OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Ready Biodegradability

INTRODUCTION

1. In this Guideline six methods are described that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium. They are:

301 A: DOC Die-Away
301 B: CO_2 Evolution (Modified Sturm Test)
301 C: MITI (I) (Ministry of International Trade and Industry, Japan)
301 D: Closed Bottle
301 E: Modified OECD Screening
301 F: Manometric Respirometry

Method 301 A is similar to the ISO Standard 7827-1984 and replaces the Modified AFNOR method; AFNOR has adopted the ISO standard. Methods 301 B, 301 D and 301 E are modified versions of the earlier OECD Guidelines adopted in 1981. Method 301 C is virtually identical with earlier Guideline 301 C (MITI I). Method 301 F is new; it is similar to 301 C differing mainly in the inocula employed.

2. Much experience has accumulated with the six methods over the years including an OECD inter-laboratory comparison exercise (ring test) in 1988. The accumulated experience, and the ring test, have confirmed that the methods may be used for the assessment of ready biodegradability. However, depending on the physical characteristics of the substance to be tested, a particular method may be preferred.

3. General considerations including those common to all six methods are given hereafter. Details of individual methods are given under separate headings (301 A to F). Throughout the text the reader is referred to the Annexes which contain definitions (Annex I), formulas and useful guidance material.

GENERAL PRINCIPLE OF THE TESTS

4. A solution, or suspension, of the test substance in a mineral medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC in the test solution due to the inoculum should be kept as low as possible compared with the amount of organic carbon due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blanks with inoculum but without test substance, although the endogenous activity of cells in the presence of a chemical will not exactly match that in the endogenous control. A reference compound is run in parallel to check the operation of the procedures.

5. In general, degradation is followed by the determination of parameters such as DOC, CO_2 production and oxygen uptake and measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation. With automatic respirometers the measurement is continuous. DOC is sometimes measured in addition to another parameter but this is usually done only at the beginning and end of the test. Specific chemical analysis can also be used to assess primary degradation of the test substance and to determine the concentration of any intermediate substances formed. It is obligatory in the MITI method (301 C).

6. Normally, the test lasts for 28 days. Tests however may be ended before 28 days, i.e. as soon as the biodegradation curve has reached a plateau for at least three determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but that the plateau has not been reached by day 28, but in such cases the chemical would not be classed as readily biodegradable.

INFORMATION ON THE TEST SUBSTANCE

7. In order to select the most appropriate method, information on the chemical's solubility, vapour pressure and adsorption characteristics is essential. The chemical structure or formula should be known in order to calculate theoretical values and/or check measured values of parameters, e.g. ThOD, $ThCO_2$, DOC, TOC, and COD. Information on the purity or the relative proportions of major components of the test material is required in order to interpret the results obtained, especially when the result lies close to the pass level.

8. Information on the toxicity of the test substance to bacteria (Annex II) may be very useful for selecting appropriate test concentrations and may be essential for the correct interpretation of low biodegradation values.

APPLICABILITY AND SELECTION OF METHODS

9. Test substances which are soluble in water to at least 100 mg/l may be assessed by all methods, provided they are non-volatile and non-adsorbing. For those chemicals which are poorly soluble in water, volatile or adsorbing, suitable methods are indicated in Table 1. The manner in which poorly water-soluble chemicals and volatile chemicals can be dealt with is described in Annex III, but in the MITI method neither solvents nor emulsifying agents are to be used. Moderately volatile chemicals may be tested by the DOC Die-Away method if there is sufficient gas space in the test vessels (which should be suitably stoppered). In this case, an abiotic control must be set up to allow for any physical loss.

TABLE 1 APPLICABILITY OF TEST METHODS

Test	Analytical method	Suitability for compounds which are:		
		poorly soluble	volatile	adsorbing
DOC Die-Away (301 A)	Dissolved organic carbon	-	-	+/-
CO_2 Evolution (301 B)	Respirometry: CO_2 evolution	+	-	+
MITI (I) (301 C)	Respirometry: oxygen consumption	+	+/-	+
Closed Bottle (301 D)	Respirometry: dissolved oxygen	+/-	+	+
Modified OECD Screening (301 E)	Dissolved organic carbon	-	-	+/-
Manometric Respirometry (301 F)	Oxygen consumption	+	+/-	+

