namely that a "biodegradability spectrum" using different methods should be determined until more is known about the form in which insoluble chemicals are present in the environment.

The method of assessment to be applied to the dispersed chemical is preferably the Respiration or MITI oxygen uptake or the Sturm CO_2 evolution methods. It should not take long for these treatments and methods to be examined and agreed. However, the modified Blok or BODIS method (ISO, 1990b), in which the contents of the partially filled bottle are constantly stirred, should also be considered since it offers advantages over the other two methods. A calibration exercise (ring test) of the BODIS method was organised by ISO/TC147/WG4 and a revised method, based on the outcome of the ring test, will be reviewed at an ISO meeting in Vancouver in October 1994.

Inherent tests for poorly soluble chemicals have not been described, but in principle there is no reason why such chemicals should not be used in the SCAS test to attempt to adapt the microbial population. The chemical could be prepared in the same way as in the screening test and the exposed sludge could be used as inocula in the Sturm or respirometric method after, say, two or four weeks' exposure.

As for simulations of waste water treatment, ECETOC (1984) suggested that the Husmann, porous pot and OECD methods could be used to test insoluble chemicals, but obviously the means of introducing the chemical to the aeration vessel of the activated sludge unit would have to be investigated and the analytical methods for effluent and sludge would have to be established (Yogo, *et al.*, 1982). Mass balances of the test chemical would also be required. At least, any effect the chemical might have on the treatment process could be determined.

Table C.1   Gaps in methods

Test/type of chemical	Type of test	Solution
C.1 Insoluble chemicals	ready inherent simulation	{ISO/CD10634 (1990b) as {basis {ISO "BODIS" (1990a) SCAS – ready test Husmann/porous pot
C.2 Volatile chemicals	ready inherent simulation	adapted BOD bottle ISO N40 (1984) Hanstveit and de Kreuk (1977) SCAS – ready test Struijs and Stoltenkamp (1986) Enclosed Husmann
C.3 Anaerobic	methane production inhibition of methane production	Shelton and Tiedjie (1984) Battersby and Wilson (1988) Birch, *et al.* (1989) ISO (1985) Laake, *et al.* (1990) Woods, *et al.* (1990)
C.4 "Ready" tests C.4 Sturm test	use of pre-exposed inocula (controversial) ready	(see section A.4) 15-18d pre-exposure investigate CO_2 collection
C.5 Simulation	sewage treatment anaerobic natural waters/ sediments sea water	porous pot, rotating tube low concentration small-scale digester slurries (some references) cores/microcosms (many references) ^{14}C (some references)
C.6 Kinetics	die-away simulation	^{14}C; HPLC, GC various sludge retention times

C.2   Volatile chemicals

The need for assessing the biodegradability of volatile chemicals was questioned by ECETOC (1984) since such chemicals are not likely to persist for any length of time in the aquatic environment. By contrast, volatile chemicals such as halo aliphatic hydrocarbons and benzene are present, though in low concentrations, in the ground and river waters. But it can be agreed that tests for volatile chemicals are less urgent than those for insoluble chemicals.

Suitable tests are available based on the Closed Bottle and the MITI methods; in the former an adapted bottle or flask is used (Hanstveit and de Kreuk, 1977; ISO, 1984) while in the latter the volume of the headspace is reduced. The proposal put to ISO was not discussed. Some authors have successfully modified the DOC Die-away method by sealing the flasks and withdrawing samples as quickly as possible.

As a test for inherent biodegradability, the SCAS method can be used to expose activated sludge to the chemical prior to applying a die-away, Closed Bottle or MITI test, provided that the chemical is not so volatile that it disappears completely between additions

in the SCAS test (Struijs and Stoltenkamp, 1986a). Volatile chemicals have also been examined in the Blok or BODIS test (ISO, 1990a).

While volatile chemicals are not often examined in tests simulating activated sludge, small activated sludge units have been enclosed and volatile chemicals in the exit gas collected in traps to estimate physical loss from the system. For example, Horn, *et al.* (1970) examined tertiary butanol in this way.

C.3   Anaerobic tests

No anaerobic tests have yet been agreed internationally even though there is keen need for them, especially for chemicals which are insoluble and/or are adsorbed on to sludge and sediments. This need is clearly brought out, for example, in the EC Guidance on biodegradability testing (1986) discussed in section E.

C.3.1 Anaerobic degradation

Although not vigorously ring-tested, methods for assessing the potential for anaerobic biodegradability (methane production) have been applied to a large number of chemicals and it should be possible to reach an agreed method(s) fairly quickly. The need for such a method was stressed by the ECETOC group (ECETOC, 1988; Birch, *et al.*, 1989), who reviewed existing methods using digesting sludge. Methods have been described by Owen, *et al.* (1979), Shelton and Tiedje (1984), the Netherlands (ISO, 1985), ECETOC (1988), Birch, *et al.* (1989), Battersby and Wilson (1988), Baumann and Schefer (1990) and James, *et al.* (1990). Applications of these methods are described, for example, by Healy and Young (1979), Boyd, *et al.* (1983), Boyd and Shelton (1984) and Battersby and Wilson (1989).

Other methods, not involving sewage sludge, but using anaerobic conditions with sewage (Liu, *et al.*, 1981), in sediments (Rhee, *et al.*, 1989) and sub-surface systems (Novak, *et al.*, 1985) have been described.

Biodegradability has been assessed by the net increase in pressure of gas during incubation, and by the amount of methane produced measured by GC or by absorbing CO_2 in an alkaline suspension. Schraa (1988) has discussed the anaerobic degradation processes – denitrification, sulphate reduction, methanogenesis – and has commented on the relatively few anaerobic tests. In particular, he is critical of the high concentration of test chemical (50 mgC/*l*) used, the relatively short incubation period (eight weeks), and the lack of use of sludge acclimated to the test chemical (see below).

The two most popular methods are both based on that of Shelton and Tiedje (1984). Battersby and Wilson (1988; HMSO, 1989) modified the original method in which the net increase in pressure during incubation of 10% anaerobically digesting sludge (2-3 g/l) containing the test chemical, in serum bottles sealed with Suba-seals. Gas production was measured periodically with a hand-held precision pressure meter, previously calibrated, and the gas volume was read from the calibrated graph. Net gas production was taken as the difference between the volume formed in the presence of the test chemical and the volume from control bottles not containing an added chemical. The calculation of % theoretical gas production (% ThGP) is based on the fact that the amount of test chemical C used yields 10.5 ml gas under the conditions of the test and the distribution of C between CH_4 and CO_2 is determined by the Buswell equation (Buswell and Mueller, 1952). (For organic chemicals containing N and/or S, a modified equation has to be used, see for example Baumann and Schefer, 1990). A correction is made for the solubilities of the two gases, as given by Shelton

and Tiedje (1984) for the particular conditions of volume of sludge mixture, volume of headspace, temperature 35°C, etc. Thus % ThGP was calculated from the measured, net volume of gas produced and the corrected ThGP.

A serious drawback of the above method is that the empirical formula of the test chemical must be known in order to apply the Buswell equation. The ECETOC method (Birch *et al.*, 1989) overcomes this drawback and also avoids making assumptions about the validity of the Buswell equation and the solubility of CO_2. (The ECETOC group had obtained evidence from two laboratories that under the conditions of their test the Buswell equation was not valid for glucose and phenol.) Since both gases formed are monatomic, the C content of the gas produced can readily be calculated from its volume and the gas laws, thus avoiding the need to apply the Buswell equation or to know the empirical formula: only the C content of the test material need be known. The assumption about the solubility of CO_2 was avoided by determining DIC in the sludge mixture at the end of the incubation and adding this amount of C to that present in the headspace at that time. This requires that the original digesting sludge is initially washed and centrifuged to reduce the content of DIC; and, of course, no carbonate/bicarbonate is added to the medium. The relatively low solubility of CH_4 can be safely ignored.

Standard, or reference, chemicals proposed include ethanol, benzoate, palmitate, p-cresol and phthalic acid. The concentration of 50 mgC/l is high compared with that likely to be present and, on the other hand, eight weeks is longer than the retention time in digesters, normally 3-4 weeks. Tests are considered valid if ethanol is degraded by >80% in 7d and 4-cresol reaches the value by eight weeks; 80% is considered to indicate complete degradation. The shapes of the gas evolved-time curves show the types of behaviour of chemicals in the test; types range from easily degradable, degradable after a 'lag' period, not degradable but not-toxic, initially inhibitory and inhibitory throughout the test. Lower concentrations of the test chemical can be used, but this lowers the precision.

It has been suggested that the Shelton - Tiedje method be applied if knowledge about the identity of the test chemical is adequate and the result would give a reasonably reliable indication of the potential of the test material to be biodegraded anaerobically. Even if the empirical formula is not known or the chemical is too impure, the method would give at least an indication of the kinetics of biodegradation if gas pressure measurements were made at intervals during incubation. When a more accurate value is needed for chemicals having a known empirical formula, or if a value is required for a test material of unknown composition, but of known C content, the slightly more complex ECETOC method should be applied. Verification of the biodegradation of a test chemical may be made by carrying out tests using sterilized sludge, analysing the headspace gas for CH_4 using non-sterilized sludge and by following the disappearance of the test chemical by specific analysis.

At a meeting in September 1991, the ISO Working Group 4 decided to adopt the ECETOC method and to carry out a calibration exercise (ring test). A revised method, based on the outcome of the ring test will be discussed at an ISO meeting in Vancouver in October 1994.

Baumann and Schefer (1990) used a lower concentration of inoculum (0.5 g/l, compared with 2-3 g/l in the above methods) and overcame the problem of the solubility of CO_2 by adding NaOH to the digesting mixture when gas production had reached a plateau. The alkaline mixture absorbed the CO_2 in the gas. Their flasks, fitted with stirrers, were larger than in the above methods and gas production was measured by means of a mercury manometer fitted to each flask. They also showed how to calculate the theoretical amounts of gas produced from mixtures of unknown organic chemicals from TOC and COD values.

Although some insoluble chemicals have been tested by these methods, the ECETOC (1988) group recommended that further studies of a wider range of types of such chemicals should be undertaken, since only 5 mg C are added to the 100 ml reaction mixture. The Baumann-Schefer method has an advantage here, in so far as the volume of the reaction mixture is 500 ml.

It is stressed by most authors that only the potential to degrade is measured by these tests, which could give low results because of inhibition or of lack of use of exposed inocula, or could give high results because of the long incubation period. Thus, if more detailed knowledge is required, other tests must be carried out, such as applying a laboratory-scale continuous digester test using sludge known to be free from inhibitory matter (for example, Bruce, *et al.*, 1966).

C.3.2 Tests for inhibition

The need for tests to determine the inhibition of anaerobic processes by xenobiotic chemicals is at least as great as the need for a test for degradation. In principle, any test for anaerobic biodegradability can easily be adapted to assess inhibition, for example, Owen, *et al.* (1979), Stuckey, *et al.* (1990), but it would be sensible to use the same basic procedure and apparatus for determining both degradability and inhibition. Laake, *et al.* (1990) have investigated some methods, and Woods, *et al.* (1990) have compared the macro method of Swanwick and Foulkes (1971) with one based on Battersby and Wilson's method (1988). This latter method was of acceptable precision, gave results comparable with those of the macro method and those found in the literature, and could deal with volatile chemicals. Examples of the determination of inhibition are given by Parkin, *et al.* (1983), Fedorak, *et al.* (1986) and O'Connor and Young (1989).

As with anaerobic biodegradability, agreement on a test(s) for inhibition should be fairly quickly attainable.

C.4   Changes to existing aerobic tests

A number of minor changes to existing methods have already been incorporated in the tests for ready biodegradability (OECD, 1992), but two more major aspects demand some attention.

C.4.1 Pre-exposed inocula

It has been argued, in section A.4.4, that the use of inocula previously exposed to the test chemical should be permitted in screening tests because microbial populations in the environment will be exposed to regularly-used chemicals and, as a secondary reason, so few "new" chemicals degrade in tests for ready biodegradability. There is opposition to this point of view; ready tests should remain so stringent that it can be widely accepted that chemicals passing the tests can still be assumed to degrade in the environment. This point of view would accept pre-exposure if adaptation could also be shown to occur in the environment, but would prefer to label chemicals passing in this way as inherently biodegradable. As against this, the protagonists of allowing controlled pre-exposure of inocula propose that such chemicals be classified as "readily biodegradable using pre-exposed inocula". This new class could be added to the proposed five classes (Struijs and Stoltenkamp, 1986b) discussed in section B.1, and would be a class which degraded more easily than those labelled inherently biodegradable.

At whatever level pre-exposed inocula are to be used, it is time for agreement on the conditions of pre-exposure to be applied. The most important conditions are that the source of the inocula is natural (not artificially engineered) and the duration of exposure is not excessive. The evidence in section A.4.4 suggests that 15 to 18d is not too long and that it would be preferable to expose the inocula simultaneously to the chemical plus other organic matter such as sewage. The current ISO method (revised 1991) recognizes this position.

It is pertinent to recall that in their comparison of seven tests with 44 chemicals, Gerike and Fischer (1979, 1981) exposed the inocula to the test chemical in sewage effluent for 14d but only in the Sturm CO_2 evolution test, which then continued for a further 28d. Even so, there was no significant difference in the pattern of passes and failures between the Sturm and other equivalent tests for ready biodegradability for the chemicals examined.

C.4.2 Sturm CO_2 evolution test

The evidence presented by Weytjens (1990) given in section A.7.4 strongly suggests that the rate of microbial production of CO_2 in the conventional Sturm test was greater than the rate of stripping from the medium and subsequent collection in the alkaline absorbent. Thus, although the final % $ThCO_2$ on day 28-29 would give true value because the pH of the medium is lowered to about 3, the plot of CO_2 collected (corrected for blank) against time would not be a true representation of the biodegradation curve. The kinetic rate of CO_2 production calculated from the practical curves would also be lower than the true value and the 10d window would not have the same meaning in this test as in the others.

The action necessary here is either to give the Sturm test a dispensation regarding the 10d window rate or to investigate the problem practically for verification or denial. No solution seems possible (if the problem is verified) except to determine DIC in liquid samples taken during incubation, just as Weytjens did (pers. comm.).

C.5   Simulation tests

At present, only one simulation test is described in the OECD Guidelines, namely that for waste water treatment by the activated sludge process. Other methods are available for simulating sewage treatment, and many papers have been published describing tests for simulating, to various degrees, events occurring in natural waters and sediments (see section A.9).

C.5.1 Sewage and waste water treatment

The OECD Guideline method describes the coupled-unit system using Husmann units, whereas the EC (Off. J. E. Comm., 1988) method embraces the use of porous pot units as well as the Husmann apparatus. The EC method also permits a number of choices, namely between coupled or non-coupled mode, synthetic or domestic sewage, different sewage retention times, and different methods of wasting sludge. The validity of these factors was substantiated by experimentation (for example, Painter and Bealing, 1989) and, since some of the choices offer advantages over the coupled-unit method, it is appropriate for the EC methods be adopted by the OECD.

At the same time the inclusion in the Guidelines should be considered of a treatability method (Birch 1984, 1990; HMSO, 1988) which describes the use of a range of sludge retention times to determine more precisely the conditions under which a chemical can be treated. The method also enables the calculation of kinetic constants.

The above methods describe the addition 10-20 mg/l of the test chemical, but most chemicals are present at very much lower concentrations, so that a description of the treatment of chemicals at around 10 µg/l is needed. Few papers describe methods not requiring the use of ^{14}C-labelling, for example Moos, *et al.*, 1983, Topping, 1987, Shimp, 1987, and Nyholm, *et al.*, 1992. Only primary degradation was assessed in these cases by means of HPLC or GC analyses. Gledhill (1975) used ^{14}C-labelled chemicals in a 300 ml-size apparatus; others using ^{14}C-labelling were Gardner, *et al.* 1982, Ku and Alvarez, 1982, and Harbison and Belly, 1982.

There is a need, especially for high-tonnage chemicals, for a method for determining whether all the parent chemical has been mineralized and no recalcitrant intermediates are formed (see section A.8.4). The method of Gerike, *et al.* (1984) is available for this purpose, but it is (and perhaps has to be) laborious, costly and prolonged. Thought should be given to providing an alternative, but the priority is not high.

If for any reason a simulation of fixed film reactors (percolating filters, biodiscs, etc.) is needed, the methods of Gerike, *et al.* (1980), Baumann and Schefer (1990) and HMSO (1983 – rotating tubes) are available. A few chemicals which failed to degrade well in Husmann units did so when treated in laboratory-scale filters (Gerike and Fischer, 1981); the reasons given were that filter film is more robust than activated sludge and the concentration gradient in the filter aids biodegradation.

C.5.2 Natural waters and sediments

No methods yet exist in the OECD Guidelines for tests in environmental waters and sediments. One major problem is the use of environmentally realistic concentrations which, for ultimate biodegradation, inevitably means employing ^{14}C-labelled chemicals. It is possible to use specific analysis to assess primary biodegradation; mineralization could perhaps be established in separate experiments. The ECETOC report (1984), as well as the discussion in sections A.9.2 to A.9.4, pointed out the problems of simulating the wide variety of conditions existing in the environment, making it difficult to develop standard test methods. Besides low test concentrations, there are the relatively low microbial activity and the sometimes limited and variable amounts of nutrients available.

Many papers describe what seem to be suitable methods, varying from using natural waters alone, slurries of natural water plus sediments, and natural water plus settled quiescent sediments, and natural waters overlying sediment cores, some being static, while others are more complex with continuous flow of water. Besides the papers listed in Table A.9.2 in section A.9.2, a selection of references is appended. The stream model of Shimp, *et al.* (1989), the die-away tests of Wylie, *et al.* (1982) and the microcosm of Porcella, *et al.* (1982) seem promising.

A pilot study was tentatively suggested (ECETOC, 1984) similar to the proposal of Carson, *et al.* (1990) and the implied proposal of Struijs (1991), see section B.1. A number of chemicals covering a range of biodegradability would be tested in relatively simple laboratory-scale tests with fresh-, estuarine- and sea water - sediments, using waters of known nutrient status. Candidate chemicals are LAS, TPBS, NTA, diethylene glycol and certain phenols. By relating what is known about the behaviour of the "reference" chemicals in the environment to their behaviour in the "surrogate" simulation tests, it might be possible to establish a few simple laboratory methods capable of simulating natural water/sediment systems. This would avoid having to apply cumbersome and very costly simulation tests.

Factors such as cometabolism, threshold concentrations, secondary oxidation discussed in some of the papers listed would also have to be taken into account. It would be the aim in these tests not merely to determine percentage removal but also to acquire data to enable kinetic constants to be calculated (see sections B.3, C.6).

