

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Aerobic Mineralisation in Surface Water – Simulation Biodegradation Test

INTRODUCTION

1. The purpose of this test is to measure the time course of biodegradation of a test substance at low concentration in aerobic natural water and to quantify the observations in the form of kinetic rate expressions. This simulation test is a laboratory shake flask batch test to determine rates of aerobic biodegradation of organic substances in samples of natural surface water (fresh, brackish or marine). It is based on the ISO/DIS 14592-1 (1) and it also includes elements from the OECD Guidelines 307 and 308 (2)(3). Optionally, with long test times, semi-continuous operation replaces batch operation in order to prevent deterioration of the test microcosm. The principal objective of the simulation test is to determine the mineralisation of the test substance in surface water, and mineralisation constitutes the basis for expressing degradation kinetics. However, an optional secondary objective of the test is to obtain information on the primary degradation and the formation of major transformation products. Identification of transformation products, and if possible quantification of their concentrations, are especially important for substances that are very slowly mineralised (e.g. with half-lives for total residual ^{14}C exceeding 60 days). Higher concentrations of the test substance (e.g., $>100\ \mu\text{g/l}$) should normally be used for identification and quantification of major transformation products due to analytical limitations.

2. A low concentration in this test means a concentration (e.g. less than $1\ \mu\text{g/l}$ to $100\ \mu\text{g/l}$) which is low enough to ensure that the biodegradation kinetics obtained in the test reflect those expected in the environment. Compared to the total mass of biodegradable carbon substrates available in the natural water used for the test, the test substance present at low concentration will serve as a secondary substrate. This implies that the anticipated biodegradation kinetics is first order (“non-growth” kinetics) and that the test substance may be degraded by “cometabolism”. First order kinetics implies that the rate of degradation (mg/L/day) is proportional to the concentration of substrate which declines over time. With true first order kinetics the specific degradation rate constant, k , is independent of time and concentration. That is, k does not vary appreciably during the course of an experiment and does not change with the added concentration between experiments. By definition the specific degradation rate constant is equal to the relative change in concentration per time: $k = (1/C) \cdot (dC/dt)$. Although first order kinetics are normally expected under the prescribed conditions, there may be certain circumstances where other kinetics are more appropriate. Deviations from first order kinetics may, for example, be observed if mass transfer phenomena such as the diffusion rate, rather than the biological reaction rate, is limiting the rate of biotransformation. However, the data can nearly always be described by pseudo first order kinetics accepting a concentration dependent rate constant.

3. Information on biodegradability of the test substance at higher concentrations (e.g. from standard screening tests) as well as information on abiotic degradability, transformation products and relevant physico-chemical properties should be available prior to the test to help establish the experimental planning and interpret the results. The use of ^{14}C labelled test substances and the determination of the phase distribution of ^{14}C at the end of the test, enable ultimate biodegradability to be determined. When non-labelled test substance is used, ultimate biodegradation can only be estimated if a higher concentration is tested and all the major transformation products are known.

4. See Annex 1 for definitions and units.

GENERAL PRINCIPLE OF THE TEST

5. The test is performed in batch by incubating the test substance with either surface water only (“pelagic test”) or surface water amended with suspended solids/sediment of 0.01 to 1 g/L dry weight (“suspended sediment test”) to simulate a water body with suspended solids or re-suspended sediment. The suspended solids/sediment concentration in the lower range of this interval is typical for most surface waters. The test flasks are incubated in darkness at an environmental temperature under aerobic conditions and agitation. At least two different concentrations of test substance should be used in order to determine the degradation kinetics. The concentrations should differ from each other by a factor of 5 to 10 and should represent the expected range of concentrations in the environment. The maximum concentration of the test substance should not exceed 100 µg/L, but maximum test concentrations below 10 µg/L or less are preferred to ensure that the biodegradation follows first order kinetics. The lowest concentration should not exceed 10 µg/L, but lowest test concentrations of 1-2 µg/L or less than 1 µg/L are preferred. Normally an adequate analysis of such low concentration can be achieved by use of commercially available ¹⁴C-labelled substances. Because of analytical limitations, it is frequently impossible to measure the concentration of test substance with the required accuracy, if the test substance is applied at a concentration ≤100 µg/L (see paragraph 15). Higher concentrations of test substance (>100 µg/L and sometimes >1 mg/L) may be used for the identification and quantification of major transformation products or if a specific analysis method with a low detection limit is not available. If high concentrations of test substance are tested, it may not be possible to use the results to estimate the first order degradation constant and half-life, as the degradation will probably not follow first order kinetics.

