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Water quality — Evaluation of the aerobic biodegradability of organic compounds at low concentrations —

Part 1: Shake-flask batch test with surface water or surface water/sediment suspensions

*Qualité de l'eau — Évaluation de la biodégradabilité aérobie des composés
organiques présents en faibles concentrations —*

*Partie 1: Essai en lots de flacons agités avec des eaux de surface ou
des suspensions eaux de surface/sédiments*



Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this part of ISO 14592 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 14592-1 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 14592 consists of the following parts, under the general title *Water quality — Evaluation of the aerobic biodegradability of organic compounds at low concentrations*:

- *Part 1: Shake-flask batch test with surface water or surface water/sediment suspensions*
- *Part 2: Continuous flow river model with attached biomass*

This corrected version of ISO 14592-1:2002 incorporates corrections to

- the reference given in the third item of the list in 8.2.1;
- the reference given in the penultimate line of 8.2.1;
- the reference given in the last line of the second paragraph of 8.4.1.

Introduction

This International Standard consists of two parts. Part 1 describes a die-away batch test for either surface water with or without added sediment in suspension simulating either a pelagic aquatic environment or a water-to-sediment interface. Part 2 describes a continuous flow system simulating a river with biomass attached to stationary surfaces.

This test has been specifically designed to provide information on the biodegradation behaviour and kinetics of test compounds present in low concentrations, i.e. sufficiently low to ensure that they simulate the biodegradation kinetics which would be expected to occur in natural environmental systems.

Before conducting this test, it is necessary to have information on the biodegradability behaviour of the test compound at higher concentrations (e.g. in standard biodegradation tests), and, if possible, on abiotic degradability or elimination from water, as well as relevant physico-chemical data. This information is necessary for proper experimental planning and interpretation of results.

When this test method is used with a single environmental sample of surface water (either with or without the addition of sediment), a laboratory-derived first-order biodegradation rate can be estimated for one single point in time and space. The test system may be more consistent and provide more reliable biodegradation results if it is adapted to the test compound at a specifically maintained concentration. This may be achieved using the optional semi-continuous procedural variant of the method.

Water quality — Evaluation of the aerobic biodegradability of organic compounds at low concentrations —

Part 1: Shake-flask batch test with surface water or surface water/sediment suspensions

WARNING AND SAFETY PRECAUTIONS — Activated sludge, sewage and effluent contain potentially pathogenic organisms. Therefore appropriate precautions should be taken when handling them. Toxic and dangerous test compounds and those whose properties are unknown should be handled with care. Radiolabelled compounds, if used, should be handled respecting existing rules and legislation.

1 Scope

This part of ISO 14592 specifies a test method for evaluating the biodegradability of organic test compounds by aerobic microorganisms by means of a shake-flask batch test. It is applicable to natural surface water, free from coarse particles to simulate a pelagic environment ("pelagic test") or to surface water with suspended sediments added to obtain a level of 0,1 g/l to 1 g/l dry mass to simulate a water body with suspended sediment ("suspended sediment test").

This part of ISO 14592 is applicable to organic test compounds present in lower concentrations (normally below 100 µg/l) than those of natural carbon substrates also present in the system. Under these conditions, the test compounds serve as a secondary substrate and the kinetics for biodegradation would be expected to be first order ("non-growth" kinetics).

This test method is not recommended for use as proof of ultimate biodegradation which is better assessed using other standardized tests (see ISO/TR 15462). It is also not well suited to studies on metabolite formation and accumulation which require higher test concentrations.

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this part of ISO 14592. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 14592 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO/TR 15462, *Water quality — Selection of tests for biodegradability*

3 Terms, definitions and symbols

3.1 Terms and definitions

For the purpose of this part of ISO 14592, the following terms and definitions apply.

3.1.1

ultimate aerobic biodegradation

breakdown of a chemical compound or organic matter by microorganisms, in the presence of oxygen, to carbon dioxide (CO₂), water and mineral salts of any other elements present (mineralization) and the production of new biomass

NOTE Total mineralization may be different from ultimate aerobic biodegradation in that total mineralization includes secondary mineralization of biosynthesis products. The kinetics may therefore deviate from first-order kinetics in particular towards the end of the experiment. In this part of ISO 14592, primary aerobic biodegradation is determined when using substance specific analysis and total mineralization when using radiolabelled compounds.

3.1.2

primary biodegradation

structural change (transformation) of a chemical compound by microorganisms resulting in the loss of a specific property of that compound

3.1.3

dissolved organic carbon

DOC

part of the organic carbon in a sample of water which cannot be removed by specified phase separation

NOTE Phase separation may be obtained, for example, by centrifugation of the sample of test water at 40 000 m/s² for 15 min or by membrane-filtration using membranes with pores of 0,45 µm diameter.

3.1.4

lag phase

t_{lag}

time from the start of a test until significant biodegradation (about 10 % of the maximum level) can be measured

NOTE Lag phase is expressed in days (d).

3.1.5

maximum level of biodegradation

degree of biodegradation of a chemical compound or organic matter in a test above which no further biodegradation takes place during the test

NOTE The maximum level of biodegradation is expressed as a percentage.

3.1.6

primary substrate

major carbon and energy source which is essential for growth or maintenance of microorganisms

3.1.7

secondary substrate

substrate component present at such low concentrations, that by its degradation, only insignificant amounts of carbon and energy are supplied to the competent microorganisms, as compared to the carbon and energy supplied by their degradation of primary substrates

3.1.8

degradation rate constant

k

rate constant for first-order or pseudo first-order kinetics which indicates the rate at which degradation processes occur

NOTE 1 The degradation rate constant is expressed in inverse days (d⁻¹).

NOTE 2 For a batch experiment, k is estimated from the initial part of the degradation curve obtained after the end of the lag phase. For a continuously operating test system, k can be estimated from a mass balance for the reactor using data collected under steady-state conditions.

3.1.9 degradation half-life

 $T_{1/2}$

characteristic of the rate of a first-order reaction and corresponds to the time interval necessary for the concentration to decrease by a factor of two

NOTE 1 The degradation half-life is expressed in days (d).

NOTE 2 The degradation half-life and the degradation rate constant are related by the following equation:

$$T_{1/2} = \ln 2/k$$

NOTE 3 The degradation half-life $T_{1/2}$ for first-order reactions should not be confused with the half-life time, T_{50} , which is often used to describe the environmental behaviour of pesticides and which is simply the time to reach 50% of total biodegradation. The half-life time T_{50} may be derived from degradation curves without making assumptions about the kinetics.

3.2 Symbols

Symbol	Description	Units
$A^{1)}$	activity of the ^{14}C -radiolabelled test compound	becquerels(Bq)
A_I	inorganic ^{14}C -activity ($^{14}\text{CO}_2$ evolved as a result of biodegradation)	becquerels(Bq)
A_{TO}	total organic ^{14}C -activity of the residual test compound, metabolites, particulate microbial biomass and dissolved cell constituents, measured in the liquid phase after stripping off $^{14}\text{CO}_2$	becquerels(Bq)
A_{DO}	dissolved organic ^{14}C -activity of the residual test compound, metabolites and dissolved cell constituents, measured in the liquid phase after stripping off $^{14}\text{CO}_2$ and separation of particles by membrane filtration or centrifugation	becquerels(Bq)
A_{PO}	particulate organic ^{14}C -activity of the sorbed ^{14}C of the test compound and particulate ^{14}C -biomass measured in the particulate residue after filtration or centrifugation	becquerels(Bq)
$a^{2)}$	specific activity of the test compound or of a mixture of radiolabelled and "cold" test compound	becquerels per microgram (Bq/ μg)
$c^{3)}$	residual molar concentration of the test compound	micromoles per litre ($\mu\text{mol/l}$)
c_0	initial molar concentration of the test compound	micromoles per litre ($\mu\text{mol/l}$)

1) A is the symbol for activity, expressed in bequerels, as specified in ISO 31-9-33:1992.

