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Biodegradation Testing: An Assessment of the Present Status

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CONTENTS

A. INTRODUCTION

The national legislations implementing the 6th Amendment to the EEC Directive on the Classification, Labelling and Packaging of Dangerous Substances (79/831/EEC) require data on the biodegradability of new chemicals, the extent and type of this data being governed by the tonnage marketed (Base set, Level 1, Level 2). Following the OECD testing strategy developed in 1978/79, certain biodegradation tests are classified as indicating either "ready" or "inherent" biodegradability, or as "simulation tests". In the EEC Directive the ready biodegradability tests are suggested for the Base set, inherent biodegradability tests for Level 1 and simulation tests for Level 2. In Table 1 are listed those at present accepted or under consideration by the OECD and EEC.

An Aquatic Biodegradation Group was established by ECETOC to review the present status of biodegradability testing and any future needs, under the following Terms of Reference :

"To assess the applicability, limitations in use, reproducibility and significant technical weaknesses of the aquatic biodegradation test methods in the OECD guidelines and in Annex V of the 6th Amendment. To recommend how they could be improved.

To assess, as above, tests proposed to up-date the above methods. To recommend how such proposed tests can be validated, and how their comparability to the existing methods can be determined (by ring-testing, if necessary).

To identify areas in the assessment of aquatic biodegradation where adequate test methods and methodology are lacking; to recommend work which would lead to adequate test methods/methodology; and to assess (as above) new methods, completed or under development, which are relevant".

TABLE 1

OECD/EEC METHODS : SITUATION AT NOVEMBER 1983

	EEC 6TH AMENDMENT				OECD
	Annex V	Annex VII	Annex VIII	MPD	TEST GUIDELINE No.
Ready Biodegradability					
Modified AFNOR Test	/	/		/	301 A
Modified Sturm Test	/	/		/	301 B
Modified MITI Test (I)	/	/		/	301 C
Closed Bottle Test	/	/		/	301 D
Modified OECD Screening Test	/	/		/	301 E
Inherent Biodegradability					
Modified SCAS Test			u.d.*		302 A
Modified Zahn-Wellens Test			u.d.*		302 B
Modified MITI Test II			u.d.*		302 C
Simulation Test - Aerobic Sewage Treatment					
Coupled Units Test			u.d.*		303 A
OECD Confirmatory Test			u.d.*		
Porous Pot Test			u.d.*		
Biodegradability Test in Soil					304 A

* under discussion

/accepted guideline

In this report the three types of biodegradation testing are considered under the following aspects : general considerations, assessment of test guidelines, recommended improvements to the tests. Subsequently, areas where further investigations are necessary are indicated.

It should be noted that for some products biodegradability may be irrelevant from an environmental viewpoint, and that in some instances ease of degradation would effectively render the product useless for its intended function.

B. READY BIODEGRADABILITY

1. General Considerations

For ready biodegradability four methods have been selected, the results from which are acceptable for Base Set submissions throughout the EEC (Table 1) :

- Modified AFNOR Test
- Modified Sturm Test
- Closed Bottle Test
- Modified OECD Screening Test

A fifth method, the MITI test, is still under consideration.

The four accepted methods are characterised by low concentrations of both microbial inoculum (10^2 to 10^6 microbes/ml) and test substance in a simple salts medium under batch, die-away conditions. Those substances which degrade to inorganic and cellular products under the stringent test conditions are classed as "readily biodegradable", the assumption being that they are likely to degrade rapidly and completely in a wide range of aerobic aqueous environments. These tests are intended as simple, economical, fail-safe screens of biodegradability. They are not definitive since many organic compounds which do not degrade under the test conditions may undergo biodegradation in the actual environment. Such compounds may require testing beyond the Base-set to allow their biodegradability to be assessed under more favourable test conditions.

2. Assessment of Test Guidelines

The four tests currently accepted by the OECD and EEC (Table 1) are acceptable for their purpose although it should be noted that they are applicable only to water-soluble compounds (see section E of present report).

