been treated with the test substance or its structural analogues within the previous four years, these should not be used for transformation studies (10)(12).

27. The soil should be freshly collected from the field (from the A horizon or top 20 cm layer) with a soil water content which facilitates sieving. For soils other than those from paddy fields, sampling should be avoided during or immediately following long periods (> 30 days) of drought, freezing or flooding (12). Samples should be transported in a manner which minimises changes in soil water content and should be kept in the dark with free access of air, as much as possible. A loosely-tied polyethylene bag is generally adequate for this purpose.

28. The soil should be processed as soon as possible after sampling. Vegetation, larger soil fauna and stones should be removed prior to passing the soil through a 2 mm sieve which removes small stones, fauna and plant debris. Extensive drying and crushing of the soil before sieving should be avoided (12).

29. When sampling in the field is difficult in winter (soil frozen or covered by layers of snow), it may be taken from a batch of soil stored in the greenhouse under plant cover (e.g. grass or grass-clover mixtures). Studies with soils freshly collected from the field are strongly preferred, but if the collected and processed soil has to be stored prior to the start of the study storage conditions must be adequate and for a limited time only (4 ± 2°C for a maximum of three months) to maintain microbial activity[3]. Detailed instructions on collection, handling and storage of soils to be used for biotransformation experiments can be found in (8)(10)(12)(23)(24).

30. Before the processed soil is used for this test, it should be pre-incubated to allow germination and removal of seeds, and to re-establish equilibrium of microbial metabolism following the change from sampling or storage conditions to incubation conditions. A pre-incubation period between 2 and 28 days approximating the temperature and moisture conditions of the actual test is generally adequate (12). Storage and pre-incubation time together should not exceed three months.

PERFORMANCE OF THE TEST

Test conditions

Test temperature

31. During the whole test period, the soils should be incubated in the dark at a constant temperature representative of the climatic conditions where use or release will occur. A temperature of 20 ± 2 °C is recommended for all test substances which may reach the soil in temperate climates. The temperature should be monitored.

32. For chemicals applied or released in colder climates (e.g. in northern countries, during autumn/winter periods), additional soil samples should be incubated but at a lower temperature (e.g. 10 ± 2 °C).

[3] Recent research results indicate that soils from temperate zones can also be stored at –20°C for more than three months (25)(26) without significant losses of microbial activity.

Moisture content

33. For transformation tests under aerobic conditions, the soil moisture content[4] should be adjusted to and maintained at a pF of between 2.0 and 2.5 (3). The soil moisture content is expressed as mass of water per mass of dry soil and should be regularly controlled (e.g. in 2 week intervals) by weighing of the incubation flasks and water losses compensated by adding water (preferably sterile-filtered tap water). Care should be given to prevent or minimise losses of test substance and/or transformation products by volatilisation and/or photodegradation (if any) during moisture addition.

34. For transformation tests under anaerobic and paddy conditions, the soil is water-saturated by flooding.

Aerobic incubation conditions

35. In the flow-through systems, aerobic conditions will be maintained by intermittent flushing or by continuously ventilating with humidified air. In the biometer flasks, exchange of air is maintained by diffusion.

Sterile aerobic conditions

36. To obtain information on the relevance of abiotic transformation of a test substance, soil samples may be sterilised (for sterilisation methods see references 13 and 26), treated with sterile test substance (e.g. addition of solution through a sterile filter) and aerated with humidified sterile air as described in paragraph 35. For paddy soils, soil and water should be sterilised and the incubation should be carried out as described in paragraph 38.

Anaerobic incubation conditions

37. To establish and maintain anaerobic conditions, the soil treated with the test substance and incubated under aerobic conditions for 30 days or one half-life or DT_{50} (whichever is shorter) is then water-logged (1-3 cm water layer) and the incubation system flushed with an inert gas (e.g. nitrogen or argon)[5]. The test system must allow for measurements such as pH, oxygen concentration and redox potential and include trapping devices for volatile products. The biometer-type system must be closed to avoid entrance of air by diffusion.

Paddy incubation conditions

38. To study transformation in paddy rice soils, the soil is flooded with a water layer of about 1-5 cm and the test substance applied to the water phase (9). A soil depth of at least 5 cm is recommended. The

[4] The soil should neither be too wet nor too dry to maintain adequate aeration and nutrition of soil microflora. Moisture contents recommended for optimal microbial growth range from 40-60% water holding capacity (WHC) and from 0.1-0.33 bar (6). The latter range is equivalent to a pF-range of 2.0 – 2.5. Typical moisture contents of various soil types are given in Annex 3.