2) In accordance with ISO 31-9-34:1992, a is defined as the symbol for specific activity, expressed in bequerels per kilogram. It may be common practice sometimes to use the symbol σ for specific activity, but this is not in accordance with ISO 31-10-3:1992 where " σ " is defined as the cross-section for a specified target entity and for a specified reaction or process produced by incident charged or uncharged particles of specified type and energy.

3) In ISO 31-8-13:1992, c is defined as the symbol for "molar concentration", expressed in moles per litre and in ISO 31-8-11.2:1992, ρ is defined as the symbol for "mass concentration", expressed in kilograms per litre. Note that in ISO 31, "concentration" of the test compound in solution is expressed in two ways:

— " ρ " refers to the mass of the test compound per unit volume;

— " c " is specifically used to mean the number of moles of the test compound per unit volume.

$c_A^{4)}$	residual activity concentration	becquerels per millilitre (Bq/ml)
c_{A0}	initially added activity concentration	becquerels per millilitre (Bq/ml)
$c_{A_{\text{plateau}}}$	activity concentration at the plateau of the transition between the degradation curve and the subsequent "tailing"	becquerels per millilitre (Bq/ml)
F_T	flasks containing the test compound examined	
F_B	flasks containing the blank sample	
F_C	flasks for check the test performance with a reference compound	
F_S	sterile flask for checking possible abiotic degradation or other non-biological removal	
k	biodegradation rate constant	inverse days (d^{-1})
k^*	pseudo first-order rate constant for disappearance of activity	inverse days (d^{-1})
$k_{\text{non-adapted}}$	first-order rate constants and associated half-lives derived from portions of the curve showing no significant growth	inverse days (d^{-1})
k_{adapted}	pseudo first-order rate constants representing adapted environments	inverse days (d^{-1})
t	time	days (d)
t_{lag}	lag phase	days (d)
$T_{1/2}$	degradation half-life	days (d)
V	reaction volume in the reactor	litres (l)
α	fraction of ^{14}C converted to $^{14}\text{CO}_2$	
$\rho^{3)}$	residual mass concentration of the test compound	micrograms per litre ($\mu\text{g/l}$)
ρ_0	initial mass concentration of the test compound	micrograms per litre ($\mu\text{g/l}$)

4 Principle

The test is carried out by batch-wise incubation of the test compound with a sample of either surface water or surface water and sediment. When surface water alone is used, the test is referred to as a "pelagic test" and when sediment is added to obtain a suspension, the test is referred to as a "suspended sediment test". Incubation takes place at an environmental temperature under agitation by means of a system of flasks on a mechanical shaker.

Test compounds, present in lower concentrations than the natural carbon substrates also present in the system, will serve as secondary substrates. Biodegrading microorganisms obtain the major part of their energy and carbon from primary substrates and not from secondary substrates. Under these conditions, the kinetics for biodegradation would be expected to be first order ("non-growth kinetics"). First-order kinetics implies that the specific rate of degradation is constant and independent of the concentration of the test compound.

4) c_A is the symbol for volumetric activity, expressed in bequerels per cubic metre, as specified in ISO 31-9-35. a is sometimes used for volumetric activity, but is not in accordance with ISO 31.

The test compound is added at two different concentration levels. The concentrations are chosen to be within the microgram-per-litre range (preferably < 100 µg/l) so as to obtain first-order degradation kinetics and an estimated half-life independent of the test concentration. Concentrations should normally be chosen to be as low as practically possible with respect to the sensitivity of the available measurement techniques. These concentrations need not be as low as those expected in the environment to ensure the same type of degradation kinetics.

The test mixture is transferred to closed flasks with an air headspace. Flasks are incubated in the dark or in diffuse light at either the field temperature or at a temperature of 20 °C to 25 °C as commonly used in biodegradation tests. Agitation by means of continuous shaking or stirring is provided to maintain particles, including microorganisms, in free suspension.

The time-course of degradation is followed by the determination of the residual concentration of test compound at appropriate intervals. The incubation time should be sufficiently long to be able to evaluate the degradation behaviour. If the degradation is found to be significant, the extent of degradation should be sufficiently high (normally greater than 15 % to 20 % degradation) to be able to estimate the first-order rate constant.

Measurement of the degradation of the test compound is carried out either by a radiotracer technique, normally using ¹⁴C-labelling and liquid scintillation counting, or by specific chemical analysis, if a sufficiently sensitive analytical method is available. Using the ¹⁴C technique and labelling the most persistent part of the molecule with ¹⁴C, total mineralization or ultimate biodegradation can be assessed, while only primary biodegradation can be measured with specific analysis.

5 Reagents and media

5.1 Reagents

Use only reagents of recognized analytical grade and radiolabelled compounds of high radiochemical purity.

5.1.1 Deionized water, for preparing stock solutions of the test compound and the reference substance.

This water shall have a DOC content of no more than 1 mg/l DOC (use e.g. ISO 8245 for determination) and be free from inhibitory concentrations of toxic substances.

5.1.2 Volatile organic solvent (optional), for dissolving test compounds of low solubility.

5.1.3 Mercury(II) chloride (HgCl₂) (optional), added to a mass concentration of 100 mg/l in the sample of test water containing the test or reference compound and used for stopping all biological activity.

5.1.4 Sodium azide (NaN₃), (optional), added to a mass concentration of 10 g/l in the sample of test water containing the test or reference compound and used for stopping all biological activity.

5.2 Media

5.2.1 Surface water, for use in the "pelagic test".

Collect a sample of suitable surface water in a thoroughly cleansed container. Remove coarse particles, for example, by filtration through a nylon filter of about 100 µm mesh size, a coarse paper filter, or by sedimentation. Keep the sample of surface water in an aerobic environment (e.g. by keeping sufficient headspace in the flask) during the transport and until the start of the test in the laboratory. Start the test preferably within 1 d after collection. During transportation and storage, the temperature of the sample of surface water should not be permitted to exceed significantly the temperature to be used in the test. Cool to 4 °C if transportation times exceed a few hours. Ensure this water does not freeze.

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Identify the sampling location precisely and describe it in terms of its pollution and nutrient status. Provide the following minimum information for the surface water taken for the test:

- a) date and time of collection and delay between collection and use in the laboratory test;
- b) depth of collection;
- c) appearance of sample (e.g. turbidity);
- d) temperature and pH at the place and time of collection;
- e) in the case of sea water and brackish water, salinity or conductivity;
- f) in the case of a turbid sample, the amount of suspended solids;
- g) number of colony-forming microorganisms determined on a suitable growth medium according to standard methods;

and optionally, in addition:

- h) DOC and TOC concentration;
- i) inorganic nutrients such as total phosphorus, dissolved orthophosphate, total nitrogen, nitrate, nitrite or ammonium nitrogen;
- j) chlorophyll-a concentration;
- k) total microbial number using staining (e.g. by acridine orange) and epifluorescence microscopy after ultrasonic treatment or dispersion by other means;
- l) other characteristics relating to the microbial biomass and activity such as ATP (adenosine triphosphate), protein, heterotrophic carbon assimilation activity, and determination of the number of organisms capable of degrading the test compound (e.g. determined using a most probable number method).