3. Recommended Improvements of the Tests

The prime reason for up-dating the accepted OECD and EEC tests should be that the modification or new test gives a better prediction, e.g. yields fewer false negative results. Modifications to existing tests would be

valuable if they simplify the procedure, improve the reproducibility, improve the reliability, or reduce costs.

From our present knowledge and experience of existing tests, the following proposals for improvements can be made :

i) The tests for ready biodegradability do not really reflect the biodegradation potential of a chemical in the actual aquatic environment, i.e. a certain number of false negative results (lack of biodegradability) occur. On the other hand, we certainly want to make use of the biodegradation potential of the environment (including sewage treatment plants) to the fullest possible extent. One way of rendering the tests for ready biodegradability more potent would be to introduce a pre-acclimatization procedure in such a way that their important feature is maintained, i.e. that they do not yield false positive (environmentally irrelevant) results. Research ought to be dedicated to this goal.

ii) Further possibilities for the harmonisation of tests among the family of tests for ready biodegradability, should be sought. This pertains especially to the composition of the mineral nutrient and the inocula.

iii) The influence of nitrification on the evaluation of biodegradability in BOD tests should be studied, since the oxidation of ammonia occurs erratically during such tests, and leads to an increased oxygen consumption which is not related to the oxidation of carbon. This occasionally gives abnormally high BOD values and a false impression of the biodegradability of the substance under test. This subject is also mentioned in the OECD Up-Dating Programme.

C. INHERENT BIODEGRADABILITY

1. General Considerations.

Substances which do not prove to be readily biodegradable may be examined in a test for inherent biodegradability with the aim of assessing their intrinsic potential to be biodegraded, i.e. their vulnerability to breakdown by the action of micro-organisms under conditions favourable for

biodegradation. The Modified SCAS Test, Modified Zahn-Wellens Test, and Modified MITI Test II are at present available for determining inherent biodegradability (Table 1). The last two have been evaluated in ring tests for their applicability and reproducibility. The status of the MITI Test II will be further defined by a forthcoming EEC ring test. The Modified SCAS Test has not been subjected to a ring test.

A method for assessing biodegradability in soil has been proposed in the OECD (cf. Table 1). The present authors consider that such a test should be seen as a simulation test. As this report deals only with aquatic biodegradability, a further discussion of this test falls outside of its scope.

2. Assessment of Test Guidelines

An inherently biodegradable chemical is unlikely to persist in the environment for an indeterminate period. The test, however, does not normally allow the rate of degradation to be predicted under aerobic environmental conditions. In the absence of inhibition by the test substance, a negative result in one of the more powerful inherent biodegradability tests (SCAS, Zahn-Wellens) is usually taken to mean that biodegradation will probably not occur, or will be only an insignificant pathway for eliminating the substance from the environment.

During inherent biodegradation testing, the degree of ultimate biodegradation (i.e. to CO_2 and H_2O) could be determined by means of the "summary parameters"*,i.e. DOC, COD, CO_2 evolution and O_2 consumption. If analytical methods specific to the substance are available, the degree of primary biodegradation (the breakdown of a molecule by the action of microorganisms leading to the disappearance of at least one specific functional group) can also be determined. The OECD recommends that when using summary parameters, degradation higher than 20% is considered as a proof of inherent biodegradability. A figure of more than 70% degradation

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* Results from the application of analytical methods to any kind of organic chemical and mixtures thereof.

calculated from the summary parameters is regarded as evidence for ultimate biodegradation.

Because there is a high microorganism (sludge) concentration in these tests, adsorption of the substance without consequent biodegradation can and does occur. It is thus important to distinguish between removal by adsorption and by biodegradation. Respirometric parameters (CO_2, $^{14}CO_2$ generation, and O_2 consumption) are more effective means of distinguishing between biodegradation and adsorption, or other kinds of elimination, than are DOC or parent-compound analysis.The tests in Table 1 can be used provided that they distinguish adequately between volatile loss and adsorption, in either primary or ultimate biodegradation.