[5] Aerobic conditions are dominant in surface soils and even in sub-surface soils as shown in an EU sponsored research project [K. Takagi et al. (1992). Microbial diversity and activity in subsoils: Methods, field site, seasonal variation in subsoil temperatures and oxygen contents. Proc. Internat. Symp. Environ. Aspects Pesticides Microbiol., 270-277, 17-21 August 1992, Sigtuna, Sweden]. Anaerobic conditions may occur only occasionally during flooding of soils after heavy rainfalls or when paddy conditions are established in rice fields.

system is ventilated with air as under aerobic conditions. pH, oxygen concentration and redox potential of the aqueous layer should be monitored and reported. A pre-incubation period of at least two weeks is necessary before commencing transformation studies (see paragraph 30).

Test duration

39. The rate and pathway studies should normally not exceed 120 days[6] (3)(6)(8), because thereafter a decrease of the soil microbial activity with time would be expected in an artificial laboratory system isolated from natural replenishment. Where necessary to characterise the decline of the test substance and the formation and decline of major transformation products, studies can be continued for longer periods (e.g. 6 or 12 months) (8). Longer incubation periods should be justified in the test report and accompanied by biomass measurements during and at the end of these periods.

Treatment and application of test substance

40. About 50 to 200 g of soil (dry weight basis) are placed into each incubation flask (see Figures 1 and 2 in Annex 4) and the soil treated with the test substance by one of the methods described in paragraphs 19-22. When organic solvents are used for the application of the test substance, they should be removed from soil by evaporation. Then the soil is thoroughly mixed with a spatula and/or by shaking of the flask. If the study is conducted under paddy field conditions, soil and water should be thoroughly mixed after application of the test substance. Small aliquots (e.g. 1 g) of the treated soils should be analysed for the test substance to check for uniform distribution. For alternative method, see paragraph 42.

41. The treatment rate should correspond to the highest application rate of a crop protection product recommended in the use instructions and uniform incorporation to an appropriate depth in the field (e.g. top 10 cm layer[7] of soil). For example, for chemicals foliarly or soil applied without incorporation, the appropriate depth for computing how much chemical should be added to each flask is 2.5 cm. For soil incorporated chemicals, the appropriate depth is the incorporation depth specified in the use instructions. For general chemicals, the application rate should be estimated based on the most relevant route of entry; for example, when the major route of entry in soil is through sewage sludge, the chemical should be dosed into the sludge at a concentration that reflects the expected sludge concentration and the amount of sludge added to the soil should reflect normal sludge loading to agricultural soils. If this concentration is not high enough to identify major transformation products, incubation of separate soil samples containing higher rates may be helpful, but excessive rates influencing soil microbial functions should be avoided (see paragraphs 8 and 19).

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[6] Aerobic studies might be terminated much before 120 days provided that ultimate transformation pathway and ultimate mineralisation are clearly reached at that time. Termination of the test is possible after 120 days, or when at least 90% of the test substance is transformed, but only if at least 5% CO_2 is formed.

[7] Calculation of the initial concentration on an area basis using the following equation:

$$C_{soil}[mg/kg_{soil}] = \frac{A[kg/ha] \bullet 10^6[mg/kg]}{l[m] \bullet 10^4[m^2/ha] \bullet d[kg_{soil}/m^3]}$$

C_{soil} = Initial concentration in soil [mg • kg^{-1}]

A = Application rate [kg • ha^{-1}]; l = thickness of field soil layer [m]; d = dry bulk density of soil [kg • m^{-3}].

As a rule of thumb, an application rate of 1 kg • ha^{-1} results in a soil concentration of approximately 1 mg • kg^{-1} in a 10 cm layer (assuming a bulk density of 1 g • cm^{-3}).

42. Alternatively, a larger batch (i.e. 1 to 2 kg) of soil can be treated with the test substance, carefully mixed in an appropriate mixing machine and then transferred in small portions of 50 to 200 g into the incubation flasks (for example with the use of sample splitters). Small aliquots (e.g. 1 g) of the treated soil batch should be analysed for the test substance to check for uniform distribution. Such a procedure is preferred since it allows for more uniform distribution of the test substance into the soil.

43. Also untreated soil samples are incubated under the same conditions (aerobic) as the samples treated with the test substance. These samples are used for biomass measurements during and at the end of the studies.

44. When the test substance is applied to the soil dissolved in organic solvent(s), soil samples treated with the same amount of solvent(s) are incubated under the same conditions (aerobic) as the samples treated with the test substance. These samples are used for biomass measurements initially, during and at the end of the studies to check for effects of the solvent(s) on microbial biomass.

45. The flasks containing the treated soil are either attached to the flow-through system described in Figure 1 or closed with the absorption column shown in Figure 2 (see Annex 4).