5.2.2 Surface water and sediment, for use in the "suspended sediment test".

Collect a sample of surface water as described in 5.2.1. In addition, collect a grab sample of aerobic surface sediment using an appropriate sampling method. Sample, for example, a number of sediment cores using a tube of transparent plastic and slice off the upper aerobic layer immediately after sampling. Transport the sample in a container with sufficient air headspace to keep the sediment aerobic, and aerate the sample of surface water following arrival at the laboratory until use. First determine the level of suspended solids in the sediment sample, then choose a mass concentration level between 100 mg/l and 1 000 mg/l and adjust the level of suspended solids of the sample to this predetermined level.

Identify the sampling location precisely and describe it in terms of its pollution and nutrient status. Provide the same information for the sample of water medium as described for the pelagic test (5.2.1) and provide the following information for the sediment:

- a) date and time of collection and delay between collection and use in the laboratory test;
- b) depth of collection;
- c) appearance of the sample, such as coloured, muddy, silty, or sandy;
- d) dry mass in grams per litre of the suspended solids;
- e) TOC concentration or loss of ignition as a measure of the content of organic matter;

and optionally:

- f) sediment particulate characteristics (fractions of silt and sand);
- g) inorganic nutrients such as total phosphorus, dissolved orthophosphate, total nitrogen, nitrate, nitrite, or ammonium nitrogen in the pore water;
- h) microbial colony count on an appropriate growth medium as obtained after gentle treatment with ultrasound;
- i) total microbial number using staining (e.g. by acridine orange) and epifluorescence microscopy after treatment with ultrasonic or dispersion by other means;
- j) any other characteristic relating to the microbial biomass and activity.

A preliminary dry mass determination can conveniently be carried out by drying a portion of this sediment in a microwave oven, e.g. for 15 min. The estimate so obtained can be used for the purpose of calculating the amount of sediment to be added to the surface water in order to obtain a suspension with the desired content of solids dry mass. The accurate solid content is calculated subsequently using a more precise dry mass determination, e.g. obtained after drying overnight at 105 °C in a conventional oven.

6 Apparatus

Ordinary laboratory equipment and the following.

6.1 Conical or cylindrical flasks, of appropriate capacity, for example 0,5 l or 1 l, with silicone or rubber stoppers, or serum flasks with CO₂-tight lids (e.g. with butyl rubber septa).

For non-volatile test compounds that are not radiolabelled, gas-tight stoppers or lids are not required. Thoroughly clean test flasks and stoppers before use and ensure they contain no traces of detergents. To be sure that no bacterial contamination occurs, the flasks may be sterilized by heating or autoclaving.

6.2 Shaking table or magnetic stirrers, for continuous agitation of the test flasks.

6.3 Centrifuge, capable of rotating at a rate of 40 000 m/s².

6.4 pH meter.

6.5 Oven or microwave oven, for dry-mass determinations.

6.6 Membrane filtration apparatus.

6.7 Autoclave or oven (optional), for heat-sterilization of glassware and for stopping all biological activity in the aqueous media.

6.8 Radiotracer facilities and equipment, for handling and measuring ¹⁴C-labelled compounds if used, (see also annex A).

6.9 Analytical equipment, for the determination of organic test compounds if specific chemical analysis is used (e.g. gas chromatograph, high pressure liquid chromatograph).

7 Test environment and conditions

7.1 Semi-continuous incubation (optional)

Very long incubation times (several months) may be necessary if long lag times occur before significant degradation can be measured. In such cases, the microbial community of the sample of surface water may

deteriorate and the similarity of the test system to natural environmental conditions decreases. Such deterioration may affect the degradation of the test compound causing it to cease or to slow down. A likely explanation is the disappearance of slowly growing competent microorganisms from the test system. These may die out due to various loss processes, i.e. before degradation effectively starts or at a later stage if the pressure of selection exerted by the test compound, present at a low concentration, is insufficient. The problem of sample deterioration is greatest for pelagic tests. It can be remedied, however, by including a pre-adaptation period with semi-continuous operation prior to the batch test. The basic principle of the semi-continuous pre-adaptation is to renew part of the surface water periodically and make up with additional test compound to approximately the starting concentration. It has been found adequate to replace, for example, one third of the volume every two weeks. The replacement surface water should be freshly collected from the same site as the original sample. As the procedure comprises both re-inoculation and compensation of any depleted primary substrates, the initial microbial diversity and substrate availability are practically restored, and the feasible duration of the batch test thereby extended from some weeks to much longer time periods. Once adaptation has taken place, the semi-continuous operation is simply discontinued and the test system becomes a batch system with time zero being the time of the last renewal. The degradation rate constant can be interpreted as a characteristic of an adapted system and is similar to rate constants obtained in a batch system after a long lag phase.

7.2 Nutrient and sludge addition (optional)

It should be emphasized that the biodegradation potential of surface water may vary considerably in time and space. For some purposes (e.g. for relative ranking of different test compounds), it may therefore be useful to generate a rate constant representing standard conditions. In such a case, it is recommended to carry out an optional test with nutrient-rich or nutrient-enriched surface water or with membrane-filtered secondary effluent of a waste water treatment plant as the substrate amended with 3 mg/l (dry mass) of activated sludge as inoculum. The sludge should be sampled from a treatment plant predominantly receiving domestic sewage and having no significant inputs of the test compound.

7.3 Temperature and lighting

Incubation shall take place in the dark or in diffuse light at a controlled (± 2 °C) temperature which is either the field temperature or a standard temperature of 20 °C to 25 °C. Field temperatures can represent either the actual temperature of the sample as collected or represent an average field temperature at the sampling site.

8 Procedure

8.1 Preparation of test and reference compounds

8.1.1 Water-soluble test compounds

Prepare a stock solution in deionized water (5.1.1) and transfer the necessary volume of the stock solution to the test flasks (6.1) in order to achieve the desired test concentration. If ¹⁴C-labelled compounds are used, see annex A for details.

8.1.2 Poorly water-soluble test compounds

Dissolve test compounds of low volatility first in a minimum amount of volatile organic solvent. Add a small volume of this solution to the test flasks. Strip off enough of the organic solvent so that it does not significantly increase the DOC concentration of the sample of surface water. Check this by a substance-specific analysis or, if possible, by DOC analysis (see e.g. ISO 8245). In certain cases, it may be feasible to prepare an intermediate solution first in deionized water (5.1.1) or in surface water (5.2.1).

Also other techniques to introduce the test compound into the test flasks may be used as described in ISO 10634.

8.1.3 Reference compound (optional)

When inexperienced with either the test itself, the sample of surface water (5.2.1) used and/or the sediment (5.2.2), it is recommendable to use a reference compound, preferably aniline in the same range of concentration as the test compound.

8.2 Test set-up

8.2.1 Pelagic test

Set up the following flasks:

- at least two test flasks (symbolized F_T) for each of the different concentrations (at least two) of the test compound examined (see 8.1.1 or 8.1.2);
- at least one flask (symbolized F_B) for the blank containing a sample of the surface water only;
- optionally, at least two test flasks (symbolized F_C) to check the test performance with a reference compound (see 8.1.3);
- optionally, at least one sterile flask for checking possible abiotic degradation or other non-biological removal (symbolized F_S).

Transfer a suitable volume of surface water (5.2.1) to the flasks and fill the flasks to about one-third of their volume but no less than about 100 ml.

The biological activity is stopped by autoclaving (6.7) the sample of test water or by adding a toxicant, such as mercury(II) chloride (see 5.1.3) or sodium azide (see 5.1.4).

8.2.2 Suspended sediment test

Set up a sufficient number of flasks in the same manner as described for a pelagic test (8.2.1). Use closed serum bottles or similarly shaped flasks. Adjust the level of the suspended solids of the surface water sediment suspension to the predetermined level (see 5.2.2) between 100 mg/l and 1 000 mg/l. Fill the flasks with this suspension to about one-third of their volume.