6. Degradation is followed at appropriate time intervals, by measuring either the residual ¹⁴C or the residual concentration of test substance when specific chemical analysis is used. ¹⁴C labelling of the most stable part of the molecule ensures the determination of the total mineralisation, while ¹⁴C labelling of a less stable part of the molecule, as well as the use of specific analysis, enables the assessment of only primary biodegradation. However, the most stable part does not necessarily include the relevant functional moiety of the molecule (that can be related to a specific property such as toxicity, bioaccumulation, etc.). If this is the case, it may be appropriate to use a test substance, which is ¹⁴C-labelled, in the functional part in order to follow the elimination of the specific property.

APPLICABILITY OF THE TEST

7. This simulation test is applicable to non-volatile or slightly volatile organic substances tested at low concentrations. Using flasks open to the atmosphere (e.g. cotton wool plugged), substances with Henry’s law constants less than about 1 Pa · m³/mol (approx. 10⁻⁵ atm · m³/mol) can be regarded as non-volatile in practice. Using closed flasks with a headspace, it is possible to test slightly volatile substances (with Henry’s law constants <100 Pa · m³/mol or <10⁻³ atm · m³/mol) without losses from the test system. Loss of ¹⁴C-labelled substances may occur, if the right precautions are not exercised, when the CO₂ is stripped off. In such situations, it may be necessary to trap CO₂ in an internal absorber with alkali or to use an external CO₂ absorber system (direct ¹⁴CO₂ determination; see Annex 4). For the determination of biodegradation kinetics, the concentrations of the test substance must be below its water solubility. It should be noted, however, that literature values of water solubility may be considerably higher than the solubility of the test substance in natural waters. Optionally, the solubility of especially poorly water-soluble test substances may be established by use of the natural waters being tested.

8. The method can be used for simulating biodegradation in surface water free of coarse particles ("pelagic test") or in turbid surface water which, for example, might exist near a water/sediment interface ("suspended sediment test").

INFORMATION ON THE TEST SUBSTANCE

9. Both radiolabelled and non-labelled test substances can be used in this test. ^{14}C -labelling technique is recommended and labelling should normally be in the most stable part(s) of the molecule (see also paragraph 6). For substances containing more than one aromatic ring, one or more carbons in each ring should preferably be ^{14}C -labelled. In addition, one or more carbons on both sides of easily degradable linkages should preferably be ^{14}C -labelled. The chemical and/or radiochemical purity of the test substance should be >95%. For radiolabelled substances, a specific activity of approx. 50 $\mu\text{Ci}/\text{mg}$ (1.85 MBq) or more is preferred in order to facilitate ^{14}C measurements in tests conducted with low initial concentrations. The following information on the test substance should be available:

- solubility in water [OECD 105] (4);
- solubility in organic solvent(s) (substances applied with solvent or with low solubility in water);
- dissociation constant (pKa) if the substance is liable to protonation or deprotonation [OECD 112] (4);
- vapour pressure [OECD 104] (4) and Henry's law constant;
- chemical stability in water and in the dark (hydrolysis) [OECD 111] (4).

When poorly water-soluble substances are being tested in seawater, it may also be useful to know the salting out constant (or "Setschenow constant") K^s , which is defined by the expression: $\log(S/S') = K^s \cdot C_m$, where S and S' are the solubility of the substance in fresh water and seawater, respectively, and C_m is the molar salt concentration.