Sampling and measurements

46. Duplicate incubation flasks are removed at appropriate time intervals and the soil samples extracted with appropriate solvents of different polarity and analysed for the test substance and/or transformation products. A well-designed study includes sufficient flasks so that two flasks are sacrificed at each sampling event. Also, absorption solutions or solid absorption materials are removed at various time intervals (7-day intervals during the first month and after one month in 14-day intervals) during and at the end of incubation of each soil sample and analysed for volatile products. Besides a soil sample taken directly after application (0-day sample) at least 5 additional sampling points should be included. Time intervals should be chosen in such a way that pattern of decline of the test substance and patterns of formation and decline of transformation products can be established (e.g. 0, 1, 3, 7 days; 2, 3 weeks; 1, 2, 3 months, etc.).

47. When using ^{14}C-labelled test substance, non-extractable radioactivity will be quantified by combustion and a mass balance will be calculated for each sampling interval.

48. In the case of anaerobic and paddy incubation, the soil and water phases are analysed together for test substance and transformation products or separated by filtration or centrifugation before extraction and analysis.

Optional tests

49. Aerobic, non-sterile studies at additional temperatures and soil moistures may be useful for the estimation of the influence of temperature and soil moisture on the rates of transformation of a test substance and/or its transformation products in soil.

50. A further characterisation of non-extractable radioactivity can be attempted using, for example, supercritical fluid extraction.

DATA AND REPORTING

Treatment of results

51. The amounts of test substance, transformation products, volatile substances (in % only), and non-extractable should be given as % of applied initial amount and, where appropriate, as $mg \cdot kg^{-1}$ soil (based on soil dry weight) for each sampling interval. A mass balance should be given in percentage of the applied initial amount for each sampling interval. A graphical presentation of the test substance concentrations against time will allow an estimation of its transformation half-life or DT_{50}. Major transformation products should be identified and their concentrations should also be plotted against time to show their rates of formation and decline. A major transformation product is any product representing ≥ 10% of applied dose at any time during the study.

52. The volatile products trapped give some indication of the volatility potential of a test substance and its transformation products from soil.

53. More accurate determinations of half-lives or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values should be obtained by applying appropriate kinetic model calculations. The half-life and DT_{50} values should be reported together with the description of the model used, the order of kinetics and the determination coefficient (r^2). First order kinetics is favoured unless $r^2 < 0.7$. If appropriate, the calculations should also be applied to the major transformation products. Examples of appropriate models are described in references 28 to 32.

54. In the case of rate studies carried out at various temperatures, the transformation rates should be described as a function of temperature within the experimental temperature range using the Arrhenius relationship of the form:

$$k = A \bullet e^{-B/T} \quad \text{or} \quad \ln k = \ln A - \frac{B}{T},$$

where ln A and B are regression constants from the intercept and slope, respectively, of a best fit line generated from linearly regressing ln k against 1/T, k is the rate constant at temperature T and T is the temperature in Kelvin. Care should be given to the limited temperature range in which the Arrehenius relationship will be valid in case transformation is governed by microbial action.

Test report

55. The report must include:

Test substance:

- common name, chemical name, CAS number, structural formula (indicating position of label(s) when radiolabelled material is used) and relevant physical-chemical properties (see paragraph 7);
- purity (impurities) of test substance;
- radiochemical purity of labelled chemical and specific activity (where appropriate);

Reference substances:

- chemical name and structure of reference substances used for the characterisation and/or identification of transformation products;

Test soils:

- details of collection site;
- date and procedure of soil sampling;
- properties of soils, such as pH, organic carbon content, texture (% sand, % silt, % clay), cation exchange capacity, bulk density, water retention characteristic, and microbial biomass;
- length of soil storage and storage conditions (if stored);

Test conditions:

- dates of the performance of the studies;
- amount of test substance applied;
- solvents used and method of application for the test substance;
- weight of soil treated initially and sampled at each interval for analysis;
- description of the incubation system used;
- air flow rates (for flow-through systems only);
- temperature of experimental set-up;
- soil moisture content during incubation;
- microbial biomass initially, during and at the end of the aerobic studies;
- pH, oxygen concentration and redox potential initially, during and at the end of the anaerobic and paddy studies;
- method(s) of extraction;
- methods for quantification and identification of the test substance and transformation products in soil and absorption materials;
- number of replicates and number of controls.

