ANNEX 4: EXAMPLE DATA SHEET FOR RECORDING MEDIUM RENEWAL, PHYSICAL/CHEMICAL MONITORING DATA, FEEDING, DAPHNIA REPRODUCTION AND ADULT MORTALITY

Experiment No: Date started: Clone: Medium: Type of food: Test Substance: Nominal conc:

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
Medium renewal (tick)																								
pH*																							new	
																							old	
O_2 (mg/l)*																							new	
																							old	
Temp (°C)*																							new	
																							old	
Food provided (tick)																								
No. live offspring**																								Total
Vessel 1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								
9																								
10																								
																							Total	
Cumulative adult mortality***																								

* Indicate which vessel was used for the experiment

** Record aborted broods as 'AB' in relevant box

*** Record mortality of any adult animals as 'M' in relevant box

ANNEX 5

EXAMPLE DATA SHEET FOR RECORDING RESULTS OF CHEMICAL ANALYSIS

(a) Measured concentrations

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

(b) Measured concentrations as a percentage of nominal

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

ANNEX 6

CALCULATION OF A TIME-WEIGHTED MEAN

Time-weighted mean

Given that the concentration of the test substance can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations experienced by the parent *Daphnia*. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic substance is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

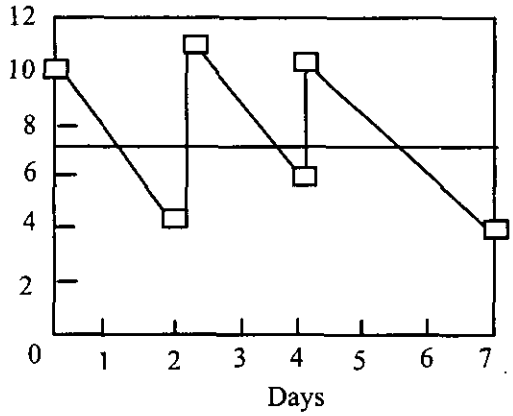

Figure 1: Example of time-weighted mean

Figure 1 shows an example of a (simplified) test lasting seven days with medium renewal at Days 0, 2 and 4.

- The thin zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.
- The 6 plotted points represent the observed concentrations measured at the start and end of each renewal period.
- The thick solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

Table 1: Calculation of Time-weighted mean

Renewal No.	Days	Conc 0	Conc 1	Ln(Conc 0)	Ln(Conc 1)	Area
1	2	10.000	4.493	2.303	1.503	13.767
2	2	11.000	6.037	2.398	1.798	16.544
3	3	10.000	4.066	2.303	1.403	19.781
Total Days:	7				Total Area:	50.092
					TW Mean:	7.156

Days is the number of days in the renewal period
Conc 0 is the measured concentration at the start of each renewal period
Conc 1 is the measured concentration at the end of each renewal period
Ln(Conc 0) is the natural logarithm of Conc 0
Ln(Conc 1) is the natural logarithm of Conc 1

Area is the area under the exponential curve for each renewal period. It is calculated by:

$$Area = \frac{Conc\ 0 - Conc\ 1}{Ln(Conc\ 0) - Ln(Conc\ 1)} \times Days$$

The time-weighted mean *(TW Mean)* is the *Total Area* divided by the *Total Days.*

Of course, for the *Daphnia* reproduction test the table would have to be extended to cover 21 days.

It is clear that when observations are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process in, in fact, exponential. A different curve would result in a different calculation for *Area*. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a word of caution is required if the chemical analysis fails to find any substance at the end of the renewal period. Unless it is possible to estimate how quickly the substance disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.

OECD/OCDE

216

Adopted:
21st January 2000

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Soil Microorganisms: Nitrogen Transformation Test

INTRODUCTION

1. This Test Guideline describes a laboratory test method designed to investigate the long-term effects of chemicals, after a single exposure, on nitrogen transformation activity of soil microorganisms. The test is principally based on the recommendations of the European and Mediterranean Plant Protection Organization (1). However, other guidelines, including those of the German Biologische Bundesanstalt (2), the US Environmental Protection Agency (3) SETAC (4) and the International Organization for Standardization (5), were also taken into account. An OECD Workshop on Soil/Sediment Selection held at Belgirate, Italy, in 1995 (6) agreed on the number and type of soils for use in this test. Recommendations for collection, handling and storage of soil samples are based on an ISO Guidance Document (7) and recommendations from the Belgirate Workshop.