10. If the test is carried out as a "suspended sediment test" the following information should also be available:

- n-octanol/water partition coefficient [OECD 107, 117] (4);
- adsorption coefficient [OECD 106] (4);

11. Other useful information may include:

- environmental concentration, if known or estimated;
- toxicity of the test substance to microorganisms [OECD 209];
- ready and/or inherent biodegradability [OECD 301, OECD 302];
- aerobic or anaerobic biodegradability in soil and sediment/water transformation studies [OECD 307, OECD 308].

REFERENCE SUBSTANCE

12. A substance, which is normally easily degraded under aerobic conditions (e.g. aniline or sodium benzoate) should be used as reference substance. The expected time interval for degradation of aniline and sodium benzoate is usually less than 2 weeks. The purpose of the reference substances is to ensure that the microbial activity of the test water is within certain limits; i.e., that the water contains an active microbial population.

QUALITY CRITERIA

Recovery

13. Immediately after addition of the test substance, each initial test concentration should be verified by measurements of ^{14}C activity, or by chemical analyses in the case of non-labelled substances, in at least duplicate samples. This provides information on the applicability and repeatability of the analytical method and on the homogeneity of the distribution of the test substance. Normally, the measured initial ^{14}C activity or test substance concentration is used in the subsequent analyses of data rather than the nominal concentration as losses due to sorption and dosing errors thereby are compensated. For ^{14}C -labelled test substance, the level of recovery at the end of the experiment is given by mass balance (see also paragraph 39). Ideally, the radiolabelled mass balance should range from 90% to 110%, whereas the analytical accuracy should lead to an initial recovery of between 70% and 110% for non-labelled test substances. These ranges should be interpreted as targets and should not be used as criteria for acceptance of the test. Optionally, the analytical accuracy may be determined for the test substance at a lower concentration than the initial concentration and for major transformation products.

Repeatability and sensitivity of analytical method

14. Repeatability of the analytical method (including the efficiency of the initial extraction) to quantify the test substance, and transformation products, if appropriate, should be checked by five replicate analyses of the individual extracts of the surface water.

15. The limit of detection (LOD) of the analytical method for the test substance and for the transformation products should be at least 1% of the initial amount applied to the test system if possible. The limit of quantification (LOQ) should be equal to or less than 10% of the applied concentration. The chemical analyses of many organic substances and their transformation products frequently require that the test substance is applied at a relatively high concentration, i.e. $>100 \mu\text{g/L}$.

DESCRIPTION OF THE TEST METHOD

Equipment

16. The test may be conducted in conical or cylindrical flasks of appropriate capacity (e.g. 0.5 or 1.0 litre) closed with silicone or rubber stoppers, or in serum flasks with CO_2 -tight lids (e.g. with butyl rubber septa). Another option is to perform the test by use of multiple flasks and to harvest whole flasks, at least in duplicate, at each sample interval (see paragraph 30). For non-volatile test substances that are not radiolabelled, gas-tight stoppers or lids are not required; loose cotton plugs that prevent contamination from air are suitable (see paragraph 29). Slightly volatile substances should be tested in a biometer-type system with gentle stirring of the water surface. To be sure that no bacterial contamination occurs, optionally the vessels can be sterilised by heating or autoclaving prior to use. In addition, the following standard laboratory equipment is used:

- shaking table or magnetic stirrers for continuous agitation of the test flasks;
- centrifuge;
- pH meter;
- turbidimeter for nephelometric turbidity measurements;
- oven or microwave oven for dry weight determinations;
- membrane filtration apparatus;
- autoclave or oven for heat sterilisation of glassware;

- facilities to handle ^{14}C -labelled substances;
- equipment to quantify ^{14}C -activity in samples from CO_2 -trapping solutions and, if required, from sediment samples;
- analytical equipment for the determination of the test (and reference) substance if specific chemical analysis is used (e.g. gas chromatograph, high-pressure liquid chromatograph).