With sediment added in high concentrations, sterile conditions are not obtained easily. In this case, repeated autoclaving (e.g. three times) is recommended. Note that information on abiotic degradation behaviour will normally be available before conducting the test and that the kinetics for abiotic degradation normally does not change at low concentrations as does that of biodegradation. Therefore, tests in sterile units normally serve the purpose of assessing sorption phenomena. Note that the sorptive properties of sediments will change by autoclaving. The sorption data obtained can therefore only be used qualitatively, for example to ascertain whether sorption is significant or not. Initial sorption may be studied quantitatively by using non-sterile sediment. If long-term studies on sorption are required, sterilization should be performed, for instance by radiation or by chemical means.

8.3 Incubation

Close the test flasks prepared in 8.2 either with stoppers or lids which are impermeable to air and especially CO_2 . In the case of a non-volatile test compound when a specific analysis is used and no radiolabelled compounds are added, a loose, cotton-wool plug may be used that prevents contamination from the atmosphere. Place the test flasks on a shaking table, or supply them with magnetic bars and place them on magnetic stirrers. Maintain a homogeneous suspension and aerobic conditions over the test period by continuous and sufficient agitation. Operate the shaking tables at about 100 r/min.

For tests with sediment, preferably place the closed test flasks horizontally on the shaker. However, this position is not feasible if sorption to the lid is significant or loose cotton-wool plugs are used. If magnetic stirring is applied to sediments, note that ordinary magnetic bars are not durable. In this case, use bars coated with glass tubing. Make sure that the stirring is vigorous enough to maintain aerobic conditions.

Place the test flasks in an environment with the selected incubation temperature (see 7.3). Withdraw samples for analysis from each of the test flasks at the beginning of the test at day 0, i.e. before degradation begins, and at suitable intervals in the course of the test. Usually, at least five sampling points in time are required to evaluate the degradation behaviour and ideally the degradation phase should be represented by at least three data points in order to estimate a reliable rate constant. No fixed time schedule for sampling can be stated, as the rate of biodegradation varies. However, in general, it is recommended to sample once a week for test compounds undergoing slow degradation and to sample once a day during the first three days and then every second or third day for readily degradable test compounds. If test samples are preserved for a specific analysis at a later time, it is advisable to take more test samples than the required minimum of five. In this case, analyse the test samples in the inverse order that they were taken, i.e. from those taken at the end of the experiment, analysed first, to those taken at beginning of the experiment, analysed last.

Keep all stored test samples cooled at 2 °C to 4 °C and keep them air-tight if analysis can be carried out within 1 d to 2 d. For longer storage, deep freezing below -18 °C or chemical preservation is necessary (see 8.4 and annex A). If it is known that the test compound will remain unaffected by acidification, acidify the test sample to pH 2 before storing. If the analytical method involves solvent extraction, perform the extraction immediately after sampling or after storing the sample refrigerated for 1 d and store the extracts only.

8.4 Determination of the remaining test compound

8.4.1 Radiochemical measurements

The amount of CO₂ evolved is determined indirectly by measuring the difference between the initial ¹⁴C-activity in the sample of surface water or the suspension containing the test compound and the total residual activity at the sampling time as measured after acidifying the sample and stripping off CO₂. Inorganic carbon is thus removed and the residual activity measured is derived from non-degraded or partially degraded test compound. For details see annex A.

With high degrees of mineralization of the test compound to CO₂, measured residual activities can be assumed to be approximately proportional to residual test compound concentrations. If the mineralization is less complete a considerable error can result if such proportionality is assumed. Therefore, if sufficient measurements have been made to estimate the residual organic activity after complete degradation of the test compound, it is necessary to make a correction to account for the fact that some carbon is not released as CO₂ but incorporated into the biomass or released as extra-cellular metabolites. For a simple correction procedure, see 9.1 and annex A.

It is recommended to make additional measurements, for example carried out at the end of the test after filtration, of particulate ¹⁴C to provide an estimate of the incorporation of carbon into the microbial biomass. Furthermore, the evolution of ¹⁴CO₂ should be measured directly in one or more test flasks in order to check the mass balance and to provide direct evidence of biodegradation as a further procedural check.

8.4.2 Specific chemical analysis

If a sensitive specific chemical analytical method is available, the primary biodegradation of the test compound may be followed by measuring the total residual test compound concentration in samples of surface water or suspensions with sediment using this method. If the test is carried out with radiolabelled test compounds, parallel specific chemical analytical measurements of primary biodegradation may provide useful additional information and serve as a further check of the procedure. For example, extract the samples for analysis with an organic solvent following the directions given in the respective analytical procedure.

Depending on the sensitivity of the analytical method, use larger test volumes than those suggested in 6.1 and 8.2.1. The test can easily be carried out with test volumes of 1 l in flasks of 2 l to 3 l capacity, allowing a sample size of for example 100 ml for analyses.

9 Calculation

9.1 Radiochemical measurements

When a ^{14}C -labelled organic compound is biodegraded, part of the ^{14}C is converted to $^{14}\text{CO}_2$, while another part is used for synthesis of new microbial biomass and/or extra-cellular metabolites. A detailed interpretation of radiochemical measurements can therefore be rather complicated. As time passes, some of the ^{14}C built into the biomass will be released again as $^{14}\text{CO}_2$ and as extra-cellular metabolites or cell fragments, which in turn are mineralized to $^{14}\text{CO}_2$ or are reused for biosynthesis. For these reasons, simple plots of residual organic ^{14}C activity (measured after stripping off CO_2) versus time will show "tailing" after degradation has been completed and the last part of the curve will be difficult to interpret. Therefore, only the initial part of the curve (less than about 50 % degradation) is used for direct estimation of a degradation rate constant.

The following simple procedure is suggested for converting activities to approximate concentrations. The procedure is briefly outlined below and is described in more detail in annex A. It is assumed that a constant fraction, α , of ^{14}C is fully mineralized to $^{14}\text{CO}_2$ during the course of degradation. The residual mass concentration, expressed in micrograms per litre ($\mu\text{g/l}$), ρ [or in molar concentration, c , expressed in micromoles per litre ($\mu\text{mol/l}$)], of the test compound can then be calculated from the measured residual activity using equation (1):

$$\begin{aligned}\rho &= \frac{1}{\alpha \cdot a} [c_A - c_{A0}(1 - \alpha)] \\ &= \frac{1}{\alpha} \left[\frac{c_A - c_{A0}(1 - \alpha)}{c_{A0}} \right] \rho_0\end{aligned}\quad (1)$$

where

- ρ_0 is the initial concentration, expressed in micrograms per litre ($\mu\text{g/l}$), of the test compound;
- a is the specific activity, expressed in becquerels per microgram ($\text{Bq}/\mu\text{g}$), of the test compound or of a mixture of radiolabelled and "cold" test compound;
- α is the fraction of ^{14}C converted to $^{14}\text{CO}_2$ (assumed to be constant);
- c_A is the residual organic activity concentration, expressed in becquerels per millilitre (Bq/ml);
- c_{A0} is the initially added activity concentration, expressed in becquerels per millilitre (Bq/ml).

An approximate estimate of α can be obtained from the activity plateau at the transition between the degradation curve and the subsequent "tailing" which reflects secondary mineralization of cell products. Assuming no residual test compound left, α is simply:

$$\alpha \approx \frac{c_{A0} - c_{A\text{plateau}}}{c_{A0}}$$

Note that with first-order kinetics, the biodegradation rate constant, k , equals $1/\alpha$ times the initial pseudo first-order rate constant for disappearance of activity, k^* as follows:

$$k = k^*/\alpha$$

If found to be independent of the added concentration, k can be interpreted as a first-order rate constant characteristic of the test compound, the environmental sample or system, and of the test temperature. The extent to which the results can be generalized or extrapolated to other systems shall be evaluated using expert judgement.