Addenda

A. A report and annex on biodegradability from Denmark (Damborg, *et al.*, 1991) has recently been received. They give very useful descriptions of principles for constructing tests which simulate the aquatic environment, largely based on Danish practical experience. The report also outlines specific methods which are consistent with the experience of other workers in this field. Separate guidelines are advocated for the various compartments and for aerobic and anaerobic conditions, but some of the details of the tests will be dependent on the purposes for which the test is carried out.

The *test system* can in some cases be simple batch (or die-away) in which a grab sample from the environment is incubated with the test chemical without further addition. But if the test chemical degrades only slowly, the naturally occurring "primary" organic substrates may be degraded before the test chemical is attacked. Thus any cometabolic effect or secondary substrate utilization may not have a chance to occur. This may be solved by periodic partial or even continuous removal by adding freshly collected environmental water samples. No inoculum should be necessary other than the micro-organisms already present in the sample, which can either be from a specific site or represent a compartment as generally as possible, according to the purpose of the test. Generally, *nutrients* should not be added unless the concentration of the test chemical added is high. If a deficiency of nutrients occurs, for example when high concentrations of naturally occurring chemicals are present, addition of nutrients would be necessary and should be made and recorded.

The concentration of *test chemical* should be that expected to be present in the compartment when the chemical is in normal use, but not usually greater than 200 μg/l. Normally, the test chemical will be at a concentration lower than its Ks value and also lower than the concentration of the naturally occurring carbon sources. Thus, the chemical may be degraded by one or other of the "cometabolic" processes. It is recognized that in some situations, for example, in sub-surface soils and groundwater, the test chemical serves as the primary substrate so that the kinetics of removal may be different. The upper limit of 200 μg/l is suggested as being suitable for specific analysis (e.g. HPLC) and for the detection limit to be sufficiently low to be able to detect about 10% of the initial concentration.

Test parameters. The concentration of test chemical can be followed by specific analysis directly or after solvent extraction in the case of sediments and soils. This would reveal only primary biodegradation, but it may be possible to demonstrate mineralization in separate experiments. DOC, CO_2 and oxygen uptake cannot be used to follow degradation of the test chemical because of the presence and degradation of relatively large amounts of uncharacterized organic carbon already present in the sample. The difference between control values (without test chemical) and those with the chemical would be too small for accurate and precise determination. But if ^{14}C-labelling were used, degradation could be followed by $^{14}CO_2$ evolution and/or by removal of ^{14}C in solution with optional analysis of ^{14}C assimilated into cells.

Some of the descriptions of these determinations are vague and others seem to be erroneous, with possible loss of $^{14}CO_2$. Probably two sub-samples are required at each time interval, one to determine ^{14}C in cells by filtration and the other to be acidified to release, and

measure, $^{14}CO_2$, followed by filtration to remove cells and to measure ^{14}C in solution, indicating test chemical remaining plus any organic intermediates. The effects of acid on cells and test chemical should be checked.

However, only if labelling is uniform could ultimate degradation (mineralization) be assessed; partial labelling would indicate mineralization for only those C atoms which were labelled.

There are sufficient data and experience in the literature to expect that surface water die-away tests, with and without suspended sediments, could be standardized fairly soon. A suggested protocol for surface water is now briefly described.

The environmental water sample is filtered to remove coarse particulate matter, which may adsorb the test chemical and such properties as pH, DO, temperature, nutrient composition, DOC and bacterial numbers are determined. The test chemical having high purity is added to triplicate aliquots of the water sample in suitable vessels, such as conical flasks. (If ^{14}C-labelling is used, closed vessels are used fitted with an internal means of absorbing $^{14}CO_2$.) The flasks are incubated preferably at the temperature of the relevant environmental compartment. Biodegradation is followed by applying specific analysis for the test chemical to samples at timed intervals, up to 200d. Analysis for known or possible intermediates could be made to help decide whether primary or ultimate degradation is occurring.

Biodegradability is expressed as percentage removal of the test chemical and the length of any lag phase, the time to reach 10% degradation, is determined. Assuming first order kinetics, the (pseudo) first order constant and the half-life period are calculated; otherwise the more complex treatment of Simkins and Alexander (1984) can be applied, as described in section B.3.

A protocol for tests with suspended sediments would include adding aerobic sediment taken from the same site as the water sample to give a 1% (w/v) suspension. The concentration of dissolved test chemical is determined about 3h after addition to decide whether adsorption on to the sediment has occurred and degradation is followed by determining dissolved test chemical.

Protocols for tests with undisturbed sediments are farther off, since factors such as the ratio of water depth to core depth and method of application of and rate of flow of air through the system will have to be investigated further and agreed.

B. The ASTM have published a protocol E 1279/89 describing the first test above and is currently finishing the first draft of the second protocol above (Boethling, priv. comm.).

C.5.3 Sea water

Screening tests for assessing biodegradability in sea water have been established (OECD, March 1991) but no simulation tests have been agreed. Nyholm, *et al.* (1992) have used a shake-flask die-away test with ^{14}C-labelled chemicals as a simulation test, finding good correlation with results of the OECD screening tests. However, only a small number of chemicals were tested (see section B.1). This method should be borne in mind when considering methods in C.5.2, since the importance of sediments in the sea is less than in estuaries, rivers and lakes.

C.5.4 Soil

Tests on soil are not in the "mainstream" of the OECD Guidelines, there being only one agreed method and this requires ^{14}C-labelled chemicals. Tests using slurries of soil and sterile water have been described, for example, by Hickman and Novak (1989), in which the concentration of the parent chemical is followed by HPLC and/or GC; this type of test could be called a screening test.

However, a number of countries (USA, Japan, Sweden, Germany) have schemes for testing pesticides and other chemicals applied to soil. For example, the German scheme is a stepwise one ranging from laboratory studies to field studies, the "triggers" being the values of t_{50} (time for 50% removal) and t_{90} (primary degradation). In addition, the complete metabolism and degradation pathways have to be investigated. Thus, it would be prudent to examine these schemes and the experience gained from using them before deciding on a Guideline method. It would also be advisable to consult with the appropriate working group of the ISO Soil Technical Committee (TC140/SC4/WG1), which hopes to have a draft method available in 1992 (Vonk, pers. comm.).

C.6   Kinetics

Kinetic data are much more useful for predictive purposes than the percentage of chemical removed. Such data have been gleaned by Birch (1984, 1990) for waste water treatment by operating porous pots receiving 10 mg test chemical/l at different sludge retention times (SRT). The concentrations of test chemical in the effluents at equilibria were plotted against the product of concentration and SRT, and μ and K_s are calculated from the slope and intercept of the resulting straight line; this method should be considered for adoption. Nyholm, *et al.* (1992) have also calculated first order rate constants for five chemicals from continuous flow and batch tests with activated sludge, using chemicals at the 5-10 μg/l.

The bacteria responsible for degradation of chemicals under conditions in the activated sludge process are eutrophic, while those in relatively unpolluted river water and sea water will probably be oligotrophic. The latter types are able to grow on lower concentrations of substrate and have lower "threshold" concentrations and K_s values. Also, most chemicals are present in the environment at the μg/l level, or even lower. Thus, test methods are needed to enable the calculation of kinetic constants from die-away curves or CO_2 production curves, with initial concentration of test chemical at around 10 μg/l. Such tests would also show whether the lack of degradation of some chemicals at higher concentrations was due to toxicity of the test chemical.

Many such methods have been described and a summary is given in B.3 (and see references given in **Table C.2**), but most employ ^{14}C-labelled chemicals; they also need more precise estimates of bacterial numbers (quantified as CFU, dry weight, ATP) than do the conventional tests, in order to calculate a "second order" rate constant thought to be independent of the concentration of bacteria (Paris, *et al.*, 1982), though this is rejected by other workers.

Although the cost of ^{14}C-labelling is prohibitive, it may now be advisable to agree on a method(s) so that optimal techniques can be pooled together in one document. The method would not be made mandatory and the technique for estimating $^{14}CO_2$ could be clarified.

Another suggestion (ECETOC, 1984) is to try to establish correlations between biodegradation in treatment plants and in surface water which would make easier the problem of predicting environmental fate, so reducing the number of simulation tests needed.

Table C.2 Additional references

Lakes, ponds

Chen and Alexander (1989)
Heitkamp, *et al.* (1987)
Hwang, *et al.* (1985)
Johnson (1980)
Kaplan and Kaplan (1985)
Klein and Alexander (1986)
Larsson, *et al.* (1988)
Novak and Alexander (1985)
Rosenberg (1984)
Rubin and Alexander (1983)
Shimp and Schwab (1991)
Spain, *et al.* (1984)
Steen, *et al.* (1986)
Subba-Rao and Alexander (1982)
Zaidi, *et al.* (1988)
Zaidi, *et al.* (1989)

River water/sediments

Anderson, *et al.* (1990)
Bailey, *et al.* (1983)
Dojlido (1979)
Evans and David (1974)
Fortmann and Rosenberg (1984)
Fushiwaki and Urano (1988)
Gledhill, *et al.* (1984)
Gonsior, *et al.* (1984)
Heitkamp, *et al.* (1986)
Knapp and Whytell (1990)
Kollig (1985)
Kondo, *et al.* (1988)
Krzeminski, *et al.* (1975)
Kuhn, *et al.* (1985)
Larson, *et al.* (1981)
Larson (1983)
Larson and Perry (1981)
Mirgrain, *et al.* (1989)
Palumbo, *et al.* (1988)
Paris and Rogers (1986)
Pitter and Chudoba (1990)
Vaishnav and Babeu (1987)
Vaishnav and Korthals (1988)
Wylie, *et al.* (1982)

Estuaries

Bartholemew and Pfaender (1983)
Larson and Ventullo (1986)
Pfaender, *et al.* (1985)
Spain, *et al.* (1980)
Spain and Van Veld (1983)

Sea/sediments

Bilstad, *et al.* (1987)
Haines and Atlas (1983)
Kondo, *et al.* (1988)
de Kreuk and Hanstveit (1981)
Massie, *et al.* (1985)
Nyholm and Kristensen (1987)

Sea water methods

Tabak (1978)
Schoberl and Mann (1976)
Ursin (1985)
Ursin and Madsen (19 ?)

Kinetics

Waste water treatment:

Blok and Booy (1984)
Cech, *et al.* (1985)
Chudoba, *et al.* (1989)
Jones (1973)
Kim, *et al.* (1981)
Knudson, *et al.* (1982)
Nyholm, Jacobsen, Petersen, Poulsen, Dambourg, Schultz (1991)
Painter and King (1983)
Stover and Kincannon (1983)

Surface waters:

Alexander (1985)
Bannerjee, *et al.* (1984)
Battersby (1990)
Boethling and Alexander (1979a)
Boethling and Alexander (1979b)
Larson (1979)
Larson (1984)
Paris, *et al.* (1981)
Paris, *et al.* (1982)
Paris, *et al.* (1986)
Paris and Rogers (1986)
Rubin, *et al.* (1982)
Schmidt, *et al.* (1985)
Shamat and Maier (1980)
Simkins and Alexander (1984)
Simkins, *et al.* (1986)
Subba Rao and Alexander (1982)
Subba Rao, *et al.* (1982)
Van Veld and Spain (1983)
Wright and Hobbie (1965)

D. New and alternative methods

The literature review has not thrown up any new method based on advances in microbiology and biochemistry.

The most urgent need is for a method to assess biodegradability at environmental concentrations (μg/l and less) and to obtain from the results some form of kinetic constants for predictive use. The use of ^{14}C-labelled chemicals would be suitable to use in these experiments but the cost would be prohibitive in routine testing. However, as mentioned in section C, it may be advisable to agree on a method to pool existing knowledge of the optimal techniques.

Indeed, the method of following primary degradation by applying specific analytical methods able to detect very low concentrations (μg/l) should also be agreed (in separate tests the chemical could be tested for ultimate biodegradation). For this purpose the modified OECD method could be further modified to accept the use of uninoculated natural waters and analytical techniques, such as HPLC and GC.

In principle, biodegradation of a chemical could be followed by determining the simultaneous increase in biomass, and the sensitivity of the method of detecting biomass will determine the lowest concentration of test chemical which can be used in the test. Techniques used have been MPN and acridine orange direct count (AODC) but they have been used only as adjuncts to other methods for assessing biodegradability. In general the numbers of bacteria increased as degradation proceeded, but the precision is poor and no author has relied on these methods. Also there is evidence that the specific activity for a given chemical varies according to the state of the cells, so that numbers alone may not be proportional to substrate removed. Biochemical methods have included determination of the energy-rich adenosine triphosphate (ATP), de-oxyribonucleic acid (DNA), protein and of various enzymes such as proteases, but more often dehydrogenase activity. The latter is determined with the aid of dyes, for example those based on triphenyltetrazonium chloride (TTC) which become reduced to a red triphenylformazan. Of these, ATP is thought to be most sensitive (Gray, 1989) although it might be difficult to interpret the results of ATP measurements in terms of biological activity, growth or biodegradation. Some miscellaneous methods for assessing biodegradability are also described for the sake of completeness. Finally, more elegant methods for determining CO_2 evolution with distinct advantages over the Sturm method are advocated.

D.1 Biochemical indicators of growth

The use of ATP to assess the quantity of active biomass was described by B.K. Jensen, *et al.* (1988) and Apoteker and Thevenot (1983). Wilson, *et al.* (1986) showed that the rate of degradation of toluene in subsurface samples was roughly proportional to the ATP content of the samples from about 0.1 ng/g up to 2 ng/g of subsurface soil; no degradation took place below about 0.05 ng/g. They found no simple relationship between ATP content and the degradation of chlorobenzene.

Gard-Terech and Palla (1986) showed that rapid degradation of LAS and branched ABS were linked with the ATP content of the sludge inocula; at ATP concentrations of 2.1 +/- 0.2 x 10^{-3} mg/mg protein degradation was rapid, whereas at 0.1-0.3 x 10^{-3} mg/mg protein the

rate of MBAS removal was much lower. (There was no such correlation between rates and DNA content.) This indicates that the general biochemical activity of inocula could be checked before use by determining their ATP activity.

Portier and Meyers (1982) found significant increased yields of ATP in river water microcosms treated with 5 mg methyl parathion/l, which was degraded, but not with 0.5 mg kepone/l, which was persistent. However, Wylie, *et al.* (1982) reported inconsistent and fluctuating results in river die-away tests with phthalic acid and its di-2-ethylhexyl ester, each added at 0.1 mg/l. They attributed the inconsistencies to a reaction to changes in conditions between the environment and the laboratory. It would seem that a period of time should elapse between sampling of natural waters and starting an experiment to allow "acclimation" to the laboratory conditions.

Van Woerkom (1983) reported a ten-fold increase, over the control, in the concentration of ATP in die-away tests when the plateau of the degradation curve of 10 mg PE/l was reached and no increase with t-butanol, which was not degraded. But the results were variable and inconsistent. Likewise Wilson, *et al.* (1990) reported a significant increase in ATP concentration with acetate at 0.4 mg C/l but found poor and inconsistent responses with PE and 4-NP. The control values were also variable. However, Struijs and Stoltenkamp (unpublished results) also had problems with inoculum and found the most consistent inoculum to be that prepared by Larson's method (1979).   Sludge was homogenized, centrifuged and the cloudy supernatant was diluted between 0.1 to 10 ml/l to give 10^2-10^3 to 10^4-10^5 CFU/ml; the study is progressing. However, even if a solution is found, the formation of non-biodegradable metabolites would have to be sought since the degree of mineralization cannot be quantified from the growth of biomass.

Besides using the AODC method for assessing bacterial population, McClellen, *et al.* (1989) determined the respiratory activity of aerobic bacterial populations degrading trichloroethylene (TCE) under "oligotropic" conditions by measuring the reduction of the dye INT, an iodine derivative of TTC. The bacteria, concentrated from shallow-well water, were re-suspended in mineral medium containing either 0.6 or 6 mg TCE/l. Although the numbers of bacteria increased by nearly two orders of magnitude in the first week of incubation and the proportion of "INT-active" cells rose from 20% to about 50% of the population, no significant removal of TCE was observed until the second and third weeks. The numbers stayed fairly constant after the first week, while the % "INT-active" population fell to 20% after the third week. Scanning electron micrographs showed an increase in rods in the period of increase in numbers, and after two and three weeks, an increase in longer rods.

Increases in protein concentration (Lowry's method) during incubation of a concentration as high as 500 mg test chemical/l in a mineral medium inoculated with activated sludge were used by Rothkopf and Bartha (1984). Three weekly transfers of 0.1 ml culture to 20 ml fresh medium were made, and one week later the protein concentration was determined. Control cultures contained 1 to 7 mg protein/l, and an increase to at least 20 mg/l was considered to indicate positive biodegradation. The test was thus not very sensitive but perhaps it could be made more sensitive by using larger volumes than the 1 ml used by the authors for the protein determination.

Another method of assessing the increase in bacterial populations involves the incorporation of 3H-thymidine in the presence of the test chemical. Gledhill (1987) calculated that the incorporation of 10 to 250 µg C/l of added test chemical could be detected when a mineral medium was inoculated with effluent to a cell density of about 10^5/ml. Glucose gave significantly increased uptake of tritium after 24-48h and as low as 5 µg/l gave a detectable

uptake. However, LAS at 50 µg C/l gave an increased uptake after 48h but the addition of 250 µg C/l did not give a proportional increase.

D.2   Other methods

A rapid screening test for primary degradation in natural waters, with no further addition of inoculum, was described by Kondo, *et al.* (1988). A solution of the test chemical (in water, acetone or dimethylsulfoxide) was added to river water or sea water containing 0.1% peptone to give a concentration of 20-100 mg/l. The concentration of the test chemical was determined after 3d at 30°C by specific analysis. They classified chemicals as "easily" degradable if 50-100% was removed, "moderately" if 15-50% was removed, and "hard" to degrade if 0-15% was removed. The removal of aniline in river waters and sea water ranged from 2 to 22% in 3d, while sorbic acid was removed by 95 to 100% in the same water samples.

By replacing chlorides with sulfates in the modified OECD medium, Steinhaser, *et al.* (1986) were able to assess the degradability of nine chloro-organic chemicals by both DOC removal and chloride production. The results showed that very little carbon was lost abiotically, but some chemicals lost chlorine in the absence of inocula while others lost both chlorine (and carbon) only on the presence of inocula.

For some years, the Co-ordinating European Council (1982) had determined the primary biodegradability of lubricant oils for two-stroke engines by a tentative method in which the removal of infra-red adsorption is followed.

Briefly, the lubricant, dissolved in 1,1,2-trichloro-trifluoroethane ($C_2Cl_3F_3$) (15% W/V), is added at 50 mg/l to a medium similar to that of the OECD and the mixture is inoculated with settled sewage or secondary effluent to give a cell density of about 10^4/ml. Blank controls are prepared with inoculum, but no oil, and inoculated calibration flasks are set up with 50 µl of a 15% (W/V) $C_2Cl_3F_3$ solution of di-isotridecyladipate and/or white oil Enerpar M2632. Abiotic controls (mercury II chloride) of the test lubricant are also prepared.