Stock solutions of test substance

17. Deionized water is used to prepare stock solutions of the test and reference substances (see also paragraph 22). The deionized water should be free of substances that may be toxic to microorganisms, and dissolved organic carbon (DOC) should be no more than 1 mg/L (5).

Collection and transport of surface water

18. The sampling site for collection of the surface water 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 must be considered. If it is known that an aquatic environment has been contaminated with the test substance or its structural analogues within the previous four years, it should not be used for the collection of test water, unless investigation of degradation rates in previously exposed sites is the express purpose of the investigator. The pH and temperature of the water should be measured at the site of collection. Furthermore, the depth of sampling and the appearance of the water sample (e.g. colour and turbidity) should be noted (see paragraph 53). Oxygen concentration and/or redox potential in water and in the sediment surface layer should be measured in order to demonstrate aerobic conditions unless this is obvious as judged from appearance and historic experience with the site. The surface water should be transported in a thoroughly cleansed container. During transport, the temperature of the sample should not significantly exceed the temperature used in the test. Cooling to 4°C is recommended if transport duration exceeds 2 to 3 hours. The water sample must not be frozen.

Storage and preparation of surface water

19. The test should preferably be started within one day after sample collection. Storage of the water, if needed, should be minimised and must in any case not exceed a maximum of 4 weeks. The water sample should be kept at 4°C with aeration until use. Prior to use, the coarse particles should be removed, for example by filtration through a nylon filter with about 100 μm mesh size or with a coarse paper filter, or by sedimentation.

Preparation of water amended with sediment [optional]

20. For the suspended sediment test, surface sediment is added to the flasks containing natural water (filtered to remove coarse particles as described in paragraph 19) to obtain a suspension; the concentration of suspended solids should be between 0.01 and 1 g/L. The surface sediment should come from the same site as that from which the water sample was taken. Dependent on the particular aquatic environment, the surface sediment may either be characterised by a high organic carbon content (2.5-7.5%) and a fine texture or by a low organic carbon content (0.5-2.5%) and a coarse texture (2). The surface sediment can be prepared as follows: extract several sediment cores using a tube of transparent plastic, slice off the upper aerobic layers (from surface to a depth of max. 5 mm) immediately after sampling and pool them together. The resulting sediment sample should be transported in a container with a large air headspace to keep the sediment under aerobic conditions (cool to 4°C if transport duration exceeds 2-3 hours). The sediment sample should be suspended in the test water at a ratio of 1:10 and kept at 4°C with aeration until use. Storage of the sediment, if needed, should be minimised and must not in any case exceed a maximum of 4

weeks.

Semi-continuous procedure [optional]

21. Prolonged incubation (several months) may be necessary if a long lag time occurs before a significant degradation of the test substance can be measured. If this is known from previous testing of a substance, the test may be initiated by using a semi-continuous procedure, which allows periodical renewal of a part of the test water or suspension (see Annex 3). Alternatively, the normal batch test may be changed into a semi-continuous test, if no degradation of the test substance has been achieved during approximately 60 days of testing using the batch procedure (see also paragraph 27).

Addition of the test (or reference) substance

22. For substances with high water solubility (>1 mg/L) and low volatility (Henry's law constants <1 Pa · m³/mol or $< 10^{-5}$ atm · m³/mol), a stock solution can be prepared in deionized water (see paragraph 17); the appropriate volume of the stock solution is added to the test vessels to achieve the desired concentration. The volume of any added stock solution should be held to the practical minimum ($<10\%$ of the final liquid volume, if possible). Another procedure is to dissolve the test substance in a larger volume of the test water, which may be seen as an alternative to the use of organic solvents.