Results:

- result of microbial activity determination;
- repeatability and sensitivity of the analytical methods used;
- rates of recovery (% values for a valid study are given in paragraph 12);
- tables of results expressed as % of applied initial dose and, where appropriate, as $mg \cdot kg^{-1}$ soil (on a dry weight basis);
- mass balance during and at the end of the studies;
- characterisation of non-extractable (bound) radioactivity or residues in soil;
- quantification of released CO_2 and other volatile compounds;
- plots of soil concentrations versus time for the test substance and, where appropriate, for major transformation products;
- half-life or DT_{50} , DT_{75} and DT_{90} for the test substance and, where appropriate, for major transformation products including confidence limits;
- estimation of abiotic degradation rate under sterile conditions;
- an assessment of transformation kinetics for the test substance and, where appropriate, for major transformation products;
- proposed pathways of transformation, where appropriate;
- discussion and interpretation of results;

- raw data (i.e. sample chromatograms, sample calculations of transformation rates and means used to identify transformation products).

Interpretation and evaluation of results

56. Although the studies are carried out in an artificial laboratory system, the results will allow estimation of the rate of transformation of the test substance and also of rate of formation and decline of transformation products under field conditions (33)(34).

57. A study of the transformation pathway of a test substance provides information on the way in which the applied substance is structurally changed in the soil by chemical and microbial reactions.

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ANNEX 1

DEFINITIONS

Test substance: any substance, whether the parent compound or relevant transformation products.

Transformation products: all substances resulting from biotic or abiotic transformation reactions of the test substance including CO_2 and products that are in bound residues.

Bound residues: "Bound residues" represent compounds in soil, plant or animal, which persist in the matrix in the form of the parent substance or its metabolite(s)/transformation products after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified in part by matrix-altering extraction methods and sophisticated analytical techniques. To date, for example, covalent ionic and sorptive bonds, as well as entrapments, have been identified in this way. In general, the formation of bound residues reduces the bioaccessibility and the bioavailability significantly (1) [modified from IUPAC 1984 (2)].

Aerobic transformation: reactions occurring in the presence of molecular oxygen (3).

Anaerobic transformation: reactions occurring under exclusion of molecular oxygen(3).

Soil is a mixture of mineral and organic chemical constituents, the latter containing compounds of high carbon and nitrogen content and of high molecular weights, animated by small (mostly micro-) organisms. Soil may be handled in two states:

(a) undisturbed, as it has developed with time, in characteristic layers of a variety of soil types;
(b) disturbed, as it is usually found in arable fields or as occurs when samples are taken by digging and used in this guideline (3).

Mineralisation is the complete degradation of an organic compound to CO_2 and H_2O under aerobic conditions, and CH_4, CO_2 and H_2O under anaerobic conditions. In the context of this guideline, when ^{14}C-labelled compound is used, mineralisation means extensive degradation during which a labelled carbon atom is oxidised with release of the appropriate amount of $^{14}CO_2$ (3).

Half-life, $t_{0.5}$, is the time taken for 50% transformation of a test substance when the transformation can be described by first-order kinetics; it is independent of the concentration.

DT_{50} (Disappearance Time 50) is the time within which the concentration of the test substance is reduced by 50%; it is different from the half-life $t_{0.5}$ when transformation does not follow first order kinetics.

DT_{75} (Disappearance Time 75) is the time within which the concentration of the test substance is reduced by 75%.

DT_{90} (Disappearance Time 90) is the time within which the concentration of the test substance is reduced by 90%.

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ANNEX 2

WATER TENSION, FIELD CAPACITY (FC) AND WATER HOLDING CAPACITY (WHC)(1)

Height of Water Column [cm]	pF[(a)]	bar[(b)]	Remarks
10^7	7	10^4	Dry Soil
$1.6 \cdot 10^4$	4.2	16	Wilting point
10^4	4	10	
10^3	3	1	
$6 \cdot 10^2$	2.8	0.6	
$3.3 \cdot 10^2$	2.5	0.33[(c)]	
10^2	2	0.1	Range of
60	1.8	0.06	Field capacity[(d)]
33	1.5	0.033	
10	1	0.01	WHC (approximation)
1	0	0.001	Water saturated soil

(a) pF = log of cm water column.
(b) 1 bar = 10^5 Pa.
(c) Corresponds to an approximate water content of 10% in sand, 35% in loam and 45% in clay.
(d) Field capacity is not constant but varies with soil type between pF 1.5 and 2.5.

Water tension is measured in cm water column or in bar . Due to the large range of suction tension it is expressed simply as pF value which is equivalent to the logarithm of cm water column.

Field capacity is defined as the amount of water which can be stored against gravity by a natural soil 2 days after a longer raining period or after sufficient irrigation. It is determined in undisturbed soil in situ in the field. The measurement is thus not applicable to disturbed laboratory soil samples. FC values determined in disturbed soils may show great systematic variances.

Water holding capacity (WHC) is determined in the laboratory with undisturbed and disturbed soil by saturating a soil column with water by capillary transport. It is particularly useful for disturbed soils and can be up to 30 % greater than field capacity (1). It is also experimentally easier to determine than reliable FC-values.