The flasks are shaken and incubated at 25+/-1°C for 21d and at 0, 7 and 21d duplicate or triplicate flasks are homogenized and acidified. The residual oil/reference compound is extracted with the solvent, and the IR adsorption at 2930 cm^{-1} (CH_3-CH_2) is measured. The residual oil contents are calculated from the mean absorption values and corrections are made for any absorption of the blank. The % primary biodegradability is calculated from the initial concentration of oil and that at 7d and 21d, allowing for any abiotic degradation.

D.3 CO_2 production

In an earlier section (A.7.4) methods using headspace analysis for CO_2 were described. An early "rapid" form of the method (Ennis and Kramer, 1975; Ennis, *et al.*, 1978) used 20 ml soil slurry containing 20 mg test chemical in 50 ml flasks, incubated at 35°C for up to 35d. Since neither shaking of the flasks nor acidification of the medium before analysing the headspace gas for CO_2 seem to have taken place, the results would have been falsely low.

The Boatman, *et al.* (1986) modification employed very small vessels (20 ml), so that there could be a dearth of competent organisms and the addition of insoluble chemicals would be difficult. On the other hand, either of the two versions, by Struijs and Stoltenkamp (1990)

or Birch and Fletcher (1991), would be suitable for insoluble and volatile chemicals, both methods giving good precision. It is highly probable that one or both versions could soon be agreed; other laboratories are gaining experience with the methods. They offer the advantages over the Sturm test of savings in space, more replicates are easily possible, greater precision, and volatile chemicals can be assessed.

E. Selection of methods; strategy, or schemes, for testing

The choice of which screening method should be used for a given chemical depends largely on its physical properties – solubility, volatility, adsorptivity. Guidance on the suitability of each test is given by ECETOC (1987) and has been incorporated in the revised OECD Test Guidelines (OECD, 1992). Briefly, chemicals which are insufficiently water-soluble cannot be assessed in DOC die-away tests (see A.1.5(a)) and neither can chemicals which adsorb significantly. Volatile chemicals cannot be tested by the Sturm test (1973), though the miniaturized, enclosed versions (e.g. Struijs and Stoltenkamp, 1990) can be applied. Also, oxygen uptake methods can be used and even DOC methods are applicable provided that, in both cases, certain precautions are taken.

The choice and application of methods are also considered in the Preface to the Test Guidelines (OECD, 1981). On the first page of each method the user's attention is drawn especially to paragraphs 3, 4, 7 and 8. Experience shows, however, that these paragraphs, which indicate that these Guidelines are not immutable and can be modified or even replaced by other methods provided that scientific justification can be made, are rarely referred to.

Struijs and Stoltenkamp (1986a) overcame the difficulty of applying the SCAS test for inherent biodegradability to volatile chemicals. Providing that the chemical was not completely lost from the system between daily additions, they used the resulting exposed (and possibly adapted) sludge as inoculum in a screening test. Vosser (in: van den Berg, 1983) proposed the use of pre-exposed inocula for testing "new" chemicals since so few degraded in the presence of unexposed inocula. For example, data taken from the MITI lists shows that in the five years 1978-1982 the proportions of chemicals tested for MITI which were biodegradable were only 10, 19, 9, 0 and 14%, respectively.

As part of the overall scheme for hazard assessment, the original OECD Report (1979) included a "flow scheme" for biodegradation testing in the form of a decision diagram (**Figure E.1**) based on the three tiers of testing – screening, or ready biodegradability, inherent biodegradability, simulation – and on consideration of possible biodegradation (intermediate) products. Chemicals which pass the first tier need not be tested further, while those which "fail" are tested by a second tier method.

At this level a higher bacterial cell density, the presence of other organic compounds and a longer duration may be used. In fact, any test may be used so long as it can be scientifically justified. Of the three formally agreed OECD methods, only the MITI II (oxygen uptake) method could deal directly with volatile and insoluble chemicals. However, although the bacterial cell density is higher (100 mg instead of 30 mg/l of "activated" sludge), there is no other organic matter added and the duration is only 28d. In the Zahn-Wellens test there is again no other organic matter added and the duration is only 28d, but the cell density is much higher at 1000 mg activated sludge/l. The test chemical is added at 50-400 mg C/l and degradation is followed by DOC removal, making allowances for adsorption. The semi-continuous activated sludge test (SCAS) is much more conducive to biodegradation than the other tests since the concentration of sludge is higher at 1-4 g/l, domestic or synthetic sewage is fed daily with the test compound and the duration can be three months or more. A control vessel, which does not receive the test chemical, has to be used. The difference in DOC between the effluents is used to calculate the % DOC removal. Both the SCAS and Zahn-Wellens tests could be applied to volatile and sparingly soluble chemicals to produce

exposed inocula to be used in tests for ready biodegradability; no results of doing this have been seen in the case of insoluble chemicals.

Those chemicals which fail (< 20%) a test at the second level are considered to be not inherently biodegraded and are not tested further. If the degree of mineralization is between 20 and 70%, any metabolites must be identified (see section A.8.5), while if it is >70%, the chemical is considered to be inherently biodegradable.

To make a hazard assessment, the predicted environmental concentration (PEC) is calculated and, in the absence of kinetic data, a reasonable "worst case" situation is considered. The PEC is estimated from annual production data, pattern of usage, route to the environment of the chemical, and daily per capita water consumption (see section B.2). If the chemical is readily biodegraded, the annual production is decreased by 90% but if it is only inherently biodegradable, it may be prudent to calculate both moderated and non-moderated values. These PEC values are compared with the lowest concentration at which the chemical causes adverse effects, or the highest observed concentration giving no effect, on aquatic animals (fish, daphnia, etc.). A judgement is then made about the effect of the chemical on the environment. Should it be necessary to know more precisely the degree of removal of a chemical, an appropriate simulation test (3rd tier) is applied and this leads to a revised estimate of the PEC.

Also for chemicals degraded by 20-70% in the inherent test and for which the remaining organic matter is not the parent compound, similar calculations are made and, if necessary, toxicity tests are carried out on the intermediate(s). More sophisticated models are available for assessing the distribution of chemicals between the atmosphere, soil and water and thence their concentration in various compartments of the aquatic environment.

The scheme produced by the EC was first indicated, rather obscurely, in the Annexes to the 6th Amendment to the Directive (Off. J .E. Comm. 1979), but was more clearly stated, with a complex decision diagram (**Figure E.2**), in a little-known publication (EC, 1986). Guiding principles are given to help in deciding which tests to carry out, and at what stage, and three categories of chemical are introduced which may be treated differently at the various stages:

- not special or not giving cause for concern,

- give cause for concern, e.g. extreme toxicity to aquatic organisms

- special cases, based on physico-chemical properties (e.g. strong adsorption) and/or use/disposal patterns.

The objective of the scheme is to reduce the uncertainty that a chemical may or may not degrade in the environment; for example, recommended further testing may involve low temperature or special environmental compartments. In some cases there are no agreed test methods, for example, anaerobic degradation for insoluble chemicals, to implement the recommendations in the scheme and the document urges that such methods soon be instituted.

Figure E.1 OECD flow scheme of biodegradation

Start

Level I

Screening test for ready biodegradability

Decision point — is compound readily biodegradable? — **Yes** → No further work needed on biodegradation

No

Level II

Test for inherent biodegradability

Decision points — >20% mineralisation? — **Yes** → Compounds not inherently biodegradable, N.F.W.*

Yes

>70% mineralisation? — **No** → Identity biodegradation products, calculate W.P.C.** levels and determine toxicity

Yes

Level III

Input information on ecotoxicity, tonnage and partition → Estimate worst possible environmental conclusion (W.P.C.) of parent compound and relate to effect concentration

is more accurate estimate of concentration of products required? — **No** → (to Estimate worst possible environmental conclusion)

is more accurate estimate of concentration required? — **No** → No further work on biod. is required

Yes → Appropriate simulation test

Yes → Appropriate simulation test

Outcome: Revised estimate of environmental concentration(s) of compound and/or biodegradation products

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* N.F.W.: No further work on biodegration testing
** W.P.C.: "Worst possible case"

Biodegradability testing is preceded by a test for inhibition to bacteria, if this property is not already known, so that the appropriate concentration of test chemical can be selected for the test. Also, if initially the chemical is thought to be not readily biodegradable, base-set (level 0) tests may be omitted and a test for inherent biodegradation may be applied.

The main criterion triggering tests in the three tiers is the annual tonnage produced, or the total tonnage over five years, of the chemical in question. An annual production of one tonne necessitates tests at the base-set level (level 0); as a very minimum the BOD and COD tests are required, but normally one of the tests for ready biodegradability is required. When the annual tonnage reaches 10 to 100 tonnes, or 50 tonnes in total (level 1a), tests for inherent biodegradability may be required, while at 100 to 1000 tonnes/y or 500 tonnes total (level 1b) such a test will be demanded. Finally, when level 2 is reached (>1000 tonnes/y), further tests to be done, for example simulation, will be agreed between the notifying manufacturer and the Competent Authority. At each level, tests appropriate to the next higher level may be asked for if the chemical is in the categories "cause for concern" or "special case". For example, a pass in the screening test does not rule out a need for further investigation if information of a more quantitative nature is required. No mention is made of metabolic intermediates, but presumably the same treatment should be given as in the OECD scheme.

Thus, it can be seen that in both the OECD and EC schemes the tests for ready biodegradability play a crucial role in hazard assessment. They should screen out chemicals that are shown not to degrade in the real environment. There are safeguards in the EC scheme. Although a chemical passing a ready test is not normally further tested until its yearly production reaches 1000 tonnes, if the chemical has a particularly high toxicity ("a chemical of concern") the Competent Authority can demand a simulation test when only 100 tonnes/y is reached (EC, 1986). That is, in UK terms, a simulation test can be demanded when the "average" PEC reaches 20 μg/l (100 tonnes/y, assuming no biodegradation) instead of waiting until 200 μg/l (1000 tonnes/y) is reached. Of course, the Competent Authority could ask for tests at even lower tonnages if the toxicity of the chemical is exceptionally high or if reasonable "worst cases" yield higher PEC values.

There are some little known aspects of the EC guidance on strategy. For example, if a chemical is judged not to be readily biodegraded, the first test applied may be one for inherent biodegradability. Also, when the annual production of a chemical reaches 100 tonnes, even if it is not inherently biodegradable, a simulation test may be called for in which any harmful effects it may have on the activated sludge process, rivers, etc. can be assessed. Other tests which can be demanded for any chemical are those for anaerobic biodegradation, degradation in soil, and photo-degradation.

As a modification, Struijs (1991) suggests that tests for ready and inherent biodegradability should be placed at the same hierarchical stage. Thus, if by applying structure-activity relationships the chemical could be considered to be not readily biodegradable, a test for inherent biodegradability should be applied. If a positive result is obtained, it would be prudent to apply a test for ready biodegradability. The next step would be to apply a test for "potential biodegradation" in a specific compartment, for example in sea water. Positive results at this "potential" level may provide sufficient information to avoid having to apply laborious and extensive simulation tests.

Another strategy should be considered, especially if a number of "new" chemicals are to be tested. Experience, based on about 150 "new" chemicals and on MITI data, has shown that the proportion of passes in the present screening tests is low, about 10%; more information on this statistic is held by Competent Authorities. Under these conditions, it would

be uneconomical and not very informative to apply tests for ready biodegradability as the first step. But more information would be forthcoming for less effort by applying an inherent test first; the results would show that the chemicals were either inherently biodegradable or non-biodegradable. A test for ready biodegradability is then applied to those chemicals shown to be inherently biodegradable. In all, fewer tests would need to be carried out and less effort would have been expended. This modified scheme would also be more advantageously applied to batches of "existing" chemicals if structure-activity relationships predicted that only a small proportion of them were readily biodegraded.

There are other suggested schemes. Gledhill, *et al.* (1980) proposed three tiers, but the first included tests for both ready and inherent biodegradability, tier 2 consisted of simulations of natural environments, and the third included field studies – sewage treatment, natural waters, soils. Grady (1985) envisaged four tiers. The first contained screening tests which eliminate those chemicals unlikely to cause problems. At this stage the inhibitory properties should be determined, preferably using a culture from the biodegradability test. Acclimation and enrichment was the object of the second tier, using chemostats and continuous flow activated systems in tests lasting up to three months to assess primary biodegradability. In the third tier the degree of biodegradation was assessed, for example by repeating a tier 1 test with inocula from tier 2, using DOC, ThOD or $ThCO_2$. Alternatively, one could use two continuous flow activated sludge reactors, one serving as control and the other dosed with test chemical. The % DOC removal could be deduced from the difference in DOC concentrations in the two effluents. If all else fails, resort is made to ^{14}C-labelled chemicals. In the last tier, kinetic constants of degradable chemicals were to be determined only if it was not obvious that the chemical degraded so fast that it would be removed in waste water treatment. The kinetic constants could be determined in continuous reactors operated at different sludge retention times (SRT): such tests were made by Birch (1984, 1990).

Kirsch, *et al.* (1986) studied "priority" pollutants, most of which were potentially not readily biodegradable, and began with acclimation to the chemical in reactors operated at high SRT. Possible removal by abiotic processes were investigated to ensure that any removal was due biodegradation. If the chemical was not biodegraded, no further tests were made; but if biodegraded, the chemical was subjected to a tier 2 test. The chemical was fed to continuous, stirred tank reactors at four SRT's, for example 3, 7, 11 and 15d, until "steady states" in the concentration of DOC were reached. If the chemical was removed in one of these reactors, treatment was considered feasible and in stage 3 its ultimate biodegradability would be investigated by determining microbial growth on the chemical as sole source of carbon or by measuring $^{14}CO_2$ from the ^{14}C-labelled chemical.

A more sophisticated scheme was put forward by Niemi and Veith (1989), who included considerations of structure-biodegradability relationships (SBR) more formally, as well as experimental steps. At each level the appropriate SBR is applied and a decision made on whether the information is adequate, inadequate or inconclusive to determine the biodegradability of the chemical. As new experimental results are acquired, they are added to the appropriate SBR data base. The first step is to consult a data base(s) and if the chemical is clearly biodegradable no further steps are needed, except to refer to degradation "by-products expert system" for a consideration of the metabolites. At the next tier the 5d-BOD SBR is consulted, and if biodegradability is not indicated, a 5d-BOD test is carried out. This procedure is repeated, if necessary, for the 28d OECD MITI test, with unacclimatised inoculum, and then, at the final stage, repeated with a river die-away test.

Figure E.2   EC scheme for biodegradability testing
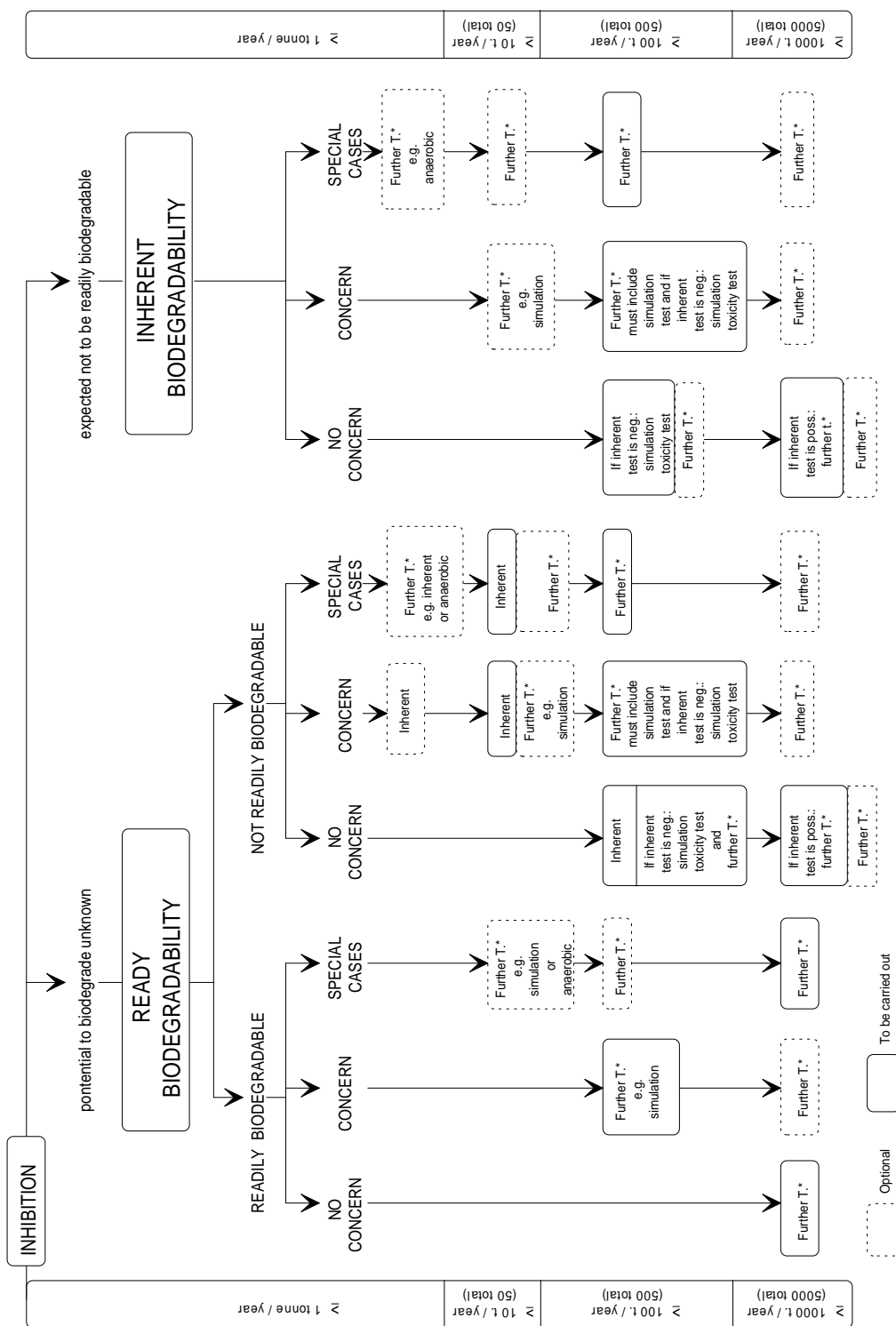
Mathiessen (1991) has proposed a tentative scheme for the investigation of the biodegrability of pesticides in natural sediment/water systems. First, the hydrolysis test is applied and only if the half-life was <2 weeks would the scheme be applied. A static sediment/water test is made in which the rate of disappearance of the pesticide is measured and metabolites would be sought. If the half-life (primary biodegradation) was <2 weeks, no further tests are needed. For other chemicals, tests with ecosystems and subsequently with mesocosms would be made in tiers 2 and 3.