INITIAL CONSIDERATIONS

2. In the assessment and evaluation of toxic characteristics of test substances, determination of effects on soil microbial activity may be required, e.g. when data on the potential side effects of crop protection products on soil microflora are required or when exposure of soil microorganisms to chemicals other than crop protection products is expected. The nitrogen transformation test is carried out to determine the effects of such chemicals on soil microflora. If agrochemicals (e.g. crop protection products, fertilisers, forestry chemicals) are tested, both nitrogen transformation and carbon transformation tests are conducted. If non agrochemicals are tested, the nitrogen transformation test is sufficient. However, if EC_{50} values of the nitrogen transformation test for such chemicals fall within the range found for commercially available nitrification inhibitors (e.g. nitrapyrin), a carbon transformation test can be conducted to gain further information.

3. Soils consist of living and non-living components which exist in complex and heterogeneous mixtures. Microorganisms play an important role in break-down and transformation of organic matter in fertile soils with many species contributing to different aspects of soil fertility. Any long-term interference with these biochemical processes could potentially interfere with nutrient cycling and this could alter soil fertility. Transformation of carbon and nitrogen occurs in all fertile soils. Although the microbial communities responsible for these processes differ from soil to soil, the pathways of transformation are essentially the same.

4. The test described in this Guideline is designed to detect long-term adverse effects of a substance on the process of nitrogen transformation in aerobic surface soils. The test method also allows estimation of the effects of substances on carbon transformation by the soil microflora. Nitrate formation takes place subsequent to the degradation of carbon-nitrogen bonds. Therefore, if equal rates of nitrate production are found in treated and control soils, it is highly probable that the major carbon degradation pathways are intact and functional. The substrate chosen for the test (powdered lucerne meal) has a favourable carbon to nitrogen ratio (usually between 12/1 and 16/1). Because of this, carbon starvation is reduced during the test and if microbial communities are damaged by a chemical, they might recover within 100 days.

5. The tests from which this Guideline was developed were primarily designed for substances for which the amount reaching the soil can be anticipated. This is the case, for example, for crop protection products for which the application rate in the field is known. For agrochemicals, testing of two doses relevant to the anticipated or predicted application rate is sufficient. Agrochemicals can be tested as active ingredients (a.i.) or as formulated products. However, the test is not limited to agrochemicals. By changing both the amounts of test substance applied to the soil, and the way in which the data are evaluated, the test can also be used for chemicals for which the amount expected to reach the soil is not known. Thus, with chemicals other than agrochemicals, the effects of a series of concentrations on nitrogen transformation are determined. The data from these tests are used to prepare a dose-response curve and calculate EC_x values, where x is defined % effect.

PRINCIPLE OF THE TEST

6. Sieved soil is amended with powdered plant meal and either treated with the test substance or left untreated (control). If agrochemicals are tested, a minimum of two test concentrations are recommended and these should be chosen in relation to the highest concentration anticipated in the field. After 0, 7, 14 days and 28 days of incubation, samples of treated and control soils are extracted with an appropriate solvent, and the quantities of nitrate in the extracts are determined. The rate of nitrate formation in treated samples is compared with the rate in the controls, and the percent deviation of the treated from the control is calculated. All tests run for at least 28 days. If, on the 28th day, differences between treated and untreated soils are equal to or greater than 25%, measurements are continued to a maximum of 100 days. If non agrochemicals are tested, a series of concentrations of the test substance are added to samples of the soil, and the quantities of nitrate formed in treated and control samples are measured after 28 days of incubation. Results from tests with multiple concentrations are analysed using a regression model, and the EC_x values are calculated (i.e. EC_{50}, EC_{25} and/or EC_{10}). See annex for definitions.

VALIDITY OF THE TEST

7. Evaluations of test results with agrochemicals are based on relatively small differences (i.e. average value ± 25 %) between nitrate concentrations in control and treated soil samples, so large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ± 15%.

DESCRIPTION OF THE METHOD

Apparatus

8. Test containers made of chemically inert material are used. They should be of a suitable capacity in compliance with the procedure used for incubation of soils, i.e. incubation in bulk or as a series of individual soil samples (see paragraph 24). Care should be taken both to minimise water loss and to allow gas exchange during the test (e.g. the test containers may be covered with perforated polyethylene foil). When volatile substances are tested, sealable and gas-tight containers should be used. These should be of a size such that approximately one quarter of their volume is filled with the soil sample.