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ANNEX 3

SOIL MOISTURE CONTENTS (g water per 100 g dry soil) OF VARIOUS SOIL TYPES FROM VARIOUS COUNTRIES

Soil Type	Country	Soil Moisture Content at		
		WHC[1]	pF = 1.8	pF = 2.5
Sand	Germany	28.7	8.8	3.9
Loamy sand	Germany	50.4	17.9	12.1
Loamy sand	Switzerland	44.0	35.3	9.2
Silt loam	Switzerland	72.8	56.6	28.4
Clay loam	Brazil	69.7	38.4	27.3
Clay loam	Japan	74.4	57.8	31.4
Sandy loam	Japan	82.4	59.2	36.0
Silt loam	USA	47.2	33.2	18.8
Sandy loam	USA	40.4	25.2	13.3

[1] Water Holding Capacity

ANNEX 4

Figure 1

Example of a flow-through apparatus to study transformation of chemicals in soil (1)(2)

1: needle valve

2: gas washing bottle containing water

3: ultramembrane (sterile conditions only), pore size 0.2 µm

4: soil metabolism flask (water-logged only for anaerobic and paddy conditions;)

5: ethylene glycol trap for organic volatile compounds

6: sulphuric acid trap for alkaline volatile compounds

7, 8: sodium hydroxide trap for CO_2 & other acidic volatiles

9: flow meter.

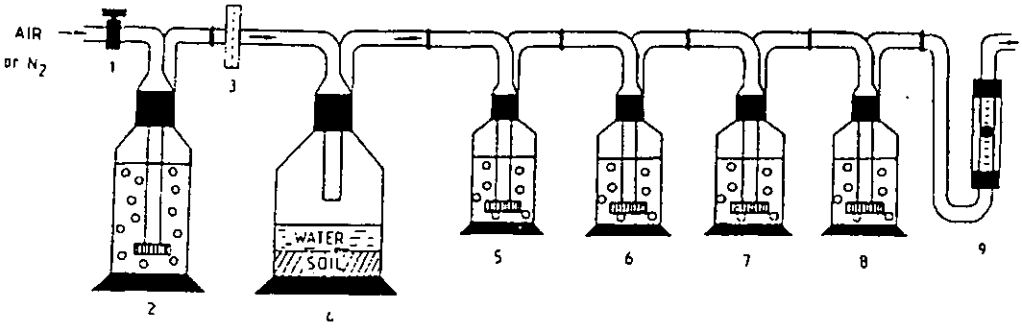
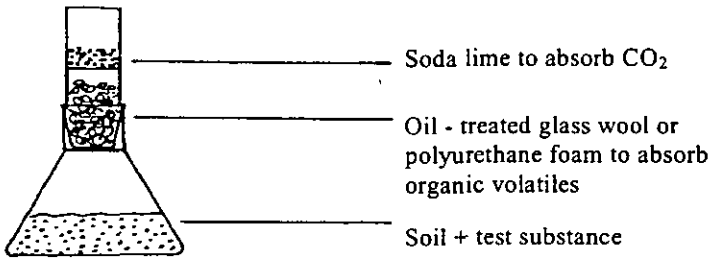


Figure 2

Example of a biometer-type flask for studying the transformation of chemicals in soil (3)



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OECD/OCDE 308

Adopted:
24th April 2002

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Aerobic and Anaerobic Transformation in Aquatic Sediment Systems

INTRODUCTION

1. Chemicals can enter shallow or deep surface waters by such routes as direct application, spray drift, run-off, drainage, waste disposal, industrial, domestic or agricultural effluent and atmospheric deposition. This Guideline describes a laboratory test method to assess aerobic and anaerobic transformation of organic chemicals in aquatic sediment systems. It is based on existing Guidelines (1)(2)(3)(4)(5)(6). An OECD Workshop on Soil/Sediment Selection, held in Belgirate, Italy in 1995 (7) agreed, in particular, on the number and type of sediments for use in this test. It also made recommendations relating to collection, handling and storage of sediment samples, based on the ISO Guidance (8). Such studies are required for chemicals which are directly applied to water or which are likely to reach the aqueous environment by the routes described above.

2. The conditions in natural aquatic sediment systems are often aerobic in the upper water phase. The surface layer of sediment can be either aerobic or anaerobic, whereas the deeper sediment is usually anaerobic. To encompass all of these possibilities both aerobic and anaerobic tests are described in this document. The aerobic test simulates an aerobic water column over an aerobic sediment layer that is underlain with an anaerobic gradient. The anaerobic test simulates a completely anaerobic water-sediment system. If circumstances indicate that it is necessary to deviate significantly from these recommendations, for example by using intact sediment cores or sediments that may have been exposed to the test substance, other methods are available for this purpose (9).