F. Structure and biodegradability

As in other fields, links between chemical structure and biodegradability have been sought since a knowledge of any such relationship would be of use in the synthesis of new, biodegradable chemicals as well as in predicting the biodegradability of untested chemicals. Also, perhaps insights would be given into the biochemical mechanisms of at least the first stage of microbial attack on the chemical. As an aid to what changes occur to chemicals of environmental importance, the lists given by Alexander (1981) of transformations, cleavages, conjugations and other reactions should be very useful.

In most practical biodegradability studies, the object has been the obvious one of determining whether or not the chemicals are biodegraded. Few practical studies have the object of relating degradation with structure. The former investigations were made using a wide variety of experimental conditions and, since the results are often dependent on such factors as source and density of the inoculum, concentration of test chemical and duration, use of the data in activity-structure relationships by later investigators has been problematical.

Recent, well-documented reviews of all aspects of structural effects have been given by Kuenemann and Vasseur (1988), Kuenemann, *et al.* (1990b), Vasseur, *et al.* (1990) and Pitter and Chudoba (1990). A selection of some chemical groups studied for qualitative effects is given in **Table F.1**; other groups are given in the reviews cited.

In what Kuenemann, *et al.* (1990b) term a boolean classification, there are lists of chemicals including polymers which are very recalcitrant (Alexander, 1973). Kawasaki (1980) classified about 120 chemicals as either biodegradable or non-biodegradable, based on results with the MITI test, and later Kitano (1983) followed with about 500 chemicals (but not all based on the MITI test). This led to the conclusion that some structures were non-biodegradable or were very difficult to degrade, for example quaternary C bondings; nitro, amino and sulfonic substituents on phenol, benzoic acid, aniline and toluene.

The next approach was to rank chemicals according to their potential for biodegradation. For example, Urano and Kato (1986b) studied a very mixed batch of 78 chemicals and showed that the ease of degradation of the following chemicals decreased in the order (a) to (d):

(a)   carboxylic acids, alcohols, esters;

(b)   ketones, olefine sulfates, olefine sulfonates;

(c)   diamines, amino-, nitro-, sulfonic aromatic acids;

(d)   alkyl ethers, halo-, amino-, nitro-sulfonic benzenes and phenols.

Others have studied derivatives of a similar basic structure, which is a more fruitful approach. In reviewing these studies, Kuenemann, *et al.* (1990) have summarized part of a complex situation in eight pithy sentences, sometimes with qualifications.

With regard to the number of C atoms in aliphatic chains, two situations occur:

1) An increased number of C atoms favours biodegradation.

   This applies to imides and amides, C_3-C_{10} aliphatic ketones, C_1 to C_8 alcohols, C_1 to C_8 2,4-D esters and to diamines.

Conversely:

2) An increased number of C atoms reduces biodegradation.

   This applies to linear alkanes, fatty acids, adipic acid esters, C-C_{15} aliphatic alcohols, phthalates and to C_{11} to C_{14} alkyl benzene sulfonates. However, members of the latter group, plus the C_{10} homologue, were recently found by Larson (1990) to degrade at virtually the same rate. The pseudo first-order reaction constants at 10-100 ug/l in river waters and sediments were 0.7+/-0.11d^{-1}. The original data from which the statement was derived related to much higher concentrations and to primary degradation.

Regarding the number of rings in cyclic compounds, many studies indicate that:

3) An increased number of rings impedes biodegradation. However, Oudot (1984) reported some discrepancies, such as that 3- and 4-ring homologues were degraded less well than 5- and 6-ring alkanes. Heterocyclics with sulfur in the ring are more resistant than their non-sulfur analogues.

Results with dyestuffs indicate that:

4) An increased number of azo bonds reduces biodegradation.

In both aromatic and aliphatic structures it appears that:

5) An hydroxyl or carboxyl group enhances biodegradation, but other substituents have different effects so that:

6) Amino and, above all, chloro, nitro and sulfonic groups generally decrease biodegradation.

The influence of the number of substituents can be summed up as:

7) An increased number of substituents decreases the ease of biodegradation, although the positions of the substituents sometimes greatly affects the biodegradability of aliphatic as well as aromatic chemicals.

Finally, the influence of structural complexity was summed up, perhaps rather crudely, as:

8) A higher molecular weight results in a lower biodegradability.

Of course, many other factors influence the susceptibility of chemicals to degradation and all of these factors must be reflected in any more complicated models relating structure with biodegradability.

F.1   Modelling and statistical analysis of biodegradability data

The boolean and ranking approaches have limited application, since no rates can be predicted, though this is partly a reflection of the lack of quantitative kinetic data. Quantitative relationships were sought and a number have been published; the present position of these attempts has been reviewed by Vasseur and her colleagues (see earlier 1988, 1990), Pitter and Chudoba (1990) and by Parsons and Govers (1990). In an earlier review (Painter, 1983) in which it was suggested that Mudder (1981) had coined the term QSBR, relatively few studies were recorded. It was foreseen, wrongly as it turns out, that the "frontier-orbital" approach (such as HOMO – highest occupied molecular orbit, and LUMO 3 – lowest unoccupied molecular orbit; Fukui, 1981) perhaps illustrated graphically in colour and three dimensions (Richards and Sackwild, 1982) might soon be used in QSBR. No evidence of this was seen.

Kuenemann, *et al.* (1990b) identify two major sorts of relationship; one is quantitative, QSBR, the other only qualitative, qual. SBR. In the former, quantitative physical properties, either macro or molecular, are related to a quantitative index of biodegradation, while in the latter a statistical treatment of the data is employed but biodegradation is expressed only as "biodegradable" or "non-biodegradable".

Table F.2 indicates virtually all the QSARs reported by the three reviewers; the equations and goodness of fit are reported by Kuenemann, *et al.* (1990b). While some authors examined their own experimental data, in nearly half of the cases authors reported on data produced by others. Ranges of parameters describing both biodegradation and physical descriptors have been used.

The results are discussed in depth by the reviewers; all agree that at present there is no general relationship between biodegradation and structure to fit all chemicals. Some discrepancies between results can be attributed to the numbers of compounds in the "training" set sometimes being too small (there should be at least five) and/or the number of molecular descriptors introduced in the regression analysis being too high for the number of chemicals. Other discrepancies arise from the paucity and poor quality of the biological data.   In particular, some authors (e.g. Dearden and Nicholson 1986, 1987a,b) used BOD5 data from various sources, which is a doubtful practice since the test is known to have poor reproducibility, especially between laboratories. A perusal of BOD5 data shows that there are three broad groups of chemicals for which the % ThOD varies from 0 to 20%, 0 to 100% and 40 to 100%, respectively.   Also, a selection of possible data was made to form the relationship, but the grounds for selection were not given. The interpretation of Dearden and Nicholson's results using BOD data is not clear. Parsons and Govers (1990) look upon the equation embracing 197 out of the 240 data set of Dearden and Nicholson (1987a,b), which gave a correlation coefficient of r_2=0.982, as interesting, but are unable to judge its significance. The results appear to indicate that the biodegradation rates of a wide range of compounds are controlled by the electronic properties of a particular reactive centre, which is not necessarily the site of attack. The fact that such good correlations were obtained with BOD as rate parameter suggests that the initial attack on these compounds is rate-determining for their complete degradation (Parsons and Govers, 1990).

In another study, Boethling (1986) correlated biodegradation data for different classes of compounds with molecular connectivity indices (MCI), which quantify molecular branching. Good correlations for a wide range of compounds were obtained, but for different structural classes different rate parameters and different order MCI values were used to achieve the best correlations. This limits their predictive utility and makes it difficult to draw general conclusions concerning the influence of molecular structure on biodegradation rates.

F.1.1 SARs

Attempts have been made to relate structure using statistical techniques of discriminant or cluster analysis on relatively large number of chemicals divided into broad classes – "biodegradable" vs. "non-biodegradable", and sometimes "non-biodegradable, qualified" for which the data are not definite. Geating and Enslein (1981) used chemicals from the literature, but were surprised that they had so much difficulty in establishing whether a chemical was degradable or not. They eventually selected 430 chemicals, of which 296 were biodegradable, but they did not give the criteria by which they assessed biodegradability. They considered three types of parameter at the outset: molecular weight; the octanol-water distribution coefficient (K_{ow}); and Wiswesser line-formula notation (WLN) based on sub-structural keys. They could not obtain accurate K_{ow} values for a sufficient number of the chemicals, so they used only molecular weight as a crude index of bulkiness and WLN-based keys taken from the CROSSBOW program (Eakin, *et al.*, 1974) for describing the chemicals.

The models were constructed using stepwise discriminant analysis and ridge regression procedures. They obtained scores of 92% correct "predictions" for degradable chemicals, but only 68% for non-degradable chemicals. Enslein, *et al.* (1984) later used MCI plus the number of atoms of H, O, etc. on 250 chemicals for which BOD values were available. Their model classified 88% of the classifiable chemicals accurately, 11% falsely positive and 1% falsely negative; 42% of the 250 chemicals were unclassifiable.

In a somewhat different approach, Niemi, *et al.* (1987) used cluster analysis to correlate MCI with BOD expressed as % ThOD of 287 chemicals. The best iteration, after calculating 54 MCI values and using five physico-chemical properties, correctly "predicted" 85% of biodegradable and 94% of persistent chemicals. They then used a second step (a heuristic step) using the results of the multivariate analysis and the literature on biodegradability to identify a new series of structural features associated with degradable and persistent chemicals. The new approach correctly "predicted" 91% of degradable and 96% of persistent chemicals.

Mudder (1981), in a thesis, used multiple linear regression to correlate the specific COD removal rates on the 54 mono- and di-substituted benzenes tested by Pitter (1976). He constructed two models, one with 14 structural descriptors and the second as the first but also with six physico-chemical variables, that is 20 variables in all. But 20 variables for 54 chemicals is too many for obtaining valid correlations; ten would have been sufficient. Partly because of this, and because of the relatively small number of chemicals (only 37 could finally be used), the results were unsatisfactory. He then tested, practically, chemicals not in Pitter's 54 by an untried modification of Pitter's method, since he chose volatile chemicals. Unfortunately, the results did not compare well with predictions made from the model. However, he was able to make useful speculations about the mechanism of degradation of phenols and anilines.

F.1.2 Other approaches

Howard, *et al.* (1987) concluded that attempts to establish valid, widely-embracing models were thwarted by insufficient rate data on too small a number of chemicals. They proposed "weight-of-evidence" evaluations. Data on individual chemicals were collected from many sources, such as screening tests, bio-treatment simulations, grab-sample tests and field studies. The data on each chemical were evaluated to form a flexible BIODEG file, which would also reflect such factors as the conclusions of the author(s) of a given report. They have recently published a book (Howard, *et al.*, 1991) containing details of aerobic and

anaerobic biodegradability of about 340 chemicals based on this approach: the book also contains data on other degradation rates, namely, photolysis, photo-oxidation and hydrolysis.

Boethling and Sabljic (1989), who required a rapid assessment, developed a method for classifying untested chemicals using some molecular properties but also opinions/judgements from 22 experts. The experts were asked to estimate the time taken for the chemical to be degraded in terms of days, weeks, months, etc. Based on environmentally relevant experimental data, the model correctly classified 36 of 40 chemicals (90%).

Another recently reported approach was made by Desai, *et al.* (1990), who calculated the contribution made by eight individual chemical groupings (CH_3, OH, etc.) using data from Urano and Kato (1986b). Details of the method for calculating the contribution, α, are given by Chitra and Govind (1986). The biodegradability of eleven compounds was measured using an electrolytic respirometer, and was expressed as pseudo-first order constant. Comparison of these determined rates with those predicted from the group contribution values showed agreement to within 20%. It was assumed that there were no interactions between contributions from different groups within a molecule. The authors stated that many more chemicals would have to be tested before coming to a conclusion on the method.

F.2   Summary

Summing up the present position, it can be said that correlations already obtained confirm the general application of linear free-energy relationships. There is general agreement that, though there is at present no general relationship embracing all chemicals, there is the prospect that improvements in the future will at least get nearer to this. For some classes of compound biodegradation rates have been successfully correlated with various descriptors. At least a partial explanation is that these structural parameters are themselves often related. Thus a better understanding of relationships between descriptors is required, as well as careful selection of the sets of compounds, to avoid this problem. There are few reports of the use of relationships to predict successfully biodegradation rates of compounds of unknown biodegradability. Also, little information has been seen on the use of QSARs based on biodegradability data from one system to predict biodegradability rates in other systems.

Table F.1   Effects of structure on biodegradability

Chemical group	References
acids	Dias and Alexander, 1971 Hammond and Alexander, 1972
alcohols	Dias and Alexander, 1971
alkylnaphthalenes	Solanas, *et al.*, 1984
amines	Rothkopf and Bartha, 1984
benzene derivatives	Alexander and Lustigman, 1966 Kitano, 1983
imides, N-substituted	Ennis, *et al.*, 1978
linear alkyl benzenes	Takada and Ishiwatari, 1990
polychlorobiphenyls	Parsons and Sijm, 1988 Parsons, *et al.*, 1988 Rhee, *et al.*, 1989; Furukawa, *et al.*, 1978
phenols	Omori and Yamada, 1973 Chambers and Kabler, 1964 Chambers, *et al.*, 1963
phthalates	Johnson and Heitkamp, 1984
polyester films	Diamond and Freeman, 1975
pyridines	Sims and Sommers, 1986
quaternary C compounds	Mohanrao and McKinney, 1962
surfactants:	Swisher 1987
anionic	Larson, 1990
nonionic	Huddleston and Allred, 1967; Fischer, 1973; Birch, 1982
cationic	Cruz, *et al.* 1979
general	Urano and Kato, 1986a, 1986b; Kitano, 1983; Kawasaki, 1980

Table F.2 Quantitative structure-activity relationships for biodegradation

	Compounds	n	Biodegradation index (a)	Descriptor (b)	Reference
1	acids	20	% ThOD	MCI/uc	Boethling, 1986
2	branched acids	10	% ThOD	MCI	Boethling, 1986
3	branched and linear acids	10	% ThOD	MCI	Boethling, 1986
4	carboxylic acids	36	BOD_5	DMAC	Dearden, Nicholson, 1986
5	carboxylic acids	40	BOD_5	DMAC	Dearden, Nicholson, 1986
6	subs. benzoates	l10	$logV_{rel}$	Hammett	Reinecke & Knackmuss, 1978
7	amino acids	8	BOD_5	DMAC	Dearden, Nicholson, 1986
8	acids and alcohols	24	% ThOD	MCI/uc	Boethling, 1986
9	linear alcohols	5	log % ThoD	$LogK_{d.oct}$	Yonezawa, Urushigawa, 1979
10	alcohols	14	% ThOD	MCI(VC)	Boethling, 1986
11	alcohols	22	$\log k_b^1$	logKd.oct	Yonezawa, Urushigawa, 1979
12	glycols	8	BOD_5	DMAC	Dearden, Nicholson, 1987b
13	alicyclic ketones and alcohols	4	$\log (rate_{cod})$	$logK_{d.oct}$	Vaishnav, *et al.*, 1987
14	alicyclic ketones and alcohols	4	$\log (rate_{cod})$	MCI(VC)	Vaishnav, *et al.*, 1987
15	alicyclic ketones and alcohols	6	$\log (rate_{cod})$	MCI(VC)	Vaishnav, *et al.*, 1987
16	alicyclic ketone	10	log (% ThOD)	$logK_{d.oct}$	Vaishnav, *et al.*, 1987
17	alicyclic ketone	5	log (% BOD_{rem})	$logK_{d.oct}$	Vaishnav, *et al.*, 1987
18	aldehydes	6	BOD_5	DMAC	Dearden, Nicholson, 1986
19	aldehydes	9	BOD_5	DMAC	Dearden, Nicholson, 1986
20	esters	19	BOD_5	DMAC	Dearden, Nicholson, 1986
21	phthalate esters	8	$\log k_b^1$	log Rt	Urushigawa, Yonezawa, 1979
22	phthalate esters	12	k_b	MCI	Boethling, 1986
23	phthalate esters	4	$\log k_b$	$\log K_{oh}$	Wolfe, *et al.*, 1980
24	phthalates and aliphaticalcohols	-	-	$\log K_{d.oct}$	Matsui, *et al.*, 1983
25	p-amino benzoate esters	8	$\log k_b^1$	$\log K_{d.hex}$	Parsons, *et al.*, 1987

	Compounds	n	Biodegra-dation index (a)	Descriptor (b)	Reference
26	2,4 esters	6	$\log k_b$	$\log K_{d.oct}$	Paris, *et al.*, 1984
27	2,4 esters	6	$\log k_b$	MCI	Boethling, 1986
28	carbamates	7	$\log \%D$	MCI	Boethling, 1986
29	carbamates	-	-	pKa	Wolfe, *et al.*, 1978
30	ethers	6	$\log \%D$	MCI(vc)	Boethling, 1986
31	amines	15	BOD_5	DMAC	Dearden, Nicholson, 1986
32	anilines	17	t_{50}	pKa	Moore, *et al.*, 1989
33	subst. anilines	-	-	r_v	Zeyer, *et al.*, 1985
34	o-,m-,p-anilines	433	$\log v$	Hammett	Pitter, 1985
35	m-anilines	7	$\log k_b$	r_v	Paris, Wolfe, 1987
36	phenols	5	BOD_5	DMAC	Dearden, Nicholson, 1986
37	phenols	20	t_{50}	pKa	Moore, *et al.*, 1989
38	phenols	-	-	MR	Yonezawa, *et al.*, 1985
39	o-,m-,p-phenols	555	$\log v$	Hammett	Pitter, 1985
40	p-subst. phenols	8	$\log k_b$	r_v	Paris, *et al.*, 1983
41	p-subst. phenols	7	$\log k_b$	r_v	Paris, *et al.*, 1983
42	chlorophenols	5	t_{50}	pKa	Moore, *et al.*, 1989
43	chlorophenols and choro-anisoles	16	$\log k_b^1$	$\log K_{d.oct}$	Bannerjee, *et al.*, 1984
44	halocatechols	7	$\log v_{max}$	Okamoto-Brown Dom, subst. constant	Knackmuss, 1978
45	halo-hydrocarbons	9	BOD_5	DMAC	Dearden, Nicholson, 1986
46	propizamide esters	40	$\log k$	Hammett, II	Cantier, *et al.*, 1986
47	azo dyes	-	-	Hammett	Zimmerman, *et al.*, 1982
48	Various, 6 groups	79	BOD_5	DMAC	Dearden, Nicholson, 1986
49	Various, 14 groups	197	BOD_5	DMAC	Dearden, Nicholson, 1986
50	Various pesticides	12	$\log k_b^1$	$\log K_{d.oct}$	Kanazawa, 1987
51	Various pesticides	12	$\log k_b^1$	$\log K_{OH}$	Kanazawa, 1987
52	Various	6	$\log k_b$	$\log K_{OH}$	Wolfe, *et al.*, 1980

Footnotes to Table F.2:

(a)   Biodegradation rate parameters: V: biodegradation rate; V_{rel}: relative biodegradation rate; V_{max}: maximum rate of enzyme reactions; k_b: second order rate constant; K^1_b: pseudo-first order rate constant; $rate_{Cod}$: rate of removal of COD; BOD_{rem}: removal of BOD; % ThOD: percent theoretical oxygen demand; BOD_5: 5d biochemical oxygen demand; % D: percent parent compound degraded; t_{50}: time for removal of 50% of parent compound.