If α cannot be estimated or measured, state this and report k as the initial pseudo first-order rate constant for disappearance of activity.

9.2 Interpretation of specific chemical analytical measurements

Only primary biodegradation can be determined by a specific analysis and the proof of ultimate biodegradation can only be obtained by other methods (see ISO/TR 15462).

9.3 Evaluation of biodegradation curves

Round sampling times to whole hours but not to whole days. Plot the estimates of the residual concentration of test compound, and reference substance if included, against time, using both a linear and a semi-logarithmic plot. Treat the data from each individual flask separately. If degradation has taken place and sterile controls have been included, compare the results with those from the flasks F_S . Compare them also with the results from the non-sterile experiments in test flasks F_T and examine also the degradation curves obtained with the reference substance in test flasks F_C . If the results from the test flasks F_S and F_T appear similar, it can be assumed that the degradation observed is predominantly abiotic. If the degradation in test flasks F_S is low, the figures may be used to correct the figures obtained with test flasks F_T by subtraction to estimate the extent of biodegradation.

9.4 Test result descriptors

9.4.1 Identification of regimes for quantification of rate constants

9.4.1.1 Conditions of the biological matrix (adapted or non-adapted)

Identify, if possible, two different regimes, non-adapted and adapted and name the rate constants accordingly.

$k_{\text{non-adapted}}$ These are first-order rate constants and associated half-lives derived from portions of the curve showing no significant growth. Such constants are taken as representative of a non-adapted environment.

k_{adapted} These are pseudo first-order rate constants representing adapted environments and as far as possible estimated as initial rates after onset of a second phase degradation.

Note that many test results will not allow such a split of the degradation curve and the results are to be assigned to one of the categories adapted or non-adapted. Adaptation may or may not take place during the course of the test or may have occurred in advance as a result of exposure of the laboratory system to full-scale field exposure.

9.4.1.2 Concentration level of test compound

A distinction is necessary between

- the low concentration range with true order (non-growth) kinetics, and
- higher or medium concentration where growth takes place and the rate constant therefore increases with time.

9.4.2 Lag phase and degradation rate constant

Estimate the lag phase duration, t_{lag} , from the degradation curve (logarithmic plot) by extrapolating its linear part to zero degradation or alternatively by reading the time for approximate 10 % degradation. For positive degradation results, estimate a degradation rate constant, k , assuming first-order kinetics. Plot the data using a semi-logarithmic plot and estimate k and its associated standard error by linear regression of $\ln \rho$ (residual test compound mass concentration) versus time. Use as far as possible only data belonging to the first linear portion of the curve. Calculate the half-life $T_{1/2} = \ln 2/k$. If too few data points (less than 3) fall on the linear portion of the curve, estimate $T_{1/2}$ using the best judgement from an eye-fitted curve, and calculate k as $\ln 2/T_{1/2}$. Alternatively, estimate k directly as the slope of a line connecting two adjacent data points which are judged to be representative. Make separate calculations for each test flask and calculate averages from replicate flasks only if results are similar. Otherwise report the range of results.

If specific analysis is used record also the maximum level of primary biodegradation at the end of the test.

With ^{14}C measurements, only the extent of mineralization to CO_2 can be estimated. As the proportion of carbon in a test compound converted to CO_2 varies, depending largely on the concentration of test compound and other substrates available, the test conditions and the aquatic microbial community, the method does not allow the determination of ultimate biodegradation. In this case, use a method described in ISO/TR 15462.

10 Validity of the test

If the, optionally tested, reference substance is not sufficiently degraded within the expected time interval, the test is suspect and its validity shall be further verified, or alternatively the test shall be repeated using a new sample of water medium. In an interlaboratory test for aniline degradation, rate constants ranged from $0,3 \text{ d}^{-1}$ to $1,7 \text{ d}^{-1}$.

11 Test report

The test report shall contain at least the following information:

- a) a reference to this part of ISO 14592, i.e. ISO 14592-1;
- b) a description of the test system (pelagic or suspended sediment test);
- c) all necessary information for the identification, characterization (e.g. type of labelling) and use of the test and of the reference compound;
- d) all relevant information pertaining to the surface water and sediments (5.2) in accordance with clause 7, such as sampling time and location, transport, storage, and pre-treatment;
- e) the test conditions, including the incubation temperature and the test duration;
- f) all information on the analytical techniques and the methods used for radiochemical measurements and for a mass balance check and measurements of phase distribution;
- g) all measured data and calculated values in tabular form and the degradation curves as follows:
 - 1) for each test concentration and for each replicate flask, the estimated lag phase and a first-order or pseudo first-order rate constant and corresponding degradation half-life for the subsequent degradation;
 - 2) the system categorized as either non-adapted or adapted as judged from the appearance of the degradation curve and from the magnitude of the rate constant (if possible, using different curve segments, estimate a rate constant for both adapted and non-adapted conditions);
 - 3) the influence of the test concentration on the rate constants;
 - 4) the results of any phase distribution measurements, and if ^{14}C technique is used, also the results of the final mass balance check and the fraction of ^{14}C mineralized and, if specific analysis is used in addition, the final maximum level of primary biodegradation;
- h) any alteration of the standard procedure and any observations and circumstances that may have affected the results and the reasons in the event of rejection of the test.

Annex A (informative)

Guidance on the use of ^{14}C -labelled compounds

WARNING AND SAFETY PRECAUTIONS — The use of ^{14}C -labelled and other radiolabelled isotopes requires special authorization and their handling must comply with national safety regulations for such compounds.

A.1 General considerations

This annex provides guidance on the use of ^{14}C radiotracer technique to assess biodegradation of organic test compounds in water at low concentrations. It is limited to the description of the use of ^{14}C as the radiotracer because this is the radioisotope most commonly used. For general information on uses of radioactive isotopes reference is made to the appropriate technical and scientific literature on the subject. ^{14}C is a soft β -emitter with a half-life of 5 730 a. The maximum energy of the β particles is 0,156 MeV. Most commonly the radiation is quantified by means of liquid scintillation counting which is the method referred to in this part of ISO 14592.

A.2 Materials and equipment

A.2.1 ^{14}C -radiolabelled test compounds

While suppliers of radioisotopes stock rather few ^{14}C -labelled organic chemicals, the compound of interest can usually be synthesized in the labelled form from ^{14}C -labelled lower molecular mass precursors. Synthesis services are offered commercially.

It is necessary for a ^{14}C -labelled test compound to meet certain quality criteria for use in a biodegradation test. Biodegradation is quantified as released $^{14}\text{CO}_2$ and it is not possible to distinguish between $^{14}\text{CO}_2$ originating from degradation of test compound and from radioactive impurities. Therefore, it is necessary for the radiochemical purity to be relatively high and as a rough guidance at least 95 % is required depending on the extent of biodegradation. It is necessary to specify which carbon atom(s) in the molecule bear the ^{14}C -label. Generally uniformly ^{14}C -labelled compounds can be used where all carbon atoms are of ^{14}C , but also partially labelled compounds are possible. In such a case, the test compound should be labelled ideally in a position ensuring that the liberation of $^{14}\text{CO}_2$ unequivocally indicates either mineralization or a specific partial degradation. Normally, it is preferable to have the label associated only with a molecular substructure that is metabolized late in the sequence of degradation steps so that liberation of $^{14}\text{CO}_2$ signifies mineralization, for instance uniformly ring-labelled aromatic compounds. If the objective is to ascertain primary or functional biodegradation on the other hand, the label should be positioned only at a site (for instance in a side chain on an aromatic ring) where the breakdown of the molecule starts or which is associated with the functional unit of the molecule.