PRINCIPLE OF THE TEST

3. The method described in this guideline employs an aerobic and an anaerobic aquatic sediment system (see Annex 1) which allows:

(i) the measurement of the transformation rate of the test substance in a water-sediment system,
(ii) the measurement of the transformation rate of the test substance in the sediment,
(iii) the measurement of the mineralisation rate of the test substance and/or its transformation products (when ^{14}C-labelled test substance is used),
(iv) the identification and quantification of transformation products in water and sediment phases including mass balance (when labelled test substance is used), and
(v) the measurement of the distribution of the test substance and its transformation products between the two phases during a period of incubation in the dark (to avoid, for example, algal blooms) at constant temperature.

Half-lives, DT_{50}, DT_{75} and DT_{90} values are determined where the data warrant, but should not be extrapolated far past the experimental period (See Annex 2 for definitions).

4. At least two sediments and their associated waters are required for both the aerobic and the anaerobic studies respectively (7). However, there may be cases where more than two aquatic sediments should be used, for example, for a chemical that may be present in freshwater and/or marine environments.

APPLICABILITY OF THE TEST

5. The method is generally applicable to chemical substances (unlabelled or labelled) for which an analytical method with sufficient accuracy and sensitivity is available. It is applicable to slightly volatile, non-volatile, water-soluble or poorly water-soluble compounds. The test should not be applied to chemicals which are highly volatile from water (e.g. fumigants, organic solvents) and thus cannot be kept in water and/or sediment under the experimental conditions of this test.

6. The method has been applied so far to study the transformation of chemicals in fresh waters and sediments, but in principle can also be applied to estuarine/marine systems. It is not suitable to simulate conditions in flowing water (e.g. rivers) or the open sea.

INFORMATION ON THE TEST SUBSTANCE

7. Non-labelled or isotope-labelled test substance can be used to measure the rate of transformation although labelled material is preferred. Labelled material is required for studying the pathway of transformation and for establishing a mass balance. ^{14}C-labelling is recommended, but the use of other isotopes, such as ^{13}C, ^{15}N, ^{3}H, ^{32}P, may also be useful. As far as possible, the label should be positioned in the most stable part(s) of the molecule[1]. The chemical and/or radiochemical purity of the test substance should be at least 95%.

8. Before carrying out a test, the following information about the test substance should be available:

(a) solubility in water [OECD Guideline 105] (10);
(b) solubility in organic solvents;
(c) vapour pressure [OECD Guideline 104] (10) and Henry's Law constant;
(d) n-octanol/water partition coefficient [OECD Guidelines 107 and 117] (10);
(e) adsorption coefficient (K_d, K_f or K_{oc}, where appropriate) [OECD Guideline 106] (10);
(f) hydrolysis [OECD Guideline 111] (10);
(g) dissociation constant (pK_a) [OECD Guideline 112] (10).
(h) chemical structure of the test substance and position of the isotope-label(s), if applicable.

Note: The temperature at which these measurements were made should be reported.

9. Other useful information may include data on toxicity of the test substance to microorganisms, data on ready and/or inherent biodegradability, and data on aerobic and anaerobic transformation in soil.

10. Analytical methods (including extraction and clean-up methods) for identification and quantification of the test substance and its transformation products in water and in sediment should be available (see also paragraphs 14 and 15).

[1] For example, if the substance contains one ring, labelling on this ring is required; if the test substance contains two or more rings, separate studies may be needed to evaluate the fate of each labelled ring and to obtain suitable information on formation of transformation products.

REFERENCE SUBSTANCES

11. Reference substances should be used for the identification and quantification of transformation products by spectroscopic and chromatographic methods.

DEFINITIONS AND UNITS

12. See Annex 2.

QUALITY CRITERIA

Recovery

13. Extraction and analysis of, at least, duplicate water and sediment samples immediately after the addition of the test substance give a first indication of the repeatability of the analytical method and of the uniformity of the application procedure for the test substance. Recoveries for later stages of the experiments are given by the respective mass balances (when labelled material is used). Recoveries should range from 90% to 110% for labelled chemicals (6) and from 70% to 110% for non-labelled chemicals.

Repeatability and sensitivity of analytical method

14. Repeatability of the analytical method (excluding the initial extraction efficiency) to quantify test substance and transformation products can be checked by duplicate analysis of the same extract of the water or the sediment samples which were incubated long enough for formation of transformation products.

15. The limit of detection (LOD) of the analytical method for the test substance and for the transformation products should be at least 0.01 $mg \cdot kg^{-1}$ in water or sediment (as test substance) or 1% of the initial amount applied to a test system whichever is lower. The limit of quantification (LOQ) should also be specified.