(b)   Chemical structure descriptors: $K_{d.oct}$: octanol water partition coefficient; $k_{d.hex}$: hexane-water partition coefficient; R_t: reversed-phase HPLC retention time; k_{OH}: alkaline hydrolysis rate constant; r_v: van der Waals radius of substituents; MCI: molecular connectivity index; MCI(vc): molecular connectivity index corrected for valency; DMAC: difference in the modulus of atomic charge of a selected bond; pKa: ionization constant; MR: molar refraction; mp: melting point; II; hydrophobicity factor. n = number of chemicals in the "training" set.

G. Discussion and conclusions

G.1 Introduction

Above all else it must be remembered that, because methods for assessing biodegradability are only one of many types tests which have to be applied to very large numbers of chemicals, the methods are compromises between the economic need for relative simplicity and the scientific requirement that the results obtained by their use should be valid and relevant. The test method most urgently required is one mimicking conditions in environmental waters, with and without sediments, such that the chemical is tested for mineralization at around 1 μg/l, without the addition of micro-organisms or nutrients. However, the literature review has revealed no new methods which would allow this to be done without the use of ^{14}C-labelling. Nevertheless, it may now be time to formalize and standardize these ^{14}C methods, capitalizing on the large amount of experience in this field without making method(s) obligatory.

There are methods in the literature which are capable of either filling gaps in the OECD Test Guidelines or improving or replacing existing methods. For example, there are methods for assessing poorly soluble and volatile chemicals as well as reliable, well-tried methods for assessing anaerobic biodegradability and inhibition to methane production, all of which should be included in the Test Guidelines. The Sturm test in its present form is incapable of yielding an accurate representation of the microbial production of CO_2 with time and it could with advantage be replaced by one or other of the elegant headspace methods, involving the determination of gaseous CO_2, or CO_2 plus DIC. Of the oxygen uptake methods, the respirometric method requires expensive equipment and the Closed Bottle test will accept only small amounts of insoluble chemicals, which are difficult to weigh out accurately. The two-phase Closed Bottle, however, is able to deal with more manageable amounts of chemical, because of the large reservoir of oxygen in the headspace. The method uses relatively cheap apparatus and its addition to the Guidelines deserves consideration.

There are also some established methods for simulating the aerobic treatment of sewage (the porous-pot technique for activated sludge simulation and the rotating tube simulating bio-filters) and these are candidates for addition to the "coupled-units" method. Similarly, simulations of the anaerobic treatment of sewage sludge are available.

Tests simulating the natural environment are more problematic, although much information has been gathered in the past decade or so about events occurring in various parts of the aquatic environment, including quantitative aspects. While it has been relatively simple to set up laboratory models of waste water treatment units, it is much more difficult to conceive of laboratory models of rivers, lakes and the sea which are practicable. Here it seems that some information will be gleaned from field tests, e.g. small ponds, but much more will be obtained from simple batch or continuous tests which will act as surrogates for larger-scale simulation methods.

The strategy and philosophy of testing adopted by the OECD and EC have been scrutinized and proposals have been made for changes, some of which possess definite advantages. Attempts to relate biodegradability with chemical structure and/or properties of the chemical have not yet been successful in achieving an all-embracing relationship, though working relationships within limited families of chemicals have been set up.

G.2   Existing methods

Turning to existing test methods, little criticism has been levelled at the procedures *per se*; it is their relevance to the environment which has been criticized, except perhaps their relevance to sewage treatment. It seems that the conditions in the methods in the revised OECD Test Guidelines (OECD, 1992), which stipulate the use of 2 to 100 mg/l of test chemical, are probably optimal and possible interferences due to nitrification have been reduced. The media probably cannot be improved except to confirm that the artificially high concentrations of phosphate buffer, rather than the environmental carbonate buffer, do not affect the results. On the other hand, the "synthetic" sewage used in the simulation of the activated sludge process requires improvement, though it is difficult to see how this is to be done in view of attempts already made. It contains relatively too much nitrogen, produces "bulking" sludges which interfere with the process, and fails to allow degradation of some chemicals which degrade well in natural sewage.

G.3   Inoculum

The inoculum continues to be the main source of uncertainty in screening tests, and the variability is especially high when the cell density in the medium is low. By using higher cell densities, but not more than the equivalent of 30 mg activated sludge solids/l (about 10^6 cells/ml), more reproducible results are obtained. This maximum density has been shown to be "safe" in screening tests, but cell densities as high as 1 g solids/l, as in the Zahn-Wellens test, can give "false" positive results in "ready" tests. Also, if the cell density is higher than about 10^6/ml, the blank (inoculum only) values of oxygen uptake, etc. are higher and this reduces the precision of the tests. Struijs (pers. comm.) has calculated that in the respirometric methods (manometric, BODIS) the maximum should be only about one half of that recommended, or 15 mg solids/l. Pre-conditioning (aeration) of the inoculum material in the medium without test chemical for up to one week is advantageous only in some cases in reducing blank values. (Pre-exposure to the test chemical is dealt with later.)

Checks on the inoculum could be made before use by determining the ATP content by a relatively straightforward method, and perhaps by determining the species composition, though this is a more tedious process. A normal content of ATP indicates the inoculum has adequate general metabolic activity, but not that the chemical under test will necessarily be degraded.

G.4   Chemicals and reference chemicals

The recent guidelines by ISO (ISO, 1990b) are useful in preparing insoluble and poorly soluble chemicals for introduction into the test medium. Similarly, there are methods described for assessing the biodegradability of volatile chemicals (e.g. ISO, 1984). These ISO guidelines should be considered for inclusion. Adsorption of chemicals in screening tests appears not to have given rise to problems, but the revised Guidelines (OECD, 1992) suggest how to deal with chemicals which adsorb.

The question of simultaneously subjecting a reference chemical, known to be readily biodegradable, to "check" the activity of the inoculum should be carried out with chemicals which are less readily degradable than those used at present (benzoate, acetate, aniline). Alternatively, the media for the reference chemical should be sterilized before inoculation, since uninoculated media have been shown to allow degradation of the present reference chemicals by virtue of the bacteria present in the deionized water.

G.5 Inhibitors

The desire to avoid the use of the environmentally damaging mercury (II) chloride, for so long used to check for abiotic degradation and to preserve samples for analysis, may be met by the use of sodium azide or formaldehyde for assessing abiotic degradation and by freezing filtered or centrifuged samples, if they have to be stored before analysis.

G.6 Analysis

A few analytical points are worth mentioning. The determination of DOC in solutions of volatile chemicals should be examined to ascertain whether correct values are obtained or whether significant proportions of the chemical are escaping oxidation. The determination of COD is unsatisfactory for some chemicals, and falsely low values are sometimes obtained, leading to falsely high values for biodegradability (% COD). Modifications of the COD test should be sought to remedy this situation.

Finally, the collection of CO_2 in the Sturm test has been reported to be faulty; the gas is not flushed out of solution as fast as it is produced, so that significant amounts remain in solution for several days after the plateau of removal of DOC has been reached. Consequently, the biodegradation curves are not as steep as they should be and the % $ThCO_2$ attained at the end of the 10d window is often falsely low. The 28d value is unaffected because the medium is acidified and any remaining CO_2 is determined on the 29th day. The extra CO_2 produced in the last 24h is usually negligible, because most chemicals have fully degraded by then or have not degraded at all. The difficulty of poor collection may be overcome by taking samples of the medium at the same time as CO_2 is determined in the external traps and analyzing the medium for DIC. Alternatively, the method should be used for the 28-29d value only, or it should be replaced by or augmented with a headspace method.

G.7 Comparison of the screening methods

Of the three parameters – DOC, oxygen uptake, CO_2 – used to assess mineralization, CO_2 is the most positive since it has come directly from the test chemical. Few abiotic processes lead to production of the gas, while more such processes can remove DOC. Another disadvantage of DOC is that it cannot be applied to insoluble chemicals, but CO_2 production and oxygen uptake can. Oxygen uptake methods, except the Closed Bottle test, require specialized apparatus and greater temperature control, and can suffer from interferences from nitrification. Of the three parameters, DOC is determined with greatest precision and CO_2 the least when determined by the titration method. Much better precision results when a solution of NaOH is used as the absorbent (and is not replaced during the test) and DIC is determined; care must be taken to avoid atmospheric CO_2 reaching the NaOH. However, if the precautions given in the guidelines are taken, all three methods give valid results, apart from any difficulties with the inocula. Unless inhibition occurs, the four methods using the highest cell densities – MITI, DOC die-away, Sturm, and the Respirometric – will show a higher proportion of "passes" than the Closed Bottle and Modified OECD tests, which use low cell densities.

G.8 Pass levels and stable intermediates

The pass level of 70% for DOC removal is set at a reasonable level for screening purposes, but the pass level of 60% ThOD should be lowered to 50% (or lower) because of the overwhelming evidence, especially from ring tests, that >90% DOC removal was

equivalent to 45 to 70% ThOD. There is cause for lowering the CO_2 pass level from 60% $ThCO_2$, but far fewer directly comparable data have been seen on this point. Removal values based on COD should be regarded with suspicion, since lower than theoretical oxygen demand values can be obtained and there is no way of checking the COD value.

The value of 70% DOC has been challenged as being too low to decide that no stable intermediate has been formed. Analysis for the parent compound will help to decide this. If the parent compound is absent or is present in only low concentrations, the "recalcitrant metabolite" test (Gerike, *et al.*, 1984) could be applied, especially in the case of chemicals produced at high tonnage. This quantitative test would then show whether it was worth attempting to identify intermediates. Only a few chemicals appear to have been tested by this method.

G.9   Predictability of the screening tests

The intention behind the screening tests was that chemicals were sorted out into those which passed and those which did not. Since the tests were stringent, the latter group were not to be considered non-biodegradable but were to be examined in other, less stringent tests. Those which passed were assumed to have the potential to degrade in the aquatic environment generally, but no individual test method was linked with any specific part of the environment. However, the question naturally arises as to the worth of this assumption as it affected the various compartments of the environment.

It is clear (from section B.1) that positive results from the screening tests are good predictors of the behaviour of chemicals in waste water treatment. Indeed, it was on the basis of good correlations between forerunners of the screening tests and behaviour in activated sludge simulation tests that the conditions of the OECD tests for ready biodegradability were established. These conditions included the incubation period of 28d and the 10d window being included as an additional safeguard to the screening tests. The 28d period was later justified by applying Monod kinetics to the screening tests, making conservative assumptions. It was demonstrated that the bacterial species which degraded chemicals passing the tests would be maintained in the activated sludge system and that tests with higher cell densities, but not those with lower densities, would pass chemicals which degraded at lower rates. It was also found, practically, that some chemicals which did not pass the tests were degraded in the simulation test. In these simulation tests the chemicals were mineralized; in practice, it is not easy to show that a chemical is mineralized in full-scale sewage treatment plants, but primary degradation of some high tonnage chemicals has been demonstrated on the large scale.

The "coupled units" Husmann procedure used in the OECD activated sludge simulation test (303A) has been shown to have no advantages over the "single unit" mode of operation. Also, the porous pot is a simpler and cheaper apparatus for simulating the activated sludge process and has been shown to produce results comparable with those from Husmann units. These modifications are included in the EC methods, and they should be considered for inclusion in the OECD Guidelines. Methods simulating biofilm reactors and biofilters, which have some advantages over completely mixed activated sludge units, are available.

Because of analytical difficulties, at least 5-10 mg C/l of test chemical has to be used both in screening and simulation tests, whereas very many chemicals would be present in sewage in the µg/l range. The normal kinetic approach shows that such concentrations are below the minimum values which would support growth in a continuous reactor, indicating that

such chemicals would pass through the activated sludge system unchanged. But the phenomenon of secondary substrate utilization comes into play, by which the presence of a (primary) substrate at relatively high concentration allows the oxidation of the "secondary" substrate present at relatively low concentration. Since not many chemicals have so far been tested by this means, and since some of these were not degraded by the secondary oxidation mechanism, it is advisable that at least more chemicals of importance should be tested at low concentration in activated sludge simulations.

In contrast to sewage, the necessary conditions for degradation do not always prevail in rivers, lakes, ground water and the sea. Certainly in pristine rivers and lakes the necessary bacterial species may not be present and/or there may be deficiencies of nutrients, especially of N and P. Even in more polluted waters deficiencies may be present, though the likelihood is greater that the conditions will be favourable for degradation. It is because of these factors that the degradation of a chemical has been found to vary in samples taken from the same site in a river at different times, and from different rivers. Other factors, considered in sections A.4.4 and B, also come into play, such as threshold values for growth and adaptation; effects of other substrates; interactions between organisms; differences between eutrophic and oligotrophic bacteria. The doubts about prediction will not be overcome until the relevant simulation or "surrogate" simulation tests are introduced in which the appropriate factors are present. Such tests are available and are considered in sections A.9 and C.5.

Prediction has been approached in a modified way through adopting different strategies of testing by extending the classification of chemicals (Struijs and Stoltenkamp, 1986b; Struijs, 1991) or by introducing or popularizing the idea of surrogate tests (Carson, *et al.*, 1990). Also, a new system for calculating the concentration of a test chemical based on a "practical biodegradation" model, proposed by Shimp, *et al.* (1990), is worthy of attention for its relative simplicity, as compared with the more complex methods referred to in section B.3. The residence time of the chemical in a given compartment is related to the rate of biodegradation (or estimated rate) in that compartment, assuming first-order degradation. The rate is converted to the half-life in the compartment, and it was shown that the half-life must be less than the residence time for significant biodegradation to occur in any given compartment. The approach readily allows estimates to be made of percentage removal of chemicals by graphical methods.

G.10   Gaps in methods

It is clear that quantitative predictions will be more accurate when appropriate simulation tests are agreed and in use, because the conditions of the tests would take into account at least some of the many interactions and other events which occur in the environment but which are not evident in the OECD screening tests. Such "simulation" tests are available (e.g. ASTM E/1279-89); some, being more or less ready for use, were discussed in sections A.9 and C.5. It was considered that tests simulating rivers would not necessarily consist of river models in the laboratory. Much smaller vessels containing the elements to be simulated, for example the water column, sediment, flowing water, could be used, especially if more results are obtained with a greater variety of chemicals than have already been used in the small microcosms. These results would be related to those obtained in the field tests, such as ponds or small streams. Such tests would be die-away batch, or flow-through, over relatively long periods, using natural waters with or without suspended/undisturbed sediments. The removal of the test chemical would be followed by using specific analysis; the initial concentration should be as close to the "probable environmental concentration" as possible. (Perhaps for chemicals of great concern and/or high tonnage, ^{14}C-labelling could be used to assess mineralization rates.)   It would be informative to know the properties of the

environmental samples such as pH, DOC, N, P, etc. If it is required to know the effect of naturally occurring chemicals on degradation of a xenobiotic, the water sample should be chosen with this in mind, or some such material could be added. Similarly, other additions could be made (N, P) or the water could be filtered to remove protozoa.

From the data collected, at least the pseudo first-order rate constant could be calculated. In some cases it may be possible to obtain a more detailed and more accurate representation of the degradation kinetics by fitting the removal data to one or more of the six variants of the Monod equation (section B.3). Alternatively, by counting the numbers of bacteria present, the "second order" constant may be calculated by dividing the pseudo first-order constant by the cell numbers/ml. There are chemicals which could not be subjected to these methods because of the lack of suitably sensitive specific methods of analysis.

However, given the present EC strategy of testing, a simulation test has to be applied to a chemical only when its annual production reaches 1000 tonnes (= 200 μg/l in UK sewage) or at a lower tonnage if the chemical causes some concern, that is, it is particularly toxic to aquatic organisms or has a peculiar pattern of usage (section E). But if the strategy were to be suitably changed, a simulation test could be demanded at an even earlier stage. (The OECD scheme of testing is not dependent on amounts of the chemical produced.) Thus, before further simulation tests are considered for inclusion in the Guidelines, it would be prudent to find out more about the frequency of use of the activated sludge simulation test. This information for "new" chemicals is held by Competent Authorities in the EC and presumably will be forthcoming. Also, it would be helpful to estimate the frequency with which any other simulation test would have been applied, if available. But, since biodegradability tests are also applied to "existing" chemicals, many of which are produced in high annual tonnages, it may be that tests simulating rivers, lakes, etc. should be agreed and used as soon as possible.

Because many chemicals adsorb onto sewage sludge and sediments, and insoluble chemicals settle with sludge and sediments, there has long been a need for methods to assess the potential for a chemical to produce methane anaerobically. There is also a need for a method for determining inhibition of methane production. Two headspace methods were considered in section C.3, and a ring test on the ECETOC method (ECETOC, 1988) has been ring-tested. Results will be discussed at a meeting of ISO in Vancouver in 1994. Methods for the simulation of anaerobic digestion of sludge are also in use, but a longer period would be required for their adoption than for the "potential" method.

G.11   Changes to existing methods

There are several minor changes which could be made to existing methods, such as using more relevant reference chemicals; adopting more environmentally-safe techniques for abiotic vessels/preservation of samples; use of optimal size of inocula in oxygen uptake methods; search for a more reliable test for COD determination. Two major changes should be considered, one relating to CO_2 collection, the other – much more controversial – the use of pre-exposed inocula.

The evidence, though from only one source, strongly suggests that CO_2 is not purged from the medium to the alkaline absorbent as fast as it is produced by the micro-organisms. There was also evidence from other sources that maximal or plateau values of CO_2 evolution were attained after longer periods than were needed in the case of DOC removal. Thus, the shape of the CO_2 biodegradation curve is different from the DOC curve and applying the 10-day window would fail some chemicals, using % $ThCO_2$, but pass them using % DOC

removal. The solution to the problem would be to determine DIC in the medium at the same time as determining CO_2 in the traps, or to use a headspace method, described later.