9. Standard laboratory equipment including the following is used:

- agitation device: mechanical shaker or equivalent equipment;
- centrifuge (3000 g) or filtration device (using nitrate-free filter paper);
- instrument of adequate sensitivity and reproducibility for nitrate analysis.

Selection and number of soils

10. One single soil is used. The recommended soil characteristics are as follows:

- sand content: not less than 50% and not greater than 75%;
- pH: 5.5 - 7.5;
- organic carbon content: 0.5 - 1.5 %;
- the microbial biomass should be measured (8)(9) and its carbon content should be at least 1 % of the total soil organic carbon.

11. In most cases, a soil with these characteristics represents a worst case situation, since adsorption of the test chemical is minimum and its availability to the microflora is maximum. Consequently, tests with other soils are generally unnecessary. However, in certain circumstances, e.g. where the anticipated major use of the test substance is in particular soils such as acidic forest soils, or for electrostatically charged chemicals, it may be necessary to use an additional soil.

Collection and storage of soil samples

Collection

12. Detailed information on the history of the field site from where the test soil is collected should be available. Details include exact location, vegetation cover, dates of treatments with crop protection products, treatments with organic and inorganic fertilisers, additions of biological materials or accidental contaminations. The site chosen for soil collection should be one which allows long-term use. Permanent pastures, fields with annual cereal crops (except maize) or densely sown green manures are suitable. The selected sampling site should not have been treated with crop protection products for a minimum of one year before sampling. Also, no organic fertiliser should have been applied for at least six months. The use of mineral fertiliser is only acceptable when in accordance with the requirements of the crop and soil samples should not be taken until at least three months after fertiliser application. The use of soil treated with fertilisers with known biocidal effects (e.g. calcium cyanamide) should be avoided.

13. Sampling should be avoided during or immediately following long periods (greater than 30 days) of drought or water logging. For ploughed soils, samples should be taken from a depth of 0 down to 20 cm. For grassland (pasture) or other soils where ploughing does not occur over longer periods (at least one growing season), the maximum depth of sampling may be slightly more than 20 cm (e.g. to 25 cm).

14. Soil samples should be transported using containers and under temperature conditions which guarantee that the initial soil properties are not significantly altered.

Storage

15. The use of soils freshly collected from the field is preferred. If storage in the laboratory cannot be avoided, soils may be stored in the dark at 4 ± 2 °C for a maximum of three months. During the storage of soils, aerobic conditions must be ensured. If soils are collected from areas where they are frozen for at least three months per year, storage for six months at minus 18°C to minus 22°C can be considered. The microbial biomass of stored soils is measured prior to each experiment and the carbon in the biomass should be at least 1 % of the total soil organic carbon content (see paragraph 10).

Handling and preparation of soil for the test

Pre-incubation

16. If the soil was stored (see paragraph 15), pre-incubation is recommended for a period between 2 and 28 days. The temperature and moisture content of the soil during pre-incubation should be similar to that used in the test (see paragraphs 17 and 25).

Physical-chemical characteristics

17. The soil is manually cleared of large objects (e.g. stones, parts of plants, etc.) and then moist sieved without excess drying to a particle size less than or equal to 2 mm. The moisture content of the soil sample should be adjusted with distilled or deionised water to a value between 40 % and 60 % of the maximum water holding capacity.

Amendment with organic substrate

18. The soil should be amended with a suitable organic substrate, e.g. powdered lucerne-grass-green meal (main component: *Medicago sativa)* with a C/N ratio between 12/1 and 16/1. The recommended lucerne-soil ratio is 5 g of lucerne per kilogram of soil (dry weight).

Preparation of the test substance for the application to soil

19. The test substance is normally applied using a carrier. The carrier can be water (for water soluble substances) or an inert solid such as fine quartz sand (particle size: 0.1-0.5 mm). Liquid carriers other than water (e.g. organic solvents such as acetone, chloroform) should be avoided since they can damage the microflora. If sand is used as a carrier, it can be coated with the test substance dissolved or suspended in an appropriate solvent. In such cases, the solvent should be removed by evaporation before mixing with the soil. For an optimum distribution of the test substance in soil, a ratio of 10 g of sand per kilogram of soil (dry weight) is recommended. Control samples are treated with an equivalent amount of water and/or quartz sand only.

20. When testing volatile chemicals, losses during treatment should be avoided as far as possible and an attempt should be made to ensure homogeneous distribution in the soil (e.g. the test substance should be injected into the soil at several places).