Accuracy of transformation data

16. Regression analysis of the concentrations of the test substance as a function of time gives the appropriate information on the accuracy of the transformation curve and allows the calculation of the confidence limits for half-lives (if pseudo first-order kinetics apply) or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values.

DESCRIPTION OF THE METHOD

Test system and apparatus

17. The study should be performed in glass containers (e.g. bottles, centrifuge tubes), unless preliminary information (such as n-octanol-water partition coefficient, sorption data, etc.) indicates that the test substance may adhere to glass, in which case an alternative material (such as Teflon) may have to be

considered. Where the test substance is known to adhere to glass, it may be possible to alleviate this problem using one or more of the following methods:

- determine the mass of test substance and transformation products sorbed to glass;
- ensure a solvent wash of all glassware at the end of the test;
- use of formulated products (see also paragraph 36);
- use an increased amount of co-solvent for addition of test substance to the system; if a co-solvent is used it should be a co-solvent that does not solvolyse the test substance.

18. Examples of typical test apparatus, i.e. gas flow-through and biometer-type systems, are shown in Annexes 3 and 4, respectively (11). Other useful incubation systems are described in reference 12. The design of the experimental apparatus should permit the exchange of air or nitrogen and the trapping of volatile products. The dimensions of the apparatus must be such that the requirements of the test are complied with (see paragraph 32). Ventilation may be provided by either gentle bubbling or by passing air or nitrogen over the water surface. In the latter case gentle stirring of the water from above may be advisable for better distribution of the oxygen or nitrogen in the water. CO_2-free air should not be used as this can result in increases in the pH of the water. In either case, disturbance of the sediment is undesirable and should be avoided as far as possible. Slightly volatile chemicals should be tested in a biometer-type system with gentle stirring of the water surface. Closed vessels with a headspace of either atmospheric air or nitrogen and internal vials for the trapping of volatile products can also be used (13). Regular exchange of the headspace gas is required in the aerobic test in order to compensate for the oxygen consumption by the biomass.

19. Suitable traps for collecting volatile transformation products include but are not restricted to 1 $mol \cdot dm^{-3}$ solutions of potassium hydroxide or sodium hydroxide for carbon dioxide[2] and ethylene glycol, ethanolamine or 2% paraffin in xylene for organic compounds. Volatiles formed under anaerobic conditions, such as methane, can be collected, for example, by molecular sieves. Such volatiles can be combusted, for example, to CO_2 by passing the gas through a quartz tube filled with CuO at a temperature of 900 °C and trapping the CO_2 formed in an absorber with alkali (14).

20. Laboratory instrumentation for chemical analysis of test substance and transformation products is required (e.g. gas liquid chromatography (GLC), high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), mass spectroscopy (MS), gas chromatography-mass spectroscopy (GC-MS), liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (NMR), etc.), including detection systems for radiolabelled or non-labelled chemicals as appropriate. When radiolabelled material is used a liquid scintillation counter and combustion oxidiser (for the combustion of sediment samples prior to analysis of radioactivity) will also be required.

21. Other standard laboratory equipment for physical-chemical and biological determinations (see Table 1, paragraph 26), glassware, chemicals and reagents are required as appropriate.

Selection and number of aquatic sediments

22. The sampling sites should be selected in accordance with the purpose of the test in any given situation. In selecting sampling sites, the history of possible agricultural, industrial or domestic inputs to the catchment and the waters upstream must be considered. Sediments should not be used if they have been contaminated with the test substance or its structural analogues within the previous 4 years.

[2] As these alkaline absorption solutions also absorb the carbon dioxide from the ventilation air and that formed by respiration in aerobic experiments, they have to be exchanged at regular intervals to avoid their saturation and thus loss of their absorption capacity.

Sediment selection

23. Two sediments are normally used for the aerobic studies (7). The two sediments selected should differ with respect to organic carbon content and texture. One sediment should have a high organic carbon content (2.5-7.5%) and a fine texture, the other sediment should have a low organic carbon content (0.5-2.5%) and a coarse texture. The difference between the organic carbon contents should normally be at least 2%. "Fine texture" is defined as a [clay + silt][3] content of >50% and "coarse texture" is defined as a [clay + silt] content of <50%. The difference in [clay + silt] content for the two sediments should normally be at least 20%. In cases, where a chemical may also reach marine waters, at least one of the water-sediment systems should be of marine origin.

24. For the strictly anaerobic study, two sediments (including their associated waters) should be sampled from the anaerobic zones of surface water bodies (7). Both the sediment and the water phases should be handled and transported carefully under exclusion of oxygen.