The second major change would be to use, in screening test procedures, inocula which have been pre-exposed to the test chemical, as is permitted in the revised versions of the ISO methods. The arguments for and against have been aired over the years; many facts about exposure of micro-organisms to chemicals in sewage treatment and in natural waters have been given in section A.4.4. The proposal would be that inocula, pre-exposed for no longer than 15-18d in the presence of naturally occurring organic matter (sewage, sludge, river water, sediments), could be used in the screening test procedures. There is fairly strong evidence that chemicals passing such tests would also be degraded in sewage treatment (though more should be investigated), but further evidence is needed in the case of river waters, sediments, etc.

Chemicals reaching the accepted pass levels (e.g. 70% DOC removed) only with exposed inocula would be separately classified as "readily biodegradable using exposed inocula". This class could be added to the five classes proposed by Struijs and Stoltenkamp (1986b) as class 2a, between class 2, "readily biodegradable", and class 3, "intermediate in biodegradability". As more data are collected on the degradation of chemicals in the environment, it will be revealed whether chemicals in class 2a degrade in the environment just as do chemicals in class 2 and class 1, "highly biodegradable".

G.12   Other methods

The need for cheap and simple methods, applicable to poorly soluble and volatile chemicals, has led to a modification of the repetitive die-away method (Blok, 1979). The proposed method, BODIS (BOD of insoluble chemicals), or two-phase Closed Bottle test, uses BOD bottles and oxygen electrodes. The advantage for insoluble chemicals is that the headspace, normally one third of the total volume, acts as a reservoir of oxygen, since the concentration of oxygen in air is many times that in saturated water. This in turn permits the use of higher concentrations of the test chemical than can be used in the Closed Bottle method, which makes adding a weighed amount of insoluble chemical a practical proposition. The test can equally well be applied to soluble and, providing certain precautions are taken, to volatile chemicals. A calibration exercise (ring test) was organised by ISO and a revised method will be discussed at an ISO meeting in Vancouver in October 1994.

The search for a substitute for the Sturm test, prompted by the large volume (3 litres) of medium needed for each test and the inability of the method to accommodate volatile chemicals, has resulted in a number of headspace methods. They use apparatus similar to that in the anaerobic methane production method. At each time interval, vessels are sacrificed by acidification and measurement of the CO_2 present in the headspace. No formal protocol has yet been prepared, but this should present no problem. There will be an additional reason for such a method if it is confirmed that the absorption of CO_2 in the Sturm test is faulty, since the latter test in its present form is unable to yield valid kinetic data.

It was disappointing that no new methods have been derived from biochemical indicators of growth such as ATP and the cellular incorporation of 3H-thymidine; the attempts made were unsuccessful, but studies are continuing. On the other hand, a method for determining the primary biodegradability of oils by following the disappearance of absorption in the UV has been successfully used in the oil industry for some years.

G.13   Strategies for testing

The strategy for testing adopted by the OECD is based on three levels of testing, namely:

- ready biodegradability, or screening;

- inherent biodegradability;

- simulation of environmental compartments.

There is also a requirement to examine the possible presence of stable intermediates at the second level if only partial mineralization occurs. The conditions provided by the tests at the first two levels are progressively less stringent.   The EC scheme is similar, in that progression from one level to the next is governed by the results at the lower level, but it differs from the OECD scheme in that the timing of the application of a test in the next tier is also dependent on the annual and total tonnage of the chemical of interest (see Figure E.2, section E). Further, chemicals are divided into three groups: those of no particular concern; those giving rise to concern; and those regarded as special cases.   Concern relates to ecotoxicity, potential for bioaccumulation and effects on human health, while special cases relate to physico-chemical properties and disposal patterns.   Chemicals of concern or regarded as special cases may have to be tested by higher tiered-tests at lower annual or total tonnages than chemicals of no special concern.

The EC scheme also introduces a "judgement" factor at the outset. If the chemical is judged, before any practical tests are applied, to be not readily biodegradable, a test for inherent biodegradability may be the first test to be applied. Further, tests for anaerobic biodegradation, degradation in soil, and toxicity in simulation tests may be required to be carried out.

Thus, this more complex system, which seems not to have been sufficiently publicised, accommodates some of the suggestions made in the literature. Both Gledhill, *et al.* (1980) and Struijs (1991) proposed that inherent and ready tests should be included in the first tier and Niemi and Veith (1989) proposed a scheme involving the re-iterative use of structure-degradability relationships at each stage. Struijs (1991) additionally proposed that the second step should be to apply a test for "potential biodegradability" in a specific compartment (a "surrogate" test) which, if positive, might give sufficient information to avoid having to apply a more costly simulation test.

Along with considerations of establishing new and modified tests, there should be a discussion on the strategies of testing with the aim of updating the OECD strategy and the EC guidance document and to include the use of QSBR and SBR, and "surrogate" tests. The resulting scheme should be more widely publicized, as should the optional use of any other method, providing that it can be justified scientifically.

G.14   Structure-activity relationships

A brief review of the literature on the various types of structure-activity relationships has shown that many attempts made, with a wide variety of molecular descriptors and various measures of biodegradation, have so far failed to produce a successful relationship which includes a large number of a variety of chemicals. Probably the biggest problem is the lack of reliable, uniform biodegradation data. However, successful working relationships for small numbers of chemicals with the same functional group have been established. Workers in this field are optimistic that satisfactory relationships will be found.

G.15 Tailpiece

A bewildering array of mechanisms has been described in the literature which can influence the degradation of a chemical in the environment. Because of this, no single test using samples even from a given site in, for example, a river would indicate the kinetic fate of the chemical on all occasions. Similarly, the factors affecting adaptation of a microbial population to allow it to degrade a chemical which it initially could not attack are complex; the factors determining the ability of the population to retain the acquired ability are not well understood.

At the present time there is no easy way of determining how any given chemical would behave under the varied conditions in the aquatic environment. If this information is required, all that can be done at present, in a routine way, is to test chemicals of concern under two or three sets of conditions to discover the capacity of the environment to deal with them and at what kinetic rates.

Overall, the present scheme and strategy of testing, as well as the test methods themselves, have so far served us well in assessing the possible hazards that chemicals may cause in the environment: it is now time to consider improvements in the strategy and changes and additions to the methods. While there is a clear need for some of the methods described earlier, the need for others is debatable. For example, the need for a test for anaerobic biodegradation is urgent, as is the requirement to adapt methods for use with insoluble chemicals. But the need and usefulness of methods which produce detailed kinetic data for degradation in lakes and rivers are by no means established. Although there have been suggestions and demands to include such methods, it is debatable whether the knowledge of the detailed kinetics in various environmental compartments is of any great importance at the present time. It is not clear how easy it is to distinguish practically between the various kinetic equations, nor how accurately all the relevant equations relating to low concentrations in rivers, etc. can be approximated to first-order kinetics. Put another way, what error would be involved if first-order kinetics were assumed, bearing in mind that the objective of "routine" biodegradability testing is the estimation of the probable environmental concentration in hazard assessment to be compared with the maximum "no observed effect" concentration? This question must be resolved before complex kinetic methods are included in the OECD Test Guidelines.

Appendix I

List and Synopses of Existing Methods

I.1   List of the methods

Some of the present methods (**Table App. I.1**) have their origins in the OECD tests for surfactants, in which degradation was followed by specific chemical tests, methylene blue reactivity for anionic surfactants, and the Dragendorf bismuth reagent for nonionics. While specific analysis may still be applied in the new methods as an optional addition, the major analytical tools are DOC (or COD), oxygen uptake and CO_2 evolution which indicate ultimate biodegradation. When oxygen uptake or CO_2 evolution is used, DOC may be applied at the start and end of incubation as an additional parameter.

The media used have now been harmonized (**Table App. I.2**), so that the only differences now are that in the Closed Bottle method the mineral medium is one tenth of the concentration of that used in the other "ready" tests and the MITI I test employs a slightly different medium at pH 7.0 instead of 7.4. In the inherent and simulation tests the same medium, synthetic sewage or domestic sewage, is used.

The tests for ready biodegradability are conducted at 22+/-2°C, except for the respirometric method which technically requires a narrower tolerance of +/-1°C and the MITI test, which is held at 25+/-1°C. The normal duration of the tests is 28d.

Except for the MITI test, the inocula are drawn from activated sludge, sewage effluent, surface waters or soil extracts, and recommendations are made on the approximate bacterial cell densities to be used. The inocula may be pre-conditioned (= pre-acclimated or acclimatized) to the test conditions by aerating the inocula in the medium in the absence of the test chemical to attempt to reduce the oxygen uptake and CO_2 evolution of the "blank-controls". In the ISO methods only, pre-exposure to the test chemical is also permitted with the object of adapting the population so that it will degrade the chemical within the 28d test period. The reported results must be accompanied by a description of the details of the pre-exposure.

Certain conditions have to be met in these tests to establish their validity. First, a reference chemical (benzoate, aniline, acetate) must have been degraded by 70% DOC (60% ThOD or $ThCO_2$) within specified periods. This condition checks the whole procedure but not the activity of the inoculum, since the reference chemicals at present used are so readily attacked that they degrade even in uninoculated media. Next, the control vessels – inoculated, but containing no added organic chemical – should not take up more than 30 mg 0_2/l or produce more than 40 mg CO_2/l in 28d. Also, in the CO_2 evolution test the concentration of inorganic carbon at the start of incubation should not be more than 5% of the total carbon concentration. In the Closed Bottle test, the blank control oxygen uptake should be less than 1.5 mg/l in 28d and the concentration of dissolved oxygen in all bottles must not be less than 0.5 mg/l. There are also restrictions on the final pH values of the media.

Table App. I.1   List of methods

A   OECD Test Guidelines – Ready Biodegradability (1992)

        301A DOC die-away
        301B CO_2 Evolution Test
        301C Modified MITI* Test (I)
        301D Closed Bottle Test
        301E Modified OECD Screening Test
        301F Manometric Respirometry Test

B   EC Methods – Ready Biodegradability

        C.4.A Dissolved organic carbon (DOC) die-away
        C.4.B Modified OECD Screening Test
        C.4.C Carbon dioxide (CO_2) (Sturm)
        C.4.D Manometric respirometry
        C.4.E Closed Bottle
        C.4.F Modified MITI (I)
        C.5   Biochemical Oxygen Demand

        (Off. J. E. Comm. Vol. L383 A, 29-12-1992, pp. 187-227)

C   ISO Methods (not differentiated between ready and inherent biodegradability)

        ISO 7827 Method by analysis of dissolved organic carbon (1985), 1991
        ISO 9408 Method by determining the oxygen demand in a closed respirometer
                    (1991)
        ISO 9439 Method by analysis of released carbon dioxide (1991)
        ISO/TC147/SC5 Closed Bottle (not yet completed) WG4/N152,N160

        CD 10,634 Guidance on evaluating biodegradability of insoluble chemicals

D   OECD Test Guidelines – Inherent Biodegradability

        302A Modified Semi-Continuous Activated Sludge (SCAS) Test (1981)
        302B Modified Zahn-Wellens/EMPA** Test (1992)

E   EC Methods – Inherent Biodegradability

        Modified SCAS
        Modified Zahn-Wellens

        (Off. J. E. Comm. L133, Vol 31, 30th May 1988)

F   ISO Methods (not differentiated)

        ISO 9887 SCAS (1991)
        ISO 9888 Zahn-Wellens (1991)

G   OECD Test Guidelines – Simulations (1981)

        303A Aerobic Sewage Treatment: Coupled Units Test

H   EC Methods (see Ref. in E above) – Simulations

Activated Sludge Simulation Tests

I   ISO

ISO/TC147/SC5/WG4 N140 (1991)

Activated Sludge Simulation Tests

 * *Ministry of International Trade and Industry (Japan)*
** *EMPA: Swiss Federal Laboratories for Materials Testing and Research*

Table App. I.2 Conditions in the OECD tests for ready biodegradability

Test	DOC die-away	CO_2 evolution	Manometric respirometry	Modified OECD Screening	Closed bottle	MITI (I)
Concentrations of test substance:						
mg/l			100		2-10	100
mg DOC/l	10-40	10-20		10-40		
mg ThOD/l			50-100		5-10	
Concentration of inoculum:						
mg/l SS		≤30				30
ml effluent/l		≤100		0.5	≤5	
approx. cells/l	$10^7 - 10^8$	$10^7 - 10^8$		10^5	$10^4 - 10^6$	$10^7 - 10^8$
Concentration of elements in mineral medium (in mg/l):						
P			116		11.6	29
N			1.3		0.13	1.3
Na			86		8.6	17.2
K			122		12.2	36.5
Mg			2.2		2.2	6.6
Ca			9.9		9.9	29.7
Fe			0.05-0.1		0.05-0.1	0.15
pH			7.4 ± 0.2			preferably 7
Temperature °C			22 ± 2			25 ± 1

DOC = Dissolved Organic Carbon   *ThOD = Theoretical Oxygen Demand*   *SS = Suspended Solids*

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Finally, the extremes of replicate values of percentage removal should not differ by more than 20%; however, it is found that some chemicals tested with low cell densities often give wider variations than this.

In the OECD and EC tests the "pass" levels have to be reached within 10d of the removal reaching 10%, called the "ten-day window".

I.2 Synopses of the methods

I.2.1 Ready biodegradability

DOC die-away test

The test chemical is added as the sole source of carbon, at 10 to 40 mg C/l, to a mineral salts medium containing salts of Na, K, Mg, Ca, Fe, NH_4, Cl, SO_4 and PO_4; the medium is buffered by the phosphate salts at pH 7.4. The medium is inoculated to give an increased cell density between about 10^2-10^3/ml (Modified OECD test) and 10^4-10^6/ml (OECD, ISO). At least duplicate vessels are set up containing the chemical plus inoculum and another inoculated pair containing no added test chemical to act as controls. A further inoculated flask is set up containing a reference chemical (aniline, benzoate, acetate) at 20 mg C/l to check the procedure. Abiotic controls are set up, if required, containing a sterilized (e.g. $HgCl_2$) uninoculated solution of the chemical. A further inoculated vessel containing the test and reference chemicals is set up if the inhibitory property of the chemical is to be determined.

The flasks are incubated in the dark at 22+/-2°C (20-25°C, ISO) with shaking. Frequent samples are taken during the 28d incubation so that an adequate biodegradation curve may be drawn. The samples are either membrane-filtered or centrifuged to remove bacterial cells, and DOC is determined in duplicate on the filtrate or supernatant. Filtered samples may be stored at 2-4°C for up to 2d or below -18°C for a longer period.

The removal of DOC is calculated as:

$$\% \text{ removal} = \frac{\text{DOC at day 0 - DOC at day t}}{\text{DOC at day 0}} \times 100$$

In all cases the DOC values are corrected for those of the blank controls.

A plot is made of the average percentage removal against time. The lag time is defined as the time from inoculation until the removal has reached 10% of the starting concentration. This lag time (which is recorded; ISO only) is often highly variable and poorly reproducible. The maximum level of degradation is defined as the approximate level above which no further degradation takes place during the test. Degradation time is defined as the time from the end of the lag period to the time when about 90% of the maximum level of degradation has been reached (ISO only). The "pass" level (OECD and EC only) is 70% DOC and must be reached within 10d of the end of the lag period.

CO$_2$ Evolution (Modified Sturm) test

Batches of 3l of the mineral medium (used in the DOC die-away test) contained in enclosed vessels are inoculated normally with 30 mg/l activated sludge, or homogenised sludge at 1%, giving about 10^5 to 10^6 cells/ml. The inoculated mixtures are aerated with CO_2-free air overnight to purge the system of CO_2. The test chemical and reference chemical are added, separately, to give 10-20 mg DOC or TOC/l; some vessels – control blanks – receive the equivalent amounts of water to equalize the volume in all vessels. As in the DOC die-away test, inhibition and abiotic controls may be set up. Absorber vessels are partially filled with solutions of barium hydroxide or sodium hydroxide.

CO_2-free air is bubbled through the vessels held at 22+/-2°C in the dark, at roughly constant rates between 30 and 100 ml/min. Frequently, the CO_2 trapped is determined either by titration of the unreacted barium hydroxide in the trap nearest to the reaction vessel, or by withdrawing, say, 100 μl of the sodium hydroxide solution by syringe and injecting into the inorganic part of a carbon analyzer. Samples are taken at least at two- to three-day intervals in the first part of the incubation and then at least every five days. On day 28, 1 ml concentrated HCl is added and the medium is aerated overnight to drive off any residual CO_2 into the traps.

Usually 100 ml of 0.0125 M barium hydroxide is contained in each trap and the HCl solution is 0.05 M. Hence the mass of CO_2 trapped is given by 1.1 (50-V) mg, where V is the volume of HCl used for the titration. The percentage degradation is given by:

$$\frac{\text{mg } CO_2 \text{ in test trap - mg } CO_2 \text{ in control trap x 100}}{\text{ThCO}_2},$$

where ThCO$_2$ = $\frac{44}{12}$ x concn. of test chemical C x vol. of test solution.

For NaOH in traps,

% degradation = $\frac{\text{mg IC from test flask - mg IC from blank}}{\text{mg TOC added, as test substance}}$

Manometric respirometry

The test chemical is added to give 50-100 mg ThOD/l as the sole source of carbon to the mineral medium (as used in the DOC die-away method). Similarly, a reference chemical is added to another batch of the medium and an equivalent volume of water is added to a third batch to act as a control. If the toxicity of the chemical is to be assessed, a further solution in the medium is prepared containing both the test and reference chemicals at the same concentrations as in the individual solutions. Insoluble and poorly soluble chemicals are added at a later stage. If abiotic degradation is to be investigated, a solution containing the test chemical is sterilized by the addition of a toxic substance (e.g. mercuric chloride).

The specific ThOD (mg O_2/mg chemical) is calculated on the basis of the formation of ammonium from N-containing test chemical, unless nitrification is anticipated when the calculation is based on the formation of nitrate.

Known volumes of each solution are added to the respective respirometer flasks, at least in duplicate, contained in a water bath or incubator at 22+/-1°C, and it is at this stage that insoluble and poorly soluble chemicals are added (see ISO CD 10 634). The CO_2

absorbent is then added to the absorber compartment and the flasks are inoculated with activated sludge to give a concentration of 30 mg solids/l.

The stirrers are started, the flasks are sealed when they have reached the operating temperature, and the measurement of oxygen uptake is begun. With automatic respirometers a continuous record is obtained, so that the lag period and the "ten-day window" are easily recognized; with non-automatic respirometers daily readings of the volume/pressure are adequate.

The oxygen uptake is calculated from the readings by the methods given by the manufacturers of the equipment. The specific biochemical oxygen uptake at any time interval is given by:

specific BOD = $\dfrac{\text{mgO}_2 \text{ uptake by test chemical - mgO}_2 \text{ uptake by blank}}{\text{mg test chemical in respirometer flask}}$

and the percentage biodegradation is given by:

% ThOD = $\dfrac{\text{specific BOD x 100}}{\text{specific ThOD*}}$

* COD can be used if ThOD cannot be calculated, but it is a poor substitute since some chemicals do not react fully in the COD test.