Test concentrations

21. If agrochemicals are tested, at least two concentrations should be used. The lower concentration should reflect at least the maximum amount expected to reach the soil under practical conditions whereas the higher concentration should be a multiple of the lower concentration. The concentrations of test substance added to soil are calculated assuming uniform incorporation to a depth of 5 cm and a soil bulk density of 1.5. For agrochemicals that are applied directly to soil, or for chemicals for which the quantity

reaching the soil can be predicted, the test concentrations recommended are the maximum Predicted Environmental Concentration (PEC) and five times that concentration. Substances that are expected to be applied to soils several times in one season should be tested at concentrations derived from multiplying the PEC by the maximum anticipated number of applications. The upper concentration tested, however, should not exceed ten times the maximum single application rate.

22. If non-agrochemicals are tested, a geometric series of at least five concentrations is used. The concentrations tested should cover the range needed to determine the EC_x values.

PERFORMANCE OF THE TEST

Conditions of exposure

Treatment and control

23. If agrochemicals are tested, the soil is divided into three portions of equal weight. Two portions are mixed with the carrier containing the product, and the other is mixed with the carrier without the product (control). A minimum of three replicates for both treated and untreated soils is recommended. If non-agrochemicals are tested, the soil is divided into six portions of equal weight. Five of the samples are mixed with the carrier containing the test substance, and the sixth sample is mixed with the carrier without the chemical. Three replicates for both treatments and control are recommended. Care should be taken to ensure homogeneous distribution of the test substance in the treated soil samples. During mixing, compacting or balling of the soil should be avoided.

Incubation of soil samples

24. Incubation of soil samples can be performed in two ways: as bulk samples of each treated and untreated soil or as a series of individual and equally sized subsamples of each treated and untreated soil. However, when volatile substances are tested, the test should only be performed with a series of individual subsamples. When soils are incubated in bulk, large quantities of each treated and untreated soils are prepared and subsamples to be analysed are taken as needed during the test. The amount initially prepared for each treatment and control depends on the size of the subsamples, the number of replicates used for analysis and the anticipated maximum number of sampling times. Soils incubated in bulk should be thoroughly mixed before subsampling. When soils are incubated as a series of individual soil samples, each treated and untreated bulk soil is divided into the required number of subsamples, and these are utilised as needed. In the experiments where more than two sampling times can be anticipated, enough subsamples should be prepared to account for all replicates and all sampling times. At least three replicate samples of the test soil should be incubated under aerobic conditions (see paragraph 23). During all tests, appropriate containers with sufficient headspace should be used to avoid development of anaerobic conditions. When volatile substances are tested, the test should only be performed with a series of individual subsamples.

Test conditions and duration

25. The test is carried out in the dark at room temperature of 20 ± 2 °C. The moisture content of soil samples should be maintained during the test between 40 % and 60 % of the maximum water holding capacity of the soil (see paragraph 17) with a range of ± 5 %. Distilled, deionized water can be added as needed.

26. The minimum duration of tests is 28 days. If agrochemicals are tested, the rates of nitrate formation in treated and control samples are compared. If these differ by more than 25 % on day 28, the

test is continued until a difference equal to or less than 25 % is obtained, or for a maximum of 100 days, whichever is shorter. For non-agrochemicals, the test is terminated after 28 days. On day 28, the quantities of nitrate in treated and control soil samples are determined and the EC_x values are calculated.

Sampling and analysis of soils

Soil sampling schedule

27. If agrochemicals are tested, soil samples are analysed for nitrate on days 0, 7, 14 and 28. If a prolonged test is required, further measurements should be made at 14 days intervals after day 28.

28. If non-agrochemicals are tested, at least five test concentrations are used and soil samples are analysed for nitrate at the beginning (day 0) and at the end of the exposure period (28 days). An intermediate measurement, e.g. at day 7, may be added if deemed necessary. The data obtained on day 28 are used to determine EC_x value for the chemical. If desired, data from day 0 control samples can be used to report the initial quantity of nitrate in the soil.

Analysis of soil samples

29. The amount of nitrate formed in each treated and control replicate is determined at each sampling time. Nitrate is extracted from soil by shaking samples with a suitable extraction solvent, e.g. a 0.1 M potassium chloride solution. A ratio of 5 ml of KCl solution per gram dry weight equivalent of soil is recommended. To optimise extraction, containers holding soil and extraction solution should not be more than half full. The mixtures are shaken at 150 rpm for 60 minutes. The mixtures are centrifuged or filtered and the liquid phases are analysed for nitrate. Particle-free liquid extracts can be stored prior to analysis at minus 20 ± 5 °C for up to six months.