Under suitable conditions, inherent biodegradability tests will clearly distinguish biodegradable from non-biodegradable chemicals. Failure to biodegrade may result from such factors as substrate toxicity, lack of co-metabolism and too short an acclimatisation time. The optimum conditions required for these tests are in sharp contrast to those for ready biodegradability tests where the conditions are deliberately made stringent. The three tests proposed by the OECD are reviewed below in the light of the foregoing requirements.

2.1. The MITI test II originated from the MITI test I for ready biodegradability. By reversal of the original ratio of the concentrations of substrate and microorganisms (to give in MITI-II, 30 ppm test substance and 100 ppm sludge) and the use of a mixed microbial inoculum, the toxic effects of the substance were reduced and the biodegradation potential enhanced. The test was thus transformed from a ready into an inherent biodegradability test. Despite these changes, this test has not the optimal characteristics of an inherent biodegradability test as outlined above, since the sludge density is still comparatively low. It is in principle suitable for the study of chemicals of low solubility and high volatility.

2.2. The SCAS test provides very favourable conditions for degradation, including a long test period, daily addition of settled domestic sewage, the presence of additional organic nutrients, and a concentration of test material corresponding to 20 mg carbon. $liter^{-1}$ which is lower than normal and thus reduces the risk of toxic effects. The

test, however, lacks analytical sensitivity because of the low amount of added substrate compared with the organic carbon content of the sewage. It also fails adequately to distinguish adsorption from true biodegradation and has presented serious difficulties in performance at some (but not all) laboratories. Daily analysis, and the statistical evaluation of raw data, allow a better interpretation of the results. This test gives fewer false negative results than do the other two. It is suitable only for the testing of water-soluble, non-volatile chemicals.

2.3. The Zahn-Wellens test has a number of advantages similar to those of the SCAS test (e.g. high sludge concentration, long test period). A higher initial concentration of test substance is used, and there is no regular addition of further carbon substrate since it is a batch die-away test. It gives a good indication of inherent biodegradability and permits acclimatisation phenomena to be detected from the recorded degradation-time relationship. The drawback of this test lies in its high initial concentration of test substance (possible toxic effects on inoculum). Like the SCAS test, the Zahn-Wellens test is suitable only for water-soluble, non-volatile chemicals.

3. Recommended Improvements of the Tests.

If the tests for inherent biodegradability in Table 1 are to demonstrate the intrinsic potential of a substance to be biodegraded, the conditions should be optimised by extending the test duration, using a higher concentration of micro-organisms and, if necessary, introducing additional nutrient to induce co-metabolism. In particular, the provision of a high concentration and diversity of micro-organisms increases the possibility of adaptation processes.

Modifications should be designed to :

i) eliminate those conditions which may limit the biodegradation potential and lead to false negative results. This can be achieved by gradually increasing the test substance concentration over a period of time by repetitive addition (this allows acclimatisation to occur and reduces toxic effects); increasing

the test duration; increasing the diversity of the inoculum; and adding suitable compounds to induce co-metabolism.

ii) improve the distinction between adsorption and biodegradation. This is achieved in the Zahn-Wellens test by starting the analysis in the early stages of the test. A respirometric test on inoculum transferred at the time when high elimination of the test material is observed would be of value in furnishing proof of biodegradability in both the SCAS and Zahn-Wellens tests.

iii) improve the sensitivity of the tests and the reproducibility of the results. The analytical sensitivity of the SCAS test could be improved by the addition of a die-away period at the end of the test (cf. EPA activated sludge die-away test). Statistical treatment of more results obtained over a longer period of time would help to define the results better, particularly in the SCAS test.

iv) increase the scope of the test (such as in ii) above) by the use of respirometric methods, or of ready biodegradability tests with microorganisms developed from the present inherent biodegradability tests. This would give a better understanding of the elimination processes in inherent biodegradation tests.