If nitrification is expected or thought to have occurred, corrections should be made for the oxygen used in oxidizing ammonium by analysing samples from the respirometer flasks at the beginning and end of incubation for nitrite and nitrate.

MITI test

The differences between this and the previous method are as follows (see Table App. I.2):

(a)   The medium contains less P, Na and K and more Mg, Ca and Fe, and the pH value is 7, not 7.4.

(b)   The temperature is 25+/-1°C.

(c)   The inoculum is prepared in a relatively complex way. Fresh samples from sewage, waste waters, rivers, lakes, seas are collected from ten sites mainly in areas where a variety of chemicals are used and discharged. Equal volumes of these ten samples are thoroughly mixed and the supernatant, after the mixture has been allowed to stand, is adjusted to pH 7+/-1. This supernatant is used to fill a "fill-and-draw" activated sludge plant, which is then operated on a 24h cycle (23h aeration, 1 hour settlement) and is fed with 0.1% each of glucose, peptone and phosphate. After operation for one month, the sludge may be used as a source of inocula for a further three months.

(d)   If biodegradation is between 20 and 60% ThOD and the C remaining is not the parent compound, intermediates of the test chemical must be sought.

Closed Bottle test

Solutions containing 2 to 5 mg/l of the test chemical and reference chemical as sole sources of carbon are prepared, separately, in previously aerated mineral medium, which is one tenth of the concentration of the medium used in other methods. A blank control solution consists of the aerated mineral medium alone. If toxicity is to be investigated, a solution is prepared containing both the test and reference chemicals.   These solutions are then inoculated with secondary effluent or surface water at the rate of 0.05 to 5ml/l to give about 10 to 10^3 added cells/ml, and each well mixed solution is carefully dispensed into a series of at least ten BOD bottles so that all bottles are completely full. If insoluble and poorly soluble chemicals have been added to the bulk solutions using a method described in ISO/CD 10 634, ensure that the contents of the containers holding the suspensions are well mixed during the dispensing operation. Otherwise such chemicals may be added directly to the BOD bottles (see ISO/CD 10 634,1990b).

Duplicate bottles of each series are analysed immediately for dissolved oxygen by the modified Winkler or electrode methods, and the remaining bottles are carefully stoppered and incubated at 22+/-1°C in the dark. Bottles of all series are withdrawn in duplicate for dissolved oxygen analysis at least weekly over the 28d incubation period. For ensuring identification of the "10-day window", sampling every three to four days should be sufficient, but this requires about 20 bottles per series.

Corrections for oxygen uptake by nitrification in the case of N-containing chemicals may be made only if the electrode method is used; after analysis for dissolved oxygen, samples are withdrawn from the bottles for analysis of nitrite and nitrate.

The specific BOD and percentage biodegradability are calculated, as indicated in the respirometric method.

I.2.2   Inherent biodegradability

Zahn-Wellens – EMPA method

It is advisable first to determine the toxicity of the test chemical towards activated sludge, so that a non-inhibitory concentration is used in the subsequent test.

A batch of 2l of the normal mineral medium is prepared containing the test chemical at 50-400 mg DOC/l and 200-1000 mg dry solids/l of previously washed activated sludge. The ratio between inoculum and test chemical (as DOC) is kept between 2.5:1 and 4:1. Other batches contain reference chemical and no added chemical to act as a control.   The suspensions are contained in glass cylinders, each equipped with a stirrer and a device for aerating the contents.

The suspensions are aerated with purified, humified air at 20-25°C in the dark or diffuse light for up to 28d. The mixture is stirred if necessary to keep the sludge in suspension and to maintain the concentration of dissolved oxygen above 1 mg/l. The pH value is frequently monitored and adjusted to pH 6.5-8, if necessary. The first sample is taken at 3+/-0.5h after addition of the chemical to estimate any adsorption of the chemical by the sludge. Then samples are taken on at least four occasions between the first and 27th days, adjusting the frequency to suit the rate of disappearance of the chemical. Finally, samples are taken on the 27th and 28th days, or on the last two days of the test run.

The samples are filtered or centrifuged and the concentration of DOC, or COD, is determined on the filtrates or supernatants. The percentage removal is given by:

$$\%R_t = \frac{DOC(\text{or COD}) \text{ at 3h} - DOC (\text{or COD}) \text{ at time } t}{DOC (\text{or COD}) \text{ at 3h}} \times 100$$

In all cases the values of DOC (or COD) are corrected for the appropriate blank value. A degradation curve is drawn by plotting % R against time. The shape of the curve, and the difference between the 3h values and the expected initial value of the DOC (or COD) concentrations, give indications of whether any disappearance is due to biodegradation or physical processes.

Semi-Continuous Activated Sludge (SCAS) test

A sufficient number of SCAS units, varying in size between 150 and 1500 ml, are set up so that there is at least one for each test chemical plus a control. The units can be measuring cylinders with a means of aeration, or the aeration vessel can be a tube containing a sealed-in air inlet tube and a tap so placed that one third of the total volume of mixed liquor remains (as settled sludge) in the vessel after draining off the settled supernatant.

The units are filled to the appropriate volume with activated sludge and aeration is begun. After about 23h, aeration is stopped for about an hour to allow the formation of a clear supernatant and a volume equivalent to two thirds the total volume of mixed liquor is discarded. Sewage or synthetic sewage is added to the settled sludge to replace the withdrawn supernatant. The fill and draw procedure is repeated daily, and the filtered effluents are analysed for DOC (or COD) two or three times per week. When the concentration of DOC attains a constant value, indicating a steady state, the test chemical is added with the sewage to the test unit(s), while only sewage continues to be added to the control unit. Analysis of the effluents is continued daily, if the DOC value from the test vessels changes significantly, until the difference between the DOC of the control and test effluents remains fairly constant over six consecutive measurements. Otherwise the effluent is analysed two or three times per week; if no removal is observed, the analysis is continued for at least 12 weeks but not more than 26 weeks.

The percentage removal is calculated from:

$$\% R = \frac{\text{Nominal DOC in chemical added} - DOC^* \text{ at end of aeration period}}{\text{Nominal DOC in chemical added}} \times 100$$

* corrected for the DOC in the control effluent, which has been shown to vary from 5.8+/-1.9 mg DOC/l for OECD synthetic sewage and 5.4+/-0.8mg/l to 13.2+/-2.8 mg DOC/l for domestic sewages.

If the plot of DOC in the test effluent against time has the typical shape of a biodegradability curve with lag and plateau phases, and if the chemical does not adsorb significantly onto sludge, the elimination of the chemical can be reasonably confidently assigned to biodegradation. In case of doubt, a respirometric method using exposed sludge from the SCAS test as the inoculum should differentiate between physical and biological processes.

I.2.3 Simulation methods

Activated sludge

Attempts to simulate the activated sludge process have taken many forms, and many are probably adequate for the purpose. The OECD has so far concentrated on one method called the "coupled-units" test (Fischer, *et al.*, 1975), which uses the Husmann apparatus (3 litre aeration vessel) from the OECD Confirmatory test for surfactant biodegradation. The EC describes the "coupled-units" mode of operation, but the single or non-coupled mode, as well as the UK porous pot system, are also described. The EC methods describe a range of a number of operation variables, including type of sewage and inoculum, mean retention time of sewage (3 to 6h), mean retention time of sludge (6 to 10d), methods of wasting sludge and of adding the test chemical.

Briefly, two units, Husmann or porous pots, are run in parallel under identical conditions. The test chemical is added to the influent synthetic or domestic sewage to one of the units, while the other receives the sewage alone. In the coupled-units mode, sludge is daily interchanged equally between the two units in an attempt to equate the microbial populations in the two sludges.

The concentration of DOC (or COD) in the effluents is determined, but the DOC due to the added chemical in the influent is calculated, not measured. The difference between the mean concentrations of the test and control effluents is assumed to be due to undegraded test chemical. Plots of DOC against time are drawn to show the progression of biodegradation, if any. If the plot for the test chemical effluent has a typical shape of a biodegradability curve with lag and plateau phases, and if the test chemical does not adsorb significantly onto sludge, the elimination of the chemical can be assumed to be due to biodegradation. This could be confirmed by applying the respirometric method using sludge from the simulation test.

The test normally lasts for no more than nine weeks; up to six weeks are allowed for adaptation of the sludge and three weeks for steady operation, during which about 14 measurements are made. The mean of these values is used to calculate the percentage elimination from:

$$\%R = \frac{\text{DOC in test effluent - DOC in control effluent}}{\text{DOC due to test chemical in influent}} \times 100$$

I.3   Comparison of the methods: accuracy and precision

The CO_2 evolution method gives the most direct evidence of oxidation of organic carbon during biodegradation; the removal of DOC can be due to processes other than biodegradation and the uptake of oxygen is only an indirect measure for assessing biodegradability. Also, only by using DOC, either in the DOC die-away test or as additional determinations in the other two methods, can an indication be obtained of the formation of any recalcitrant intermediate metabolites.

The DOC die-away method is obviously limited to soluble chemicals, with a solubility of at least 50 mg/l, and to those which are not significantly adsorbed or are volatile, although the latter can be assessed by modifying the apparatus. The respirometric method can accommodate soluble, insoluble and volatile chemicals while the CO_2 evolution method can deal with soluble and insoluble but not volatile chemicals. The Closed Bottle method can deal with volatile chemicals if the bottles are modified. Although insoluble chemicals can be

assessed by this method, the values obtained could be falsely low because mixing is extremely deficient. The concentration of test chemicals in this test is limited to 2 to 5mg/l because of the low solubility of oxygen in water.

The inherent and simulation tests are obviously suitable for soluble chemicals, but a case can be made for their application to insoluble (but not volatile) chemicals provided they are suitably dispersed. In inherent tests the resulting exposed sludge could subsequently be used in CO_2 evolution or oxygen uptake tests and in the simulation tests the effects of the insoluble chemicals on the performance (BOD removal, nitrification) of the activated sludge units could be ascertained. Volatile chemicals have been examined in simulation tests using enclosed aeration tanks.

The overall accuracy, precision and reproducibility of the various methods are adversely affected by the inconsistency and unpredictability of the inocula which, of necessity, have to be used. For chemicals which are very easily biodegraded, such as the reference chemicals, very high values of %DOC removal approaching the theoretical of 100% are consistently obtained with high precision and reproducibility. However, the %ThOD and %ThCO$_2$ obtained are always lower than % DOC removal (for all chemicals, not just the very easily degradable chemicals) because some of the carbon is converted to biomass. The proportion of the carbon used for cell synthesis varies both between species of bacteria and between chemicals so that the % ThCO$_2$ and % ThOD will vary from test to test and from chemical to chemical. Hence, there are no "accurate" values. In these tests the precision with which very easily degradable chemicals are assessed is high but not so reproducible either between tests using inocula from different sources or as for %DOC removals. For chemicals which are not so easily degraded and may require longer lag periods, the precision in the various tests is not so high as with chemicals like the reference chemicals, especially when low cell densities are used.

The chemical determinations can be carried out with a greater accuracy and precision than the tests as a whole. The determination of DOC can be made with a precision of +/-0.25 mg/l with a lower limit of about 0.5 mg/l. In the automatic respirometer the "quantum" of oxygen measured is 0.5 to 1.0 mg, equivalent to about 1 to 2 mg DO/l, although this limit could be lowered. In the Closed Bottle test the concentration of dissolved oxygen can be determined to +/-0.05 mg/l. However, measurements of CO_2 in the Modified Sturm test are considered to be less precise (than DOC and oxygen in other tests) because the relatively small amounts of CO_2 evolved in the period between measurements is determined from the difference between two relatively high titration values. For example, a blank titre could be 50 ml and the "test" titre 45 ml 0.05 M HCl so that the mass of CO_2 produced would be:

$$1.1 \times (50-45) = 5.5 \text{ mg } CO_2.$$

For this reason, Gerike and Fischer (1979,1981) carried out only one titration for a 28d test. Others have used sodium hydroxide and determined IC, at frequent intervals, with much greater precision than the determination of CO_2 by back titration.

In the SCAS test, the % DOC removal and standard deviation for aniline was 96+/-2.6% and for Marlon A 88.8+/-9.9%. In the simulation test in the uncoupled mode 1-naphthol was removed by 92+/-8.2%, pentaerythritol 84+/-11% and 18+/-19% for sulfanilic acid. In both tests the standard deviation is often much higher when poorly degradable chemicals are tested.

Appendix II

Mechanisms of Adaptation

Exposure is a process to which a mixed microbial population is subjected with the object of developing in that population the ability to degrade a substance hitherto not biodegraded, or only slightly so, by the unexposed inocula. The population is exposed for various periods of time to the substance, usually at low concentration and usually in the presence of other substances known to be degradable, often under conditions which are similar to those prevailing in the test for ready biodegradability to which the substance will be subsequently subjected. It does not follow, of course, that exposure necessarily results in a population with a new degradative ability, but when it does the population can be said to have become adapted to the new substance.

There are several mechanisms by which a mixed population can acquire new oxidative properties. The simplest is a shift in the population, which normally takes days to weeks rather than months. A small minority of species already capable of degrading a given chemical, but not at a sufficiently high rate to show significant removal in 28d, multiply sufficiently during the period of pre-exposure to produce a sufficient number of cells to degrade the substance during the die-away test by acceptable amounts (>80% test substance, >70% DOC). There may or may not be a lag in the die-away test with pre-exposed inocula; it is common experience, however, that substances which are degraded by unacclimatised inocula are usually degraded by pre-exposed inocula with shorter lags, but that the kinetic rate is often not greatly changed. It should be said, however, that kinetic rates are rarely reported.

A second mechanism, which normally takes from minutes to days, is the induction of the appropriate catabolic and permease enzymes. Here, species in the population have the necessary means of synthesising the enzyme system which will degrade the compound, but do not possess the enzymes unless the compound is present in the medium. A second compound, usually structurally similar to the first, may cause the induction of the necessary enzyme system capable of degrading the first compound and sometimes does so at a faster rate than "self" induction. This is called sequential induction. Induction is usually rapid, but if it occurs in only a relatively few cells in the population the compound will not be degraded to a significant extent in the 28d test until a population shift has occurred – the first mechanism.

Next, existing catabolic enzymes, including those involved with transport and regulatory mechanisms, can become modified by mutation to deal with a new substance. A non-normal substrate must first be converted to an intermediate which is either a recognizable component of an existing pathway or one which is a simple derivative of such a component. This initial step can take place fairly easily, but not very rapidly initially, if the enzymes involved are not very substrate-specific and if the xenobiotic compound is of similar structure to a natural substrate. The facility may increase as the cell learns to modify the structure of the enzyme accordingly. The process is repeated with each successive step until a component of an established pathway is formed, when the way is open for complete degradation to mineral products.

In the case of compounds containing, for example, halogens or sulphonic acid, alternative mechanisms are available. The group (X) can be eliminated at the beginning of the pathway, which facilitates the subsequent breakdown of the intermediate metabolite. On

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the other hand, X may be present in the intermediates and becomes less easy to eliminate, with the opportunity of "dead-end" metabolites being formed, e.g. fluoro-acetate, fluorocitrate. Such metabolites are recalcitrant and sometimes strongly inhibitory to other pathways. Compounds from which these metabolites are formed, e.g. 3-halocatechols, have been called "suicide substrates".

The mechanisms so far described can lead to adapted populations in relatively short times, from days to weeks or months. However, mutations leading to enzyme modification and production of some dehalogenases and desulphatases in adequate cellular concentration can take much longer with compounds of more complex structure. Thus, the occurrence of the adaptations in nature will also be less frequent.

The last way in which it is thought that populations become adapted, but over much longer periods of time, is by the evolution of complete metabolic pathways as opposed to a modification of an existing pathway. Since micro-organisms have had geological time-scales over which to evolve catabolic sequences to effect breakdown of naturally occurring chemicals, the time for which man-made chemicals have been in the environment (about 150 years) is extremely small by comparison. The time which can normally be allowed in the laboratory, e.g. 6-12 months as in the SCAS test, to test a "new" man-made chemical gives even less chance of a new pathway being established. How these pathways are established is a matter for debate and further research. One hypothesis is the step-wise retrograde mechanism in which gene duplication is followed by modification of one copy to produce an altered protein (enzyme) which has the required catalytic function for the next step in the pathway. Some experimental evidence supports this while other evidence does not. These theories relate to pure cultures, but another promising proposal relates to metabolic interaction between members of microbial communities. This proposal suggests that there is a much greater chance of there being an existing enzyme with fortuitous activity towards a new substance in the larger genetic pool of many different species. Similarly, a second enzyme is unlikely to be present in the same organism or species as in the first. Thus, degradation is the result of the collective activity of the metabolically structured community.

Many mechanisms exist for genetic transfer, and it may be that this will allow a substantial portion of the information within different populations to exist in a state of flux between the species. Genetic transfer (non-specific phage, plasmid, etc.) could thus result in all the relevant genetic information of the new pathway existing within a single species. The more complex the new substance, the more information would be needed and it would be expected that this would take a longer time. Also, single species would have to possess a selective growth advantage over the community and it is debatable whether this could be achieved.

It may be concluded that the more easily, quickly and reproducibly (with inocula from a wide range of sources) a chemical can bring about adaptation, by whatever mechanism, of a microbial population, the greater the chance that the chemical will be readily degraded in the aquatic environment. The potentially competent organisms would be fairly uniformly distributed throughout the environment. Conversely, if a microbial population takes many months or years to adapt to a chemical, and does so erratically both temporally and spatially, it is unlikely that the chemical will be degraded readily in all parts of the environment, because micro-organisms which have the ability to develop the necessary metabolic pathway will be sporadically and unevenly distributed in the environment.

Retention of ability

Research in this field gives very few clues as to how long, and under what conditions, the ability to degrade a new chemical will be retained by a microbial population. Retention of the newly acquired ability would of course continue so long as the substrate was continuously presented to the population. When the new substrate was withdrawn it might be expected that easily acquired abilities and the potential for their acquisition would be retained longer than would abilities acquired over a longer period, although the opposite might apply, that is, easily acquired abilities could easily be lost.

The view has been expressed that a complex multi-functional substrate would require the acquisition by an organism of so many new enzymes that the resulting mutant might be just a laboratory curiosity and would probably not be able to grow fast enough to compete with other species under conditions of mixed substrate, and/or would prefer to degrade more simple substrates.

All reviewers of these topics agree that much further research is necessary.