A.2.2 Radiolabelled reference substances

^{14}C -labelled preparations of several readily degradable chemicals, such as aniline and sodium benzoate, are commercially available and can be used as reference substances for checking the biological reagent and the test procedure.

A.2.3 Unlabelled test compounds

Radiolabelled test compounds may be used directly but are frequently mixed with unlabelled, referred to as "cold", compounds to avoid excessive count numbers and to limit the cost of the ^{14}C preparation. A suitable activity for counting is often in the range of 80 Bq to 170 Bq per sample for analysis. An activity of 15 Bq/ml to 30 Bq/ml in the test flask results in initially 75 Bq to 150 Bq with a sample size of 5 ml.

A.2.4 Necessary facilities and equipment

The following facilities and equipment are necessary for handling ^{14}C -radiolabelled test compounds:

- a designated area (e.g. ventilated hood) for handling of ^{14}C -radiolabelled test compounds;
- a set-up for stripping $^{14}\text{CO}_2$ from test samples;
- a liquid scintillation counter.

A.3 Method of radiochemical measurements

A.3.1 General principles of measurements

There are several feasible ways of performing radiochemical measurements, and the methods described here are for guidance only.

Approximate degradation rates can be calculated from measured residual organic ^{14}C in the liquid phase and the $^{14}\text{CO}_2$ produced. More detailed evaluations of results can be rather complex, on the other hand, due to a variable fate of the test compound carbon. Only a part of the ^{14}C -labelled test compound is mineralized to $^{14}\text{CO}_2$, while another part is used in the synthesis of new microbial biomass and transformed into a particulate phase (cellular material) and a further part is transformed into dissolved extra-cellular metabolites or released as colloidal material. Both pools of organic ^{14}C (dissolved and particulate) will in time be subjected to secondary mineralization to $^{14}\text{CO}_2$, which results in a "tailing" of the disappearance curve of the ^{14}C of the test compound.

It is generally possible to separate and measure the ^{14}C -activity (A) of an added water-soluble test compound, which is not volatile as follows:

- inorganic ^{14}C -activity (A_I), i.e. the $^{14}\text{CO}_2$ evolved as a result of biodegradation, measured directly after trapping $^{14}\text{CO}_2$ in an absorber or indirectly as a difference, after CO_2 has been stripped off;
- total organic ^{14}C -activity (A_{TO}), i.e. residual test compound, metabolites, particulate microbial biomass and dissolved cell constituents, measured in the liquid phase after stripping off $^{14}\text{CO}_2$;
- dissolved organic ^{14}C -activity (A_{DO}), i.e. residual test compound, metabolites and dissolved cell constituents, measured in the liquid phase after stripping off $^{14}\text{CO}_2$ and separation of particles by membrane filtration or centrifugation and can be calculated by the mass balance as follows:

$$A_{\text{DO}} = A_{\text{TO}} - A_{\text{PO}}$$

- particulate organic ^{14}C -activity (A_{PO}), i.e. sorbed ^{14}C of the test compound and particulate ^{14}C -biomass measured in the particulate residue after filtration or centrifugation.

The following basic measurements are possible.

a) Simple $^{14}\text{CO}_2$ determination by difference:

Determination of the amount of evolved $^{14}\text{CO}_2$ as a simple difference between the measured ^{14}C activity of the initially added labelled test compound and the A_{TO} (used to construct the degradation curve), the total organic ^{14}C activity of the residue, measured after acidifying the sample and stripping off CO_2 .

b) Direct $^{14}\text{CO}_2$ measurement, phase distribution and mass balance:

In order to check the procedure or for more detailed information, the routine measurements of ^{14}C in the residue (A_{TO}) should be supplemented by mass balance measurements involving a direct determination of the evolved $^{14}\text{CO}_2$ after trapping it in an absorber. In itself, a positive $^{14}\text{CO}_2$ formation is direct evidence of

biodegradation as opposed to abiotic degradation or other loss mechanisms, such as volatilization and sorption. Additional useful information characterizing the biodegradability behaviour can be obtained from measurements of the distribution of total activity (A_{TO}) between the dissolved state (A_{DO}) and the particulate state (A_{PO}) after separation of particulate by membrane filtration or centrifugation. A_{PO} consists of test compounds sorbed onto the microbial biomass and onto other particulate in addition to the test compound carbon that has been used for synthesis of new cellular material and thereby incorporated into the particulate biomass fraction. The formation of dissolved ^{14}C organic material can be estimated as the A_{DO} at the end of biodegradation in the plateau phase of the degradation curve.

There are several methods available for trapping evolved $^{14}CO_2$ in absorber systems. Gaseous CO_2 is rapidly sorbed in alkali or other sorbents because of enhancement by chemical reaction. It is important to remember that the mass transfer of CO_2 from water to air is slow, and that equilibrium between the two phases is not readily attained at neutral or basic pH. Acidification is therefore normally necessary to drive $^{14}CO_2$ out of the water.

A.3.2 Recommended specific procedures

A.3.2.1 Test samples with low particle content

For routine measurements, simply transfer unfiltered samples, for example 5 ml size to scintillation vials. A suitable activity in samples is between 80 Bq to 170 Bq initially, and a minimum initial activity is about 20 Bq. Strip off the CO_2 after acidifying the test sample between pH 2 and pH 3 with 1 drop to 2 drops of concentrated hydrochloric acid or another suitable acid. The CO_2 stripping can be performed by bubbling the test sample with air for about 0,5 h to 1 h. Alternatively, vials can be shaken vigorously for 1 h to 2 h (for instance on a microplate shaker) or with gentler shaking can be left overnight. The efficiency of the CO_2 -stripping procedure should be checked (by prolonging the aeration or shaking period). Add a scintillation liquid, suitable for counting aqueous test samples, homogenize on a whirling mixer and determine the radioactivity by liquid scintillation counting, subtracting the background activity found in the test blanks.

Unless the test sample is very coloured or contains a high concentration of particles, the test samples will normally show uniform quenching and it will be sufficient to perform quench corrections using an external standard. If the test sample is highly coloured, quench correction by means of internal standard addition may be necessary. If the concentration of particles is high, it may not be possible to obtain a homogeneous solution or gel or the quench variation between test samples may be large. In this case, the counting method described below for test slurries can be used.

Estimate the phase distribution of residual ^{14}C in selected samples by filtering samples on a 0,22 μm or 0,45 μm membrane filter of a material that does not adsorb significant amounts of the test compound (polycarbonate filters may be suitable). If sorption of the test compound onto the filter is too high to be ignored (to be checked prior to the experiment), high-speed centrifugation can be used instead of filtration.

Proceed with the filtration or centrifugation as described of unfiltered test samples. Dissolve membrane filters in a suitable scintillation fluid and count them, normally using only the external standard ratio method to correct for quenching. If centrifugation has been used, re-suspend the pellet formed of the particulate fraction in 1 ml to 2 ml of distilled water and transfer to a scintillation vial. Subsequently, wash the fraction twice with 1 ml of distilled water and transfer the washing water to the vial. If necessary, the suspension can be embedded in a gel for liquid scintillation counting (see below).

It is recommended to perform a mass balance check (see A.3.3).