PASS LEVELS

10. The pass levels for ready biodegradability are 70% removal of DOC and 60% of ThOD or $ThCO_2$ production for respirometric methods. They are lower in the respirometric methods since, as some of the carbon from the test chemical is incorporated into new cells, the percentage of CO_2 produced is lower than the percentage of carbon being used. These pass values have to be reached in a 10-d window within the 28-d period of the test, except where mentioned below. The 10-d window begins when the degree of biodegradation has reached 10% DOC, ThOD or $ThCO_2$ and must end before day 28 of the test. Chemicals which reach the pass levels after the 28-d period are not deemed to be readily biodegradable. The 10-d window concept does not apply to the MITI method. The value obtained in a 14-d window would be acceptable in the Closed Bottle method if it is considered that the number of bottles necessary to evaluate the 10-d window causes the test to become too unwieldy.

REFERENCE COMPOUNDS

11. In order to check the procedure, reference compounds which meet the criteria for ready biodegradability are tested by setting up an appropriate vessel in parallel as part of normal test runs. Suitable compounds are aniline (freshly distilled), sodium acetate and sodium benzoate. These reference compounds all degrade in these methods even when no inoculum is deliberately added. It was suggested that a reference compound should be sought which was readily biodegradable but required the addition of an inoculum. Potassium hydrogen phthalate has been proposed but more evidence needs to be obtained with this chemical before it can be accepted as a reference compound.

REPRODUCIBILITY OF TESTS

12. Because of the nature of biodegradation and of the mixed bacterial populations used as inocula, determinations should be carried out at least in duplicate. It is usually found that the larger the concentration of micro-organisms initially added to the test medium, the smaller will be the variation between replicates. Ring tests have also shown that there can be large variations between results obtained by different laboratories, but good agreement is normally obtained with easily biodegradable compounds.

GENERAL PROCEDURES AND PREPARATIONS

13. General conditions applying to the methods are summarised in Table 2. Apparatus and other experimental conditions pertaining specifically to an individual method are described later under the heading for that method.

Water

14. Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. The high purity of the test water is necessary in order to eliminate high blank values. Contamination may result from inherent impurities and also from the ion-exchange resins and lysed material from bacteria and algae. For each series of tests, use only one batch of water, previously checked by DOC analysis. Such a check is not necessary for the Closed Bottle method, but the oxygen consumption of the water must be low (see 301 D, paragraph 25).

Mineral media

15. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. Since only a very small inoculum, containing low concentrations of trace elements and growth factors, is used in the Modified OECD Screening method (301 E), the medium for this test may need to be fortified with additional compounds. The details of the stock solutions of mineral salts, trace elements and growth factors and the proportions used are given under the headings for the separate tests.

Methods of adding the test and reference substances

16. The method used for adding the test and reference substances to the reaction mixture depends upon the nature of the chemical, especially its water solubility. For substances of adequate solubility, greater than about 1 g/l, prepare stock solutions at appropriate concentrations and use aliquots to prepare the final test solution. Dissolve less soluble substances in the mineral medium to avoid diluting the buffer solution. Add substances which are even less soluble directly to the final mineral medium. Finally, refer to Annex III for the handling of poorly and insoluble substances, but note that in the MITI method (301 C) neither organic solvents nor emulsifying agents are to be used.

Inoculum

17. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents (unchlorinated); surface waters and soils; or from a mixture of these. For the DOC Die-Away (301 A), CO_2 Evolution (301 B) and Manometric Respirometry (301 F) methods if activated sludge is used, it should be taken from a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Inocula from other sources, usually yielding lower cell densities, have been found to give higher scattering of results. For the Modified OECD Screening (301 E) and Closed Bottle (301 D)

methods, a more dilute inoculum without sludge flocs is needed and the preferred source is a secondary effluent from a domestic waste water treatment plant or laboratory-scale unit. For the MITI (I) method, the inoculum is derived from a mixture of sources. Details of the sources and preparation of inocula are described under the headings of the specific test methods.

Pre-conditioning of inoculum

18. Inoculum may be pre-conditioned to the experimental conditions, but not pre-adapted to the test substance. Pre-conditioning consists of aerating activated sludge (in mineral medium) or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test methods by reducing blank values. It is considered unnecessary to pre-condition MITI (I) inoculum.