Note: *The reviewer is grateful to one or two readers of the lst draft for pointing out that this section is out of date. It was compiled in 1986. For more recent and clearer explanations, please see later reviews.*

Appendix III

Some Problem Chemicals

While it is universally agreed that some chemicals are readily biodegradable and that others are either non-degradable or are degradable only under special circumstances, there are others of relatively simple structure for which disparate results have been reported. These disparities tend to blur and confuse the effects of using the acclimatised inocula. The aberrant behaviour of some of the chemicals is discussed below.

3-Aminobenzoic acid (3AB)

Although 3AB was found to be readily degradable (Gerike and Fischer 1979, King and Painter 1985) and to be removed in the OECD Confirmatory test (Painter 1985) using domestic sewage, Gerike and Fischer (1979, 1981) found it not to be removed using synthetic sewage unless they used their more lenient "square wave feed" version of the Husmann simulation test. Pitter (1976) reported 20d-acclimatised sludge to degrade it at 7 mg COD/g/h and he would not define 3AB as readily degradable.

3-Aminophenol (3AP)

Only four out of twelve participants of a ring test found 3AP to degrade in the EC Respirometric Method (CEC 1985) using unacclimatised inocula, while Haller (1978) and Lund Rodriquez (1984) found it not to degrade even when using acclimatised inocula. In the latter case inhibition by the 1000 mg 3AP-COD/l used in the acclimatisation and testing steps was the probable reason. Pitter (1976) reported 20-day acclimatised sludge to degrade 3AP at 10.6 mg COD/gh and thus would not define it as readily biodegradable. 3AP was degraded by acclimatised inoculum in the modified OECD test, but not in the 42d AFNOR test, using fresh inoculum. In simulation tests it was degraded after lags of 14-28d (Gerike and Fischer 1979, King and Painter 1985) and wastewaters from the manufacture 3AP were successfully treated (Deshpande, *et al.* 1985). On the other hand, Zagidullina, *et al.* (1981) reported that a longer retention time of sludge than is normal was needed (but unstated) and that a lag period of at least four months was required.

Benzene 1,3 disulphonic acid (BDSA)

BDSA was not degraded in any of the screening tests (Gerike and Fisher 1979, Painter, *et al.* 1983) nor could it be degraded in the SCAS test after seven and twelve weeks' acclimatisation (Painter, *et al.* 1983, Colquhoun 1988). Pitter (1976) reported it to degrade at the very low rate of 3.4 mg COD/g/h with acclimatised inoculum, while Gerike and Fisher (1981) observed it to degrade with acclimatised sludge, taken from the Husmann unit in the Closed Bottle and modified OECD test. It was not degraded in the 42d AFNOR test using unacclimatised sludge test and, after four weeks' lag in the Husmann test, 84-87% was removed (Gerike and Fischer 1981).

Benzene sulphinic acid, sodium salt (BSA)

In the ring test of the Respirometric method, 20 out of 21 participants attained >50% ThOD (CEC 1985). BSA did not degrade in the modified OECD test, but did so in the ISO test using a larger inoculum (30 mg/l) (Painter and King 1986) when sludge from two sources was used but not when a third source was used. When BSA was subjected to a simulation test using domestic sewage, disparate results were recorded. In duplicate vessels receiving Stevenage domestic sewage, only 27-30% DOC was removed (Painter 1985); but when sewage from Hambleden (near Medmenham) was used, 74-93% DOC was removed (Painter 1986). No tests with acclimatised inocula were carried out.

t-Butanol (t-BA)

The behaviour of t-butanol is complicated because of its volatility in aqueous solution. In conditions similar to those in the Husmann method (King and Painter 1985) a solution containing 20 mg-BA-C/l lost 8% in 3h, while Colquhoun (1988) lost 80% in 24 h under conditions of the SCAS method. Because of this, flasks containing t-BA are usually not shaken or are stoppered to avoid loss of carbon. Most workers report it to be not readily degradable, though in the ring test of the Respirometric method two out of eleven participants reported more than 60% ThOD. Neither was it degraded well (~30% DOC) in activated sludge simulation tests (Gerike and Fischer 1981, King, *et al.* 1984a) but 70% DOC was removed in the more lenient "square wave feed" version (Gerike and Fischer 1981). In the German study acclimatised sludge failed to degrade t-BA; SCAS acclimatised sludge did so after 18 or more days, but not after 10d (Painter, *et al.* 1983). Effluent from the SCAS units after exposure for 67d did not degrade t-BA.

Horn, *et al.* (1970) reviewed the literature and reported that other workers said that the alcohol was not degraded even by exposed inocula. They found that after eight weeks' exposure in activated sludge units, 99% t-BA was removed, the retention time of the feed falling from 4.3d to 1d; the sludge age (probably high) was not stated. On making a mass balance, only 1% was found to be lost by evaporation. Romadina, *et al.* (1984) isolated two species of *Pseudomonas* which degraded t-BA and which continued to do so when added to activated sludge units (no details given in abstract).

2-Chlorobenzoic acid (2-CB)

It is agreed that this compound is not readily biodegradable. Even with inocula exposed by the Sturm method, Gerike and Fischer (1981) found little or no degradation in the Closed Bottle and modified OECD tests, but on one out of three occasions the Sturm CO_2 test was positive. Haller (1978) observed degradation of 16 mg 2-CB/l after a lag of 25d in waste water at 30°C, but no degradation was found in the SCAS test after seven weeks (Painter, *et al.* 1983) or 30 weeks (Colquhoun, 1988). Lund and Rodriquez (1984) also reported lack of adaption in a modified SCAS procedure over more than 30d, but as with other compounds the high concentration of 2-CB (= 1000 mg COD/l) may have been inhibitory.

In the simulation test, 93% DOC was removed, surprisingly with no lag, using OECD synthetic sewage (Gerike and Fischer 1979), but under the same conditions only 30% DOC was removed (King, *et al.* 1984a) although when domestic sewage was treated the value rose to 108% after a lag of four weeks.

3-Chlorobenzoic acid (3-CB)

Three groups (Zahn and Wellens 1980, using river water; Painter, *et al.* 1983; Colquhoun 1988, using the ISO method) reported that 3-CB is readily biodegradable with short lags (less than 7d). Gerike and Fischer (1979, 1981), however, reported that it was not degraded unless an acclimatised inoculum (Sturm) was employed. Similarly, Lund and Rodriguez (1984) did not find 3-CB to be biodegraded unless the inoculum had previously been pre-acclimatised (30d). They also found a sludge acclimatised to 3-methylbenzoic acid for 20d was not only able to degrade 3-CB but did so at a higher rate than sludges acclimatised to 3-CB.

In the UK study (King, *et al.* 1984a) 3-CB was removed extensively in the simulation test both with domestic and synthetic sewages after delays of 7d (synthetic) and, unexpectedly, 14 to 28d (domestic). However, in the German study (Gerike and Fischer 1981) 3-CB was removed by only 30% DOC (synthetic sewage) though with the less stringent "square wave feed" mode 95% DOC was removed after a lag of 16d.

Cyclopentane tetracarboxylic acid

This compound was not degraded in any of the seven tests applied by Gerike and Fischer (1979) and neither was it (in the cis, cis, cis, cis form) removed in the SCAS or simulation tests (Painter 1986). However, Gilbert and Lee (1980) report it to be degraded by about 85% after a lag of eight weeks in the SCAS test; they also found 0-30% removal in a "ready" test, and that the degree of removal depended on the stereo-isomer used, but did not state which isomer they had examined.

Hexamethylenetetramine (Hex)

Hex showed quite abnormal behaviour thought, but not proved, to be due to its ability to hydrolyse to formaldehyde and ammonia, especially under acid conditions. It is known not to be degraded readily in full-scale sewage treatment (Borne 1976), and Gomulka and Gomulka (1984) report that Hex needed an extended period of acclimatisation before being removed (details not given in abstract). In the ring test of the EC Respirometric method (CEC 1985), eight of 24 participants found Hex to degrade by >60% ThOD with corresponding high removals of DOC. However, in the Husmann simulation test, using either synthetic or domestic sewage, not more than 20-25% DOC Hex was removed during the whole nine weeks of the test (Painter and King 1986). Colquhoun and Smith (1986) have isolated strains, from SCAS sludge receiving Hex, which degrade and grow on the compound.

N-Methylaniline (NMA)

On five out of six occasions, NMA was not degraded in the ISO test (30 mg sludge/l); at the sixth attempt >90% DOC was removed and a purple colour was formed. Using sludge acclimatised for 10d or more by the SCAS procedure, consistently high removal values in the ISO test were obtained: acclimatised effluent inocula also gave high removals of NMA (King and Painter 1985). These findings were in agreement with those of Gerike and Fischer (1979) and Colquhoun (1988). However, Boatman, *et al.* (1986) found that 18d acclimatisation by transfer every 2d into a medium containing increasing concentrations (2-18 mg/1) of NMA did not produce inocula which degraded the chemical.

2-Nitrobenzoic acid (2NBA)

2NBA is readily biodegradable; the only test in which it did not degrade was the MITI respirometric method (Gerike and Fischer 1979). It was also removed, by 98%, after a 10d lag in the Husmann simulation test. However, Lund and Rodriguez (1984) were unable to produce an adapted sludge even after 30d, though when the acclimatised sludge was further treated with N,N-nitrosoguanidine the product was able to degrade 2NBA. A possible reason, as mentioned previously, for the lack of normal adaptation could be the high concentration (= 1000 mg COD/l) of the 2NBA involved, which may have been inhibitory. Pitter's (1976) acclimatised inoculum degraded 2NBA at 20 mg COD/g/h, making it readily biodegradable.

4-Nitrophenol (4NP)

It is well known that 4NP shows wide variability both inter- and intra-laboratory, in its behaviour with unacclimatised inoculum (OECD 1979, 1980), and that use of pre-adapted inocula gives much less variability in the lag period before degradation occurs (Nyholm, *et al.* 1984). Gerike and Fischer (1979) found 4NP to degrade by the AFNOR, Sturm and modified OECD methods, but not by the Closed Bottle and MITI methods; in the simulation test it degraded after a 7d lag by 100% DOC. 4NP was removed in seven out of nine attempts in modified OECD and ISO tests, even with initial concentration of 100 mg 4NP/1. Pagga, *et al.* (1982) reported that 100 mg 4NP/l was well degraded in the Zahn-Wellens test, but not at higher concentrations. This fits in with their reported EC50 values of 110-160 mg/l in three different tests. However, Boatman, *et al.* (1986) found that it did not degrade even in the presence of an 18d-acclimatised inoculum. Similarly, Lund and Rodriquez (1984) could not obtain an adapted inoculum, but their conditions (4NP at 1000 mg COD/l) were probably inhibitory. Pitter (1976) classifies 4NP as readily biodegradable since the adapted sludge degraded the compound at 20 mg COD/g/h. Freshwater eco-cores could be adapted to 4NP at 20 μg/l but not at 10 μg/l (Spain and Van Veld 1983).

Pentachlorophenol (PCP)

Using the Bunch-Chambers technique, Tabak, *et al.* (1981) reported that 5 mg PCP/1 was degraded after three transfers (21d) and 10 mg/l after four transfers (28 d), but it was unclear whether primary or ultimate degradation was determined. As against this Painter, *et al.* (1980) found that even after one year in the SCAS test treating 2 mg PCP/l no adaptation took place. Other work (e.g. Reiner, *et al.* 1978) suggests that PCP is degradable by slow-growing organisms under special conditions and is unlikely to be degraded in normal sewage treatment.

Pentaerythritol (PE)

Mohanrao and McKinney (1962), studying the acclimatisation of quaternary carbon compounds, which are difficult to degrade, found that PE did not degrade even after eleven weeks' acclimatisation, while many of the other quaternary compounds did. None of the screening tests yielded positive results (Gerike and Fischer 1979) but an acclimatised inoculum from the Zahn-Wellens test degraded PE in the Closed Bottle, modified OECD and Sturm tests (Gerike and Fischer 1981) and in the simulation test by 96% after a lag of 21d (King, *et al.* 1984).

However, in the ring test of the EC Respirometric method (CEC 1985) as many as 16 out of 25 participants reported values greater than 60% ThOD, with an average lag of 13 d. PE was removed increasingly rapidly with decreasing lag periods in the ISO test by using inocula acclimatised by the SCAS procedure for 10 to 26d; effluent (5 ml/l) taken from the SCAS unit at 67d also degraded PE (Painter, *et al.* 1983).

Polyvinyl alcohol (PVA)

This compound was degraded in the Zahn-Wellens test (Zahn and Wellens 1974) without lag when sludge acclimatised to the substrate for 13d was used in the test, indicating that PVA is inherently degradable. Hashimoto and Ozaki (1980) were also able to acclimatise activated sludge to degrade PVA after several weeks, using seeds, in separate experiments, from a number of sources but no other conditions were given in the abstract. Gerike and Fischer (1979), however, found no degradation in any test, including the Zahn-Wellens test, the simulation test and the "square wave feed" version.

Sulphanilic acid (SA)

This compound, like diethylene glycol, behaves erratically but is less degradable. In no "ready" test was SA found to be degraded (Gerike and Fischer 1979), but it did degrade successfully in the original Sturm test using acclimatised inocula. In a ring test of the EC Respirometric method only one out of eleven participants reported SA to be degraded, and it was thought that this was due to SA being present in the sewage on which that one sludge was grown (CEC 1985). King and Painter (1985) reported SA to degrade in the ISO method on two out of 13 occasions. Further, SA degraded in the 42d AFNOR test, in the Closed Bottle and modified OECD tests with Sturm-acclimatised inocula, and in the EPA activated sludge test (Gerike and Fischer 1981).

Surprisingly, SA was not degraded in the simulation test (King and Painter 1985), even when inoculated with acclimatised sludge from the Sturm procedure or the EPA procedure, but was degraded in experimental biological filters (Gerike and Fischer 1981). Although Pitter (1976) found SA to be degraded at 4.0 mg COD/g/h, indicating that it was not "readily" degradable, acclimatisation in the SCAS test over 67d did not lead to consistently greater biodegradation (Painter, *et al.* 1983).

Tetrahydrofuran (THF)

THF exemplifies problems caused by volatility. In the ring test of the Respirometer method (CEC 1985), which would be unlikely to allow loss by volatilisation, as many as eight out of 22 participants reported >60% ThOD, with an average lag of 17d. However, in a modified Closed Bottle method allowing the direct injection of THF into the reaction mixture, no oxygen was taken up (Painter 1986). This was contrary to a report (Pozdnyakova 1969) which claimed that 78% ThOD was exerted in a BOD test.

The loss by stripping of DOC in 3h under conditions of the simulation test was found to be 44%. In the actual simulation test DOC was removed from the first day, presumably by stripping, but after eight weeks the proportion removed rose to 100%, indicating that some biodegradation must have taken place (King and Painter 1985). That biodegradation can play a major part in the removal of volatile chemicals was shown by Klecka (1982) in the case of the more volatile dichloromethane.

Tetrahydrofuran tetracarboxylic acid (THFTA)

This compound was not degraded in the 28d ISO die-away test with fresh sludge, but was completely degraded with an eleven-week acclimatised inoculum from a SCAS unit. THFTA was also degraded in the SCAS test. However, in the simulation test, operated for the standard nine weeks, no degradation took place; in fact there was evidence of inhibition as there had been in the first five weeks of the SCAS test (Painter 1986). Perhaps THFTA might have been removed had the test been extended a further two or three weeks.

Note: *In all above cases, the test chemical was added at 10-15 mg C/l to sewage before treatment in the activated sludge simulation test. A better test of predictability would be to add the chemical at environmentally realistic concentrations, in the region of 1 to 10 µg/l, and determine its concentration in the effluent for periods of about six weeks.*

Appendix IV

List of Abbreviations

3-AB	3-amino-benzoic acid
ABS	alkyl benzene sulfonate
AFNOR	Association Francaise de Normalisation
3-AP	3-aminophenol
ASTM	American Society for Testing Materials
ATP	adenosine triphosphate
ATU	allylthiourea
t-B	t-butanol
BDSA	benzene disulfonic acid
BODIS	biochemical oxygen demand of insoluble substances
BODn	biochemical oxygen demand after n days
BDBP	t-butylphenyldiphenylphosphate
BSA	benzene sulfinic acid
2-CB	2-chlorobenzoic acid
3-CB	3-chlorobenzoic acid
4-CBP	4-chlorobiphenyl
CEC	Commission of the European Communities
CEC	Co-ordinating European Council – only in the infra-red method
CMT	carboxylmethyltartronate
COD	chemical oxygen demand
CP	chlorophenols
4-CP	4-chlorophenol
CTAB	cetyltrimethylammonium bromide
2,4-D	2,4-dichlorophenoxyacetic acid
DEG	diethylene glycol
DIC	dissolved inorganic carbon
DO	dissolved oxygen
DOC	dissolved organic carbon
DTMAC	dodecyltrimethylammonium chloride
EC (EEC)	European Community (European Economic Community)
EC50	effective concentration 50% – concentration giving an effect of 50%
ECETOC	European Chemical Industry Ecology and Toxicology Centre
EO	ethylene oxide
FA	fulvic acid
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
Hex	hexamethylenetetramine
HPLC	high performance (or pressure) liquid chromatography
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride
ISO	International Organisation for Standardization
LAS	linear alkylbenzene sulfonate
MCPA	2-methyl-4-chlorophenoxyacetic acid
MITI	Ministry of International Trade and Industry (Japan)
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
MMBT	2-(methylthio)benzothiazole
2-NBA	2-nitrobenzoic acid
NMA	N-methylaniline
2-NP	2-nitrophenol
4-NP	4-nitrophenol
NTA	nitrilotriacetic acid

OECD	Organisation for Economic Co-operation and Development
PCB	polychlorinated biphenyl
PCP	penta-chlorophenol
PE	pentaerythritol
PEC	predicted environmental concentration
PO	propylene oxide
PVC	polyvinylchloride
QSAR	quantitative structure-activity relationship
QSBR	quantitative structure-biodegradability relationship
RDA	repetitive die-away or river die-away
rsd	relative standard deviation
SA	sulfanilic acid
SAR	structure-activity relationship
SBR	structure-biodegradability relationship
SCAS	semi-continuous activated sludge
SDA	Soap and Detergent Association
SS	suspended solids
Sturm	American chemist giving his name to a biodegradability test in which CO_2 is determined
TCE	tetrachloroethylene
TCMP	2-chloro-6-trichloromethylpyridine
%ThCO$_2$	percentage of the theoretical carbon dioxide yield
THF	tetrahydrofuran
%ThOD	percentage of the theoretical oxygen demand
TLC	thin-layer chromatography
TPBS	tetrapropylene benzene sulfonate (branched ABS)
TTC	2,3,5-triphenyl-2H-tetrazoliumchloride
Z-W	Zahn-Wellens – German chemists giving their name to a test for inherent biodegradability

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