A.3.2.2 Sediments

Take a homogeneous 10 ml sample of sediment or test suspension and separate the phases by centrifugation at a suitable rotation rate (e.g. at 40 000 m/s^2 for 15 min). Proceed with the aqueous phase as above. Also determine the activity in the particulate phase by re-suspending it into a small volume of distilled water, transferring it to scintillation vials, and adding scintillation liquid to form a gel (special scintillation liquids are available for that purpose). Depending on the nature of the particles (e.g. their content of organic material), it may be feasible to digest the test sample overnight with a tissue solubilizer and then homogenize on a whirling mixer prior to the addition of scintillation liquid. When counting, internal standards should always be included, and it may be necessary to perform quench corrections using an internal standard addition for each individual sample.

A.3.3 Mass balance check

An isotope balance should be conducted at the end of a test using a separate set of flasks from which no samples are taken in the course of the test. Use at least one set of duplicate flasks for this purpose. The flasks should be closed with lids permeable to syringes. For example, serum bottles with butyl rubber septa are suitable.

At the end of the test, acidify the test sample between pH 2 to pH 3 by adding concentrated hydrochloric acid from a syringe and then trap the entire content of CO₂ in an absorbing medium, i.e. using either alkali (e.g. 1 mol/l NaOH solution, or a NaOH pellet), ethanolamine, or an ethanolamine-based, commercially available⁵⁾. Determine subsequently the total amounts of ¹⁴C separated as follows:

- trapped as ¹⁴CO₂ (A_1);
- dissolved in the aqueous phase (A_{DO}); and
- contained in the particulate phase (A_{PO}).

The total ¹⁴C recovery should normally be at least 90 %.

Two different CO₂ absorption methods can be applied, i.e. one based on an internal absorber and the other utilizing an external absorber as follows.

a) Internal CO₂ trap:

At the beginning of the test, suspend a trap (e.g. a scintillation vial) from the lid or stopper of the test flask, or use alternatively a so-called Gledhill flask (see Bibliography) with an internal glass tube provided with a hole to allow the exchange of air. The test system is kept closed throughout the entire test. With a serum flask, a scintillation vial may be conveniently attached using a stainless steel wire that penetrates the septum in the lid.

During the test, the trap will absorb CO₂ from the headspace, but most of the ¹⁴CO₂ formed is likely to remain in the aqueous phase because of the slow mass transfer of CO₂ from water to air. In order to trap CO₂ efficiently, the test solution or suspension should be acidified at the end of the test and the flask left agitated overnight before it is opened.

b) External CO₂ trap:

CO₂ absorption can also be accomplished using an external trap. After acidification, the test flasks are purged with nitrogen or CO₂-free humidified air and the exhaust gas is led through a set of absorber bottles. CO₂-free air can be obtained by passing air through a wash bottle with diluted (e.g. 1 mol/l) alkali solution. Serum bottles are conveniently used as test flasks, as both acidification and gas purging can be performed easily by penetrating the septum with syringes. The purging can be accomplished using pressurized gas applied upstream, but it is usually preferable to apply vacuum suction downstream after the absorber bottles. Purge for some hours or bubble a stream of gas overnight through the water or slurry test sample. Use a low flow rate of gas, e.g. 5 ml/min per 100 ml test volume, which represents a compromise ensuring efficient CO₂ absorption and minimizing losses by stripping of volatile substances and aerosol formation.

In order to trap volatile test compounds and test compounds present in aerosols, insert a separate wash bottle (e.g. containing acidified water) before the CO₂ absorber. Use at least two CO₂ absorbers in series in order to increase the efficiency of the trapping system.

5) Carbo-sorb CO₂ absorber and Permafluor E+ scintillation fluid are an examples of suitable products available commercially from Packard. This information is given for the convenience of users of this part of ISO 14592 and does not constitute an endorsement by ISO of these products.

A.4 Interpretation of measurements

A.4.1 General

When a ^{14}C -labelled organic test compound is biodegraded, part of the ^{14}C is converted to $^{14}\text{CO}_2$, while another part is used for synthesis of new microbial biomass and/or extra-cellular metabolites. At low concentrations, under which conditions the test compounds are believed to be degraded as secondary substrates, the fraction of carbon used for biosynthesis of new organic material is usually relatively small. The extent of immediate mineralization, however, varies between test compounds and is influenced by the test conditions.

As time passes in a batch experiment, the organically bound ^{14}C will be mineralized gradually by secondary processes, and also the phase distribution can be affected because the DOA fraction may both be mineralized and at the same time used for synthesis of new cellular material. For these reasons plots of residual ^{14}C activity versus time will show "tailing" and the last part of the curve will be difficult to interpret. Therefore, only the initial part of the degradation curve (less than about 50 % degradation) should normally be used for the estimation of a biodegradation rate constant.

If the extent of mineralization is high it may be a reasonably accurate approximation to assume that the total residual concentration of organic ^{14}C (A_{T0}) is proportional to the residual concentration of the test compound. Hence an approximate first-order degradation rate constant can be calculated simply from a plot of $\ln(c_A/c_{A0})$ where c_A and c_{A0} are the residual activity concentrations at time t and time zero, respectively. Experience gained from an international interlaboratory test has led to the conclusion, however, that it is generally advisable to convert activities to approximate concentrations. A procedure for this is outlined below is given for guidance.

Assuming that the fraction α of ^{14}C mineralized to $^{14}\text{CO}_2$ is constant during the entire course of degradation of the test compound, the residual test compound mass concentration, ρ , can then be calculated from the measured residual activity by combining a material balance and a radioactivity balance as follows:

$$\text{Material balance} \quad A = a \cdot \alpha (\rho_0 - \rho)V \quad (\text{A.1})$$

$$\text{Activity balance} \quad A = (c_{A0} - c_A)V \quad (\text{A.2})$$

where

A is the activity, expressed as becquerels, of the total amount of $^{14}\text{CO}_2$ evolved between time zero and time t ;

a is the specific activity, expressed as becquerels per microgram ($\text{Bq}/\mu\text{g}$) of the test compound;

α is the fraction of ^{14}C , which is converted to $^{14}\text{CO}_2$ as a result of biodegradation;

ρ_0 is the initially added mass concentration, expressed in micrograms per litre ($\mu\text{g}/\text{l}$), of the test compound;

ρ is the mass concentration, expressed in micrograms per litre ($\mu\text{g}/\text{l}$), of residual test compound at time t ;

c_A is the residual activity concentration, expressed as becquerels per litre (Bq/l), of the test compound at time t ;

c_{A0} is the initially added activity concentration, expressed as becquerels per litre (Bq/l), of the parent test compound;

V is the reaction volume, expressed in litres, in the reactor.

Combining Equations (A.1) and (A.2) give the following:

$$\text{Combination} \quad a\alpha(\rho_0 - \rho)V = (c_{A0} - c_A)V \quad (\text{A.3})$$

Rearranged and using initial condition that $c_{A0} = a\rho_0$ gives:

$$\rho = \frac{1}{a\alpha} [c_A - c_{A0}(1-\alpha)] \quad (\text{A.4})$$

An approximate estimate of α can be obtained from the activity plateau at the transition between the degradation curve and the subsequent "tailing" portion of the curve due to secondary mineralization of cell products. Assuming no residual test compound left, α is simply:

$$\alpha \approx \frac{c_{A0} - c_{A\text{plateau}}}{c_{A0}} \quad (\text{A.5})$$

The kinetic parameters can be obtained by a combination of both balances

$$\frac{dc_A}{dt} = \alpha a \frac{d\rho}{dt} \quad (\text{A.6})$$

Assuming first-order kinetics

$$\frac{d\rho}{dt} = k\rho \quad (\text{A.7})$$

where k is the biodegradation rate constant, expressed in inverse days (d^{-1}) and

$$\rho = \rho_0 e^{-kt} \quad (\text{A.8})$$

and using the initial condition

$$c_{A0} = a\rho_0 \quad (\text{A.9})$$

When Equation (A.7) is integrated, using Equations (A.8) and (A.9), equals

$$c_A = c_{A0} (ae^{-kt}) + 1 - a \quad (\text{A.10})$$

and gives when Equation (A.10) is rearranged for time zero

$$\frac{d \ln c_A}{dt} = -ak \quad (\text{A.11})$$

The biodegradation rate constant, k , is therefore equal to $1/\alpha$ times the initial pseudo first-order rate constant for disappearance of activity, k^* :

$$k = k^*/\alpha \quad (\text{A.12})$$

The true biodegradation rate constant reflecting chemical disappearance is always larger than the corresponding rate constant estimated from the disappearance of residual organic radioactivity or from measurements of formed $^{14}\text{CO}_2$ activity. However, with a high extent of mineralization the two rate constants are practically equal.