Abiotic controls

19. When required, check for the possible abiotic degradation of the test substance by determining the removal of DOC, oxygen uptake or carbon dioxide evolution in sterile controls containing no inoculum. Sterilize by filtration through a membrane (0.2-0.45 µm) or by the addition of a suitable toxic substance at an appropriate concentration. If membrane filtration is used, take samples aseptically to maintain sterility. Unless adsorption of the test substance has been ruled out beforehand, tests which measure biodegradation as the removal of DOC, especially with activated sludge inocula, should include an abiotic control which is inoculated and poisoned.

Number of flasks and samples

20. At least two flasks or vessels containing the test substance plus inoculum, and at least two containing inoculum only should be used. Single vessels suffice for reference compounds plus inoculum and, when required, for toxicity, abiotic removal and adsorption controls. The Closed Bottle and MITI (I) methods have special requirements for the number of flasks. These are given under the specific headings. It is mandatory to follow DOC and/or the other parameters in the test suspension and inoculum blanks in parallel. It is advisable to follow DOC in the other flasks in parallel as well. This may, however, not always be possible.

21. Although it is necessary to ensure that sufficient samples or readings are taken to allow the percentage removal in the 10-d window to be assessed, it is not possible to specify accurately the frequency of sampling because of the wide range of the lag phases and rates of degradation. In the MITI method (301 C) and, if an automatic respirometer is used in the Manometric Respirometry method (301 F), sampling for oxygen uptake presents no problems. In the latter method, daily readings are adequate when non-automatic respirometers are employed. Specific advice on sampling is given under the headings of the other four tests.

DATA AND REPORTING

Treatment of results

22. In the calculation of D_t, percentage degradation, the mean values of the duplicate measurement of the parameter in both test vessels and inoculum blank are used. The formulas are set out in the sections below on specific methods. The course of degradation is displayed graphically and the 10-d window is indicated where applicable. Calculate and report the percentage removal achieved and the value at the plateau, or at the end of the test, and/or at the end of the 10-d window, whichever is appropriate. In respirometric methods, N-containing chemicals may affect the oxygen uptake because of nitrification (see Annexes IV and V). Also, if the ThOD cannot be calculated because the test material is insufficiently defined, the COD value may be used to calculate the percentage degradation.

However, it must be borne in mind that the COD is often not as high as the ThOD as some chemicals are very poorly oxidised in the COD test, resulting in falsely high values for percentage biodegradation.

23. When specific chemical analytical data are available, calculate primary biodegradation from:

$$D_t = \frac{S_b - S_a}{S_b} \times 100$$

where:

D_t = % primary degradation at time t, normally 28 days;
S_a = residual amount of test chemical in inoculated medium at end of the test (mg);
S_b = residual amount of test chemical in the abiotic control at the end of the test (mg).

Validity of tests

24. A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14. If either of these conditions is not met, the test should be repeated. Because of the stringency of the methods, low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

25. If in a toxicity test, containing both the test substance and a reference compound, less than 35% degradation (based on total DOC) or less than 25% (based on total ThOD or $ThCO_2$) occurred within 14 days, the test substance can be assumed to be inhibitory (see Annex II for other toxicity tests). The test series should be repeated, using a lower concentration of test substance (if this can be done without seriously impairing the accuracy of the DOC determination) and/or a higher concentration of inoculum, but not greater than 30 mg solids/l.

26. Other conditions for the validity of test results specific to individual methods are set out under the headings for those tests.

Test report

27. The test report must include the following:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Test conditions:

- inoculum: nature and sampling site(s), concentration and any pre-conditioning treatment;
- proportion and nature of industrial waste water in sewage, if known;
- test duration and temperature;
- in the case of poorly soluble test substances, methods of preparation of test solutions/suspensions;

- test method applied; scientific reasons and explanation for any change of procedure.

Results:

- data in tabular form;
- any observed inhibition phenomena;
- any observed abiotic degradation;
- specific chemical analytical data, if available;
- analytical data on intermediates, if available;
- the graph of percentage degradation against time for the test and reference substances, the lag phase, degradation phase, the 10-d window and slope (see Annex I for definitions);
- percentage removal at plateau, at end of test, and/or after 10-d window.

Discussion of results.

TABLE 2: TEST CONDITIONS

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	$\leq$ 30					30
ml effluent/l	$\leq$ 100			0.5	$\leq$ 5	
approx. cells/l	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 $\pm$ 0.2					preferably 7
Temperature ° C	22 $\pm$ 2					25 $\pm$ 1°

DOC = Dissolved Organic Carbon ThOD = Theoretical Oxygen Demand SS = Suspended Solids