DATA AND REPORTING

Data

30. If tests are conducted with agrochemicals, the quantity of nitrate formed in each replicate soil sample should be recorded, and the mean values of all replicates should be provided in tabular form. Nitrogen transformation rates should be evaluated by appropriate and generally acceptable statistical methods (e.g. F-test, 5% significance level). The quantities of nitrate formed are expressed in mg nitrate/kg dry weight soil/day. The nitrate formation rate in each treatment is compared with that in the control, and the percent deviation from the control is calculated.

31. If tests are conducted with non-agrochemicals, the quantity of nitrate formed in each replicate is determined, and a dose-response curve is prepared for estimation of the EC_x values. The quantities of nitrate (i.e. mg nitrate/kg dry weight soil) found in the treated samples after 28 days are compared to that found in the control. From these data, the % inhibition values for each test concentration are calculated. These percentages are plotted against concentration, and statistical procedures are then used to calculate the EC_x values. Confidence limits ($p = 0.95$) for the calculated ECx are also determined using standard procedures (10)(11)(12).

32. Test substances that contain high quantities of nitrogen may contribute to the quantities of nitrate formed during the test. If these substances are tested at a high concentration (e.g. chemicals which are expected to be used in repeated applications) appropriate controls must be included in the test (i.e. soil plus test substance but without plant meal). Data from these controls must be accounted for in the EC_x calculations .

Interpretation of results

33. When results from tests with agrochemicals are evaluated, and the difference in the rates of nitrate formation between the lower treatment (i.e. the maximum predicted concentration) and control is equal to or less than 25 % at any sampling time after day 28, the product can be evaluated as having no long-term influence on nitrogen transformation in soils. When results from tests with chemicals other than agrochemicals are evaluated, the EC_{50}, EC_{25} and/or EC_{10} values are used.

Test Report

34. The test report must include the following information:

Complete identification of the soil used including:

- geographical reference of the site (latitude, longitude);
- information on the history of the site (i.e. vegetation cover, treatments with crop protection products, treatments with fertilisers, accidental contamination, etc.)
- use pattern (e.g. agricultural soil, forest, etc.);
- depth of sampling (cm);
- sand/silt/clay content (% dry weight);
- pH (in water);
- organic carbon content (% dry weight);
- nitrogen content (% dry weight);
- initial nitrate concentration (mg nitrate/kg dry weight)
- cation exchange capacity (mmol/kg);
- microbial biomass in terms of percentage of the total organic carbon;
- reference of the methods used for the determination of each parameter;
- all information relating to the collection and storage of soil samples;
- details of pre-incubation of soil if any.

Test substance:

- physical nature and, where relevant, physical-chemical properties;
- chemical identification data, where relevant, including structural formula, purity (i.e. for crop protection products the percentage of active ingredient), nitrogen content.

Substrate:

- source of substrate
- composition (i.e. lucerne meal, lucerne-grass-green meal)
- carbon, nitrogen content (% dry weight)
- sieve size (mm)

Test conditions:

- details of the amendment of soil with organic substrate;
- number of concentrations of test chemical used and, where appropriate, justification of the selected concentrations;
- details of the application of test substance to soil;
- incubation temperature;
- soil moisture content at the beginning and during the test;
- method of soil incubation used (i.e. as bulk or as a series of individual subsamples);
- number of replicates;
- sampling times;
- method used for extraction of nitrate from soil;

Results:

- analytical procedure and equipment used to analyse nitrate;
- tabulated data including individual and mean values for nitrate measurements;
- variation between the replicates in treated and control samples;
- explanations of corrections made in the calculations, if relevant;
- the percent variation in nitrate formation rates at each sampling time or, if appropriate, the EC_{50} value with 95 per cent confidence limit, other EC_x (i.e. EC_{25} or EC_{10}) with confidence intervals, and a graph of the dose-response curve;
- statistical treatment of results;
- all information and observations helpful for the interpretation of the results.

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ANNEX

DEFINITIONS

Nitrogen transformation is the ultimate degradation by micororganisms of nitrogen-containing organic matter, via the process of ammonification and nitrification, to the respective inorganic end-product nitrate.

EC_x (Effective Concentration) is the concentration of the test substance in soil that results in a x percent inhibition of nitrogen transformation to nitrate.

EC_{50} (Median Effective Concentration) is the concentration of the test substance in soil that results in a 50 percent (50 %) inhibition of nitrogen transformation to nitrate.

301

Adopted:
17.07.92

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;