Simkins and Alexander^[1] have described a more complex procedure based upon non-linear regression on the recorded activity data using an integrated equation for different kinetic formulations including first-order kinetics as presented here.

A.4.2 Examples of disappearance curves for residual radioactivity and test compound concentration

Examples of parallel disappearance curves for residual organic ^{14}C activity and test compound concentration are shown in Figures A.1 and A.2. The curves have been constructed for $\alpha = 0,95$ (high) (Figure A.1), and 0,7 (low) (Figure A.2) using the equations given in A.4.1 and are presented as semi-logarithmic plots.

The decrease in residual activity reflects both direct mineralization of the test compound and secondary mineralization of microbial products containing ^{14}C originating from the test compound. The use of ^{14}C technique, therefore, does not allow a straightforward estimation of ultimate biodegradation as, for example, in a DOC die-away test (ISO 7827) and the precision is more in line with the precision of traditional respirometric biodegradability tests.

Figures A.1 and A.2 show calculated first-order disappearance curves for residual organic activity and residual concentrations, respectively, assuming 95 % or 70 % of the carbon mineralized to CO_2 (fraction mineralized = α). The fraction of the initial concentration is plotted as function of time normalized to the number of half-lives of degradation. The absolute time is equal to the value read on the x-axis scale times the degradation half-life for the specific chemical investigated.

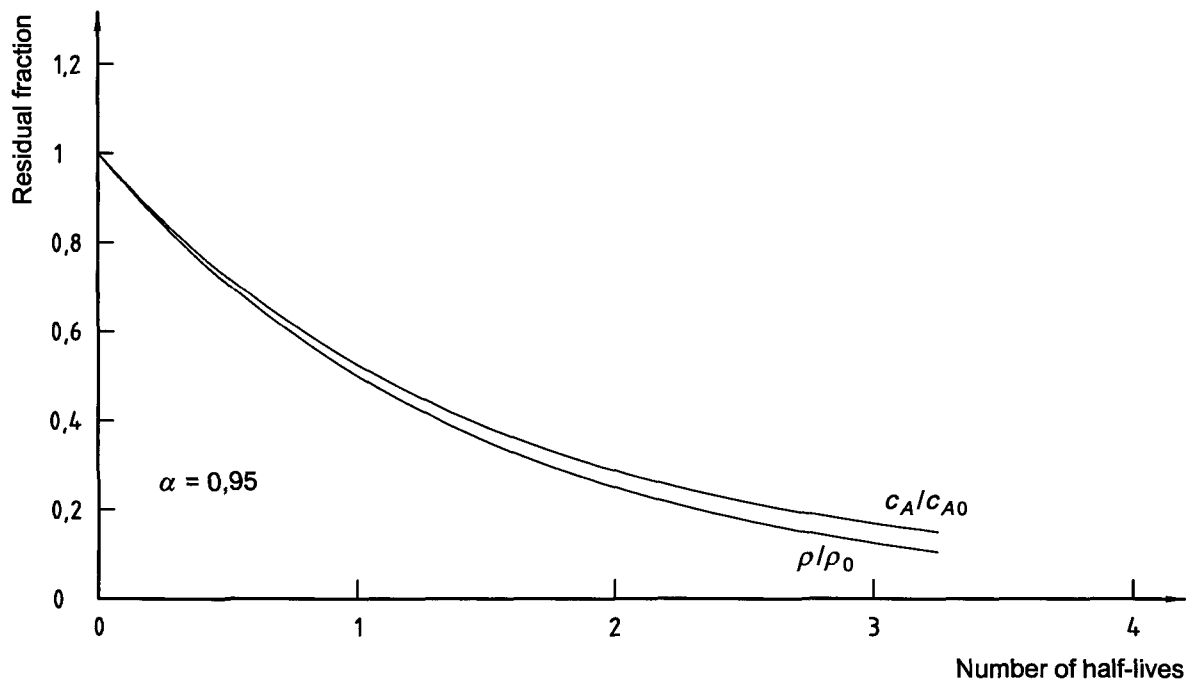


Figure A.1 — Disappearance curves for residual organic activity and residual concentrations (95 % mineralized)

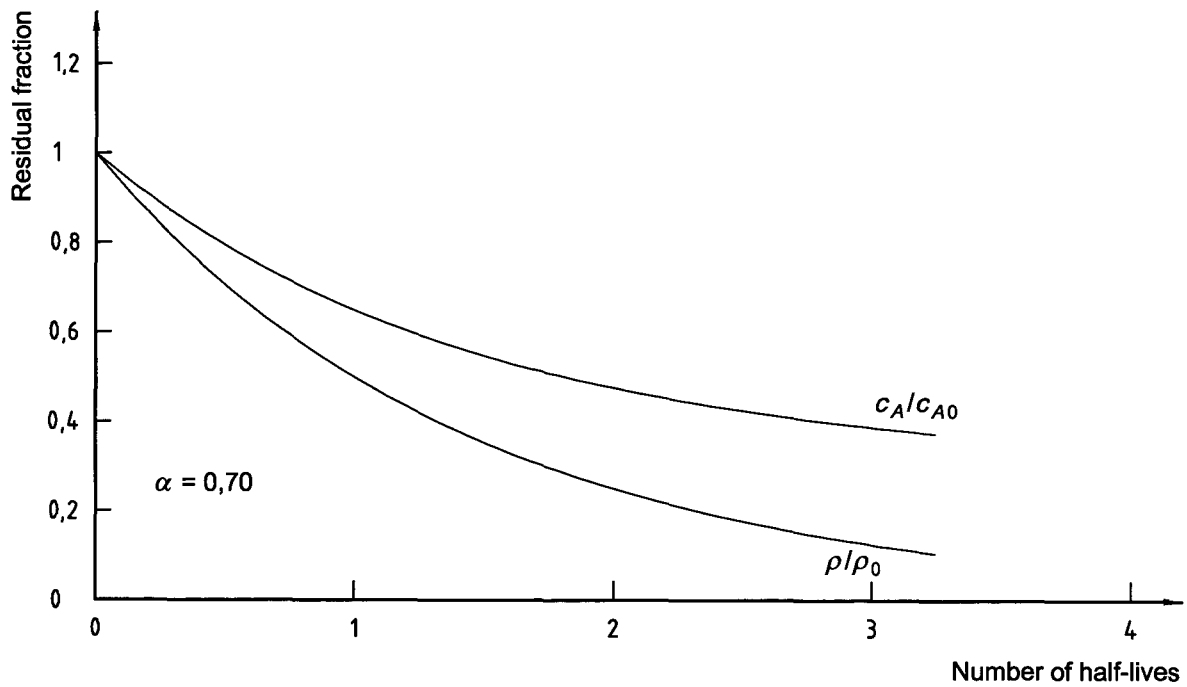


Figure A.2 — Disappearance curves for residual organic activity and residual concentrations (70 % mineralized)

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