25. Other parameters may be important in the selection of sediments and should be considered on a case-by-case basis. For example, the pH range of sediments would be important for testing chemicals for which transformation and/or sorption may be pH-dependent. pH-dependency of sorption might be reflected by the pK_a of the test substance.

Characterisation of water-sediment samples

26. Key parameters that must be measured and reported (with reference to the method used) for both water and sediment, and the stage of the test at which those parameters are to be determined are summarised in the Table hereafter. For information, methods for determination of these parameters are given in references (15)(16)(17)(18).

27. In addition, other parameters may need to be measured and reported on a case by case basis e.g. for freshwater: particles, alkalinity, hardness, conductivity, NO_3/PO_4 (ratio and individual values); for sediments: cation exchange capacity, water holding capacity, carbonate, total nitrogen and phosphorus; and for marine systems: salinity). Analysis of sediments and water for nitrate, sulfate, bioavailable iron, and possibly other electron acceptors may be also useful in assessing redox conditions, especially in relation to anaerobic transformation.

[3] [Clay + silt] is the mineral fraction of the sediment with particle size of < 50 µm.

Measurement of parameters for characterisation of water-sediment samples (7)(19)(20)

Parameter	Stage of test procedure					
	field sampling	post-handling	start of acclimation	start of test	during test	end of test
Water						
Origin/source	x					
Temperature	x					
pH	x		x	x	x	x
TOC			x	x		x
O_2 concentration*	x		x	x	x	x
*Redox potential**			x	x	x	x
Sediment						
Origin/source	x					
Depth of layer	x					
pH		x	x	x	x	x
Particle size distribution		x				
TOC		x	x	x		x
Microbial biomass**		x		x		x
Redox potential *	Observation (colour/smell)		x	x	x	x

* Recent research results have shown that measurements of water oxygen concentrations and of redox potentials have neither a mechanistic nor a predictive value as far as growth and development of microbial populations in surface waters are concerned (21)(22). Determination of the biochemical oxygen demand (BOD, at field sampling, start and end of test) and of concentrations of micro/macro nutrients Ca, Mg and Mn (at start and end of test) in water and the measurement of total N and total P in sediments (at field sampling and end of test) may be better tools to interpret and evaluate aerobic biotransformation rates and routes.

** Microbial respiration rate method (23), fumigation method (24) or plate count measurements (e.g. bacteria, actinomycetes, fungi and total colonies) for aerobic studies; methanogenesis rate for anaerobic studies.

Collection, handling and storage

Collection

28. The draft ISO guidance on sampling of bottom sediment (8) should be used for sampling of sediment. Sediment samples should be taken from the entire 5 to 10 cm upper layer of the sediment. Associated water should be collected from the same site or location and at the same time as the sediment. For the anaerobic study, sediment and associated water should be sampled and transported under exclusion of oxygen (25) (see also paragraph 24). Some sampling devices are described in the literature (8)(20).

Handling

29. The sediment is separated from the water by filtration and the sediment wet-sieved to a 2 mm-sieve using excess location water that is then discarded. Then known amounts of sediments and water are mixed at the desired ratio (see paragraph 32) in incubation flasks and prepared for the acclimation period (see paragraph 31). For the anaerobic study, all handling steps have to be done under exclusion of oxygen (26)(27)(28)(29)(30).

Storage

30. Use of freshly sampled sediment and water is strongly recommended, but if storage is necessary, sediment and water should be sieved as described above and stored together, water-logged (6-10 cm water layer), in the dark, at 4 ± 2°C[4] for a maximum of 4 weeks (7)(8)(20). Samples to be used for aerobic studies should be stored with free access of air (e.g. in open containers), whereas those for anaerobic studies under exclusion of oxygen. Freezing of sediment and water and drying-out of the sediment must not occur during transportation and storage.

Preparation of the sediment/water samples for the test

31. A period of acclimation should take place prior to adding the test substance, with each sediment/water sample being placed in the incubation vessel to be used in the main test, and the acclimation to be carried out under exactly the same conditions as the test incubation (see paragraphs 32 and 33). The acclimation period is the time needed to reach reasonable stability of the system, as reflected by pH, oxygen concentration in water, redox potential of the sediment and water, and macroscopic separation of phases. The period of acclimation should normally last between one week and two weeks and should not exceed four weeks. Results of determinations performed during this period should be reported.

PERFORMANCE OF THE TEST

Test conditions

32. The test should be performed in the incubation apparatus (see paragraphs 17-19) with a water:sediment volume ratio between 3:1 and 4:1, and a sediment layer of 2.5 cm (± 0.5 cm). A minimum amount of 50 g of sediment (dry weight basis) per incubation vessel is recommended.

[4] Recent studies have shown that storage at 4°C can lead to a decrease of the organic carbon content of the sediment which may possibly result in a decrease of microbial activity (31).