D. SIMULATION TESTS

1. General Considerations

Simulation tests are listed in Table 1. In certain circumstances, such a test carried out under conditions as close as possible to those in the environment may be required to ascertain the behaviour of a substance during sewage treatment or in natural waters. Also, when the results of testing for ready and inherent biodegradability are inconclusive, or suggest a certain hazard, simulation tests may give more precise details about the fate of a chemical in a well-defined aquatic environment. They are normally not necessary when adequate field data are available.

Three simulation test protocols are at present (end-1983) being discussed for acceptance under the 6th Amendment : the coupled units test, the porous pot test (Water Research Centre, UK), and the OECD confirmatory test with specific analysis (see Table 1). These standardised tests simulate only the activated sludge process and are applicable only to water-soluble compounds. Other simulation tests, such as the trickling filter method based on a small-scale rotating tube, and the rotating disk method are still at the development stage. It is emphasised that there are currently no well-developed tests which simulate the behaviour of chemicals in environments other than sewage plants, for example in natural waters.

Ready and inherent biodegradability tests indicate whether a chemical is, respectively, easily or potentially biodegradable. By contrast, the aim of a simulation test is to indicate whether a chemical will actually disappear, partially or completely, in an environmentally-relevant situation, and ultimately to permit an estimation of the expected environmental concentration. These tests should therefore mimic as closely as possible the real environmental conditions. If a substance has been demonstrated to be biodegradable in a simulation test, there is no need to study inherent biodegradation.

A number of aquatic environmental compartments exist - flowing and stagnant water and fresh, brackish or sea water. The conditions may be aerobic or anaerobic. It would be hopeless to try to model all of these, especially since water-insoluble and volatile compounds impose their special demands and restrictions on the method to be used. So far, standardised simulation tests have been based on the activated sludge process in order to simulate a sewage treatment plant. These tests give practical information on the possible effects of a chemical on the overall treatment. Simulation tests should also be designed to permit a very clear distinction between removal by adsorption and biodegradation.

The "ultimate" simulation test is a field trial or environmental monitoring, neither of which can be regarded as routine in view of the time, effort and expense involved.

2. Assessment of Test Guidelines

2.1. Tests under discussion. The coupled units, porous pot and OECD

confirmatory tests simulate the activated sludge process. It is generally assumed that when materials are biodegraded the same biological breakdown processes take place in sewage treatment plants as in surface waters, albeit at different rates. This may provide an argument for limiting the number of simulation tests, although further experimental proof of this is needed.

All of these tests may be criticised as being of questionable relevance to normal environmental conditions in that it is experimentally necessary to use high concentrations of the test substance. Toxic effects on the micro-organisms may be responsible for any apparent lack of degradability and should be investigated.

Testing at room temperature does not permit deductions about the influence of temperature variations on biodegradation in the environment. In sewage plants, biothermal effects may moderate the influence of the outside temperature but in other aquatic compartments this may not be the case.

The currently-recommended test durations are often insufficient to take into account the influence of biological adaptation and the age of the sludge in the test system. Prolonging the test may counteract this.

The load or food/microorganism ratio in the simulation tests may differ from that in actual activated-sludge plants.

2.2. Tests under development. These tests (trickling filter and rotating disk) suffer from the same drawbacks as the tests already standardised. As most of them have not been ring-tested and only few experimental results are available, it is premature to assess their value.

3. Recommended Improvements of the Tests.

The sludge age and retention-time are critical factors in determining the degree of removal of a compound in an activated sludge plant. By operating test units at various sludge retention times, a better estimate of

potential biodegradation under a realistic range of activated sludge conditions could be made.

In an OECD Document "Test Guidelines and Issues for Consideration in Proposed Up-Dating Programme" (1981) referring to Test Guideline 303 "Coupled Units Test", the question " should adsorbed but undegraded test material be recovered ?" is posed. For those compounds which do not chemisorb irreversibly it has been shown that this test does indicate the true biodegradation. Whilst recovery of test material from the sludge would give useful information, it is essential only when low biodegradability is found by the normal test procedure. A generally-applicable step would be trans-inoculation from the coupled units test into a respirometric test at the time when high elimination of the test material is observed. This would furnish proof of biodegradation. However, there might be cases where true biodegradation occurs in the coupled units test but the respirometric test still yields a negative result (e.g. because of lack of co-metabolism). Recovery studies are also an option to be considered.

E. FURTHER INVESTIGATIONS TO BE CONSIDERED

1. Tests for Poorly-Soluble or Insoluble Compounds

The recommended biodegradability tests as presently defined are applicable only to water-soluble, non-volatile compounds. However, it may be necessary to assess the biodegradability of certain "insoluble" (particularly lipophilic) materials, and test methods need to be developed for this purpose.

Whilst the Sturm, closed bottle, and MITI tests can in principle be used to evaluate the ready biodegradability of insoluble compounds, methods of ensuring adequate dispersion of such materials in the test containers have still to be developed, and the "pass level" for such materials requires definition.

The Blok Repetitive Die-Away test (Blok, J. and Booy, M.(1983). Internal AKZO report) and MITI test II may form the basis of an inherent biodegradability test for poorly-soluble or insoluble compounds.

The simulation tests under discussion have similarly been used only for evaluating water-soluble compounds. The possibility of adapting them for evaluating poorly-water-soluble compounds should be considered. Certain experimental details, such as means of obtaining a stable suspension or input, sampling techniques and analytical modifications, need to be elaborated. Analysis of the sludge for test material (adsorption) and the establishing of a mass balance or, more simply, trans-inoculation into tests for ready biodegradability once high elimination is observed in the simulation test, should also be specified.

Particular areas requiring study are noted below.

(a) Dispersion techniques. To ensure optimum biodegradation conditions the test substance must be dispersed in the aqueous test medium . It is unlikely that one dispersion method will be applicable to all substances and thus a number of approaches need to be evaluated :

i) Physical methods
- agitation, for dispersion of fine powders;
- emulsification or ultrasonification, for oils;
- coating the product onto inert (inorganic) fine particles.

ii) The use of chemicals such as wetters, dispersants and emulsifiers.

Physical methods have the advantage that no additional carbon source is added, thus making the results easier to assess, although difficulties may be experienced in maintaining a stable suspension/emulsion. If non-toxic, non-biodegradable dispersants can be used to form a stable dispersion in water at a level of say 10% of the substance, then only a relatively low correction need be applied to the result. The effect of an additional carbon source on the degradability of the substance, and the possible precipitation of test material after biodegradation of the dispersant, would need to be taken into account. The possibility that non-biodegradable wetters may slow down biodegradation by coating the test substance with a non-degradable surface requires evaluation.

(b) Measurement of biodegradation. The use of DOC/TOC measurements at low

concentrations of a test substance or high concentrations of wetters is not practical, and thus the following techniques could be considered :

i) respirometric techniques (O_2 consumption, CO_2 generation) ;
ii) the use of radio-labelled material;
iii) the use of specific analysis, although this would show only primary degradation, but not mineralisation, of the product.

2. Effect of Toxic Compounds on Inocula in Biodegradability Tests

In order to achieve analytical precision, biodegradability tests are operated at uncharacteristically high concentrations of test substance. In this situation a toxic but biodegradable material may be falsely identified as not biodegradable. Present methods of evaluating toxicity prior to biodegradability testing appear somewhat variable in response. Thus, the AFNOR and Blok Tests for inhibition do not wholly fulfill their function since they do not react, for example, to bactericidal quaternary ammonium compounds. It is questionable whether this is due to the base substrates (glucose and sodium acetate) or to some other feature of the tests. Research should be devoted to this question since there are grave consequences when these inhibition tests do not truly indicate toxicity, eg. the test compound may be classified as being non-biodegradable, whereas its biodegradation might merely be concentration dependent.

Although bacterial respiration-rate tests have been found to be adequate for detecting toxicity, the authors consider that the glucose/glutamic acid inhibition test may be more sensitive and reliable in operation, since the substance/micro-organism ratio is relatively high, thus enhancing possible toxic effects. This, and other approaches, should be reviewed and guidelines as to the most appropriate methods should be selected or produced.