23. If unavoidable, stock solutions of non-volatile substances with poor water-solubility should be prepared by use of a volatile organic solvent, but the amount of solvent added to the test system should not exceed 1% v/v and should not have adverse effects on the microbial activity. The solvent should not affect the stability of the test substance in water. The solvent should be stripped off to an extremely small quantity so that it does not significantly increase the DOC concentration of the test water or suspension. This should be checked by substance-specific analysis or, if possible DOC analysis (5). Care must be taken to limit the amount of solvent transferred to what is absolutely necessary and to ensure that the amount of test substance can dissolve in the final volume of test water. Other techniques to introduce the test substance into the test vessels may be used as described in (6) and (7). When an organic solvent is used for application of the test substance, solvent controls containing the test water (with no additions) and test water with added reference substance should be treated similarly to active test vessels amended with test substance in solvent carrier. The purpose of the solvent controls is to examine possible adverse effects caused by the solvent towards the microbial population as indicated by the degradation of the reference substance.

Test conditions

Test temperature

24. Incubation should take place in the dark (preferred) or in diffuse light at a controlled ($\pm 2^\circ\text{C}$) temperature, which may be the field temperature or a standard temperature of 20-25°C. Field temperature may be either the actual temperature of the sample at the sampling time or an average field temperature at the sampling site.

Agitation

25. Agitation by means of continuous shaking or stirring must be provided to maintain particles and microorganisms in suspension. Agitation also facilitates oxygen transfer from the headspace to the liquid so that aerobic conditions can be adequately maintained. Place the flasks on a shaking table (approx. 100 rpm agitation) or use magnetic stirring. Agitation must be continuous. However, the shaking or stirring should be as gentle as possible, while still maintaining a homogeneous suspension.

Test duration

26. The duration of the test should normally not exceed 60 days unless the semi-continuous procedure with periodical renewal of the test suspension is applied (see paragraph 21 and Annex 3). However, the test period for the batch test may be extended to a maximum of 90 days, if the degradation of the test substance has started within the first 60 days. Degradation is monitored, at appropriate time intervals, by the determination of the residual ^{14}C activity or the evolved $^{14}\text{CO}_2$ (see paragraphs 35-39) and/or by chemical analysis (paragraphs 40-42). The incubation time must be sufficiently long to evaluate the degradation process. The extent of degradation should preferably exceed 50%; for slowly degradable substances, the extent of degradation must be sufficient (normally greater than 20% degradation) to ensure the estimation of a kinetic degradation rate constant.

27. Periodic measurements of pH and oxygen concentration in the test system must be conducted unless previous experience from similar tests with water and sediment samples collected from the same site make such measurements unnecessary. Under some conditions, the metabolism of primary substrates at much higher concentrations within the water or sediment could possibly result in enough CO_2 evolution and oxygen depletion to significantly alter the experimental conditions during the test.

PROCEDURE**Preparation of flasks for pelagic test**

28. Transfer a suitable volume of test water to the test flasks, up to about one third of the flask volume and not less than about 100 ml. If multiple flasks are used (to allow harvesting of whole flasks at each sampling time), the appropriate volume of test water is also about 100 ml, as small sample volumes may influence the length of the lag phase. The test substance is added from a stock solution as described in paragraphs 17, 22 and 23. At least two different concentrations of test substance differing by a factor of 5 to 10 should be used in order to determine degradation kinetics and calculate the kinetic degradation rate constant. Both of the selected concentrations should be less than 100 $\mu\text{g/L}$ and preferably in the range of <1-10 $\mu\text{g/L}$.

29. Close the flasks with stoppers or lids impermeable to air and CO_2 . For non- ^{14}C -labelled non-volatile test chemicals, loose cotton wool plugs that prevent contamination from air are suitable (see paragraph 16) provided that any major degradation products are known to be non-volatile, and if indirect CO_2 determination is used (see Annex 4).

30. Incubate the flasks at the selected temperature (see paragraph 24). Withdraw samples for chemical analysis or ^{14}C measurements at the beginning of the test (i.e. before biodegradation starts; see paragraph 13) and then at suitable time intervals during the course of the test. Sampling may be performed by withdrawal of sub-samples (e.g. 5-ml aliquots) from each replicate or by harvest of whole flasks at each sampling time. The mineralisation of the test substance may either be determined indirectly or directly (see Annex 4). Usually, a minimum of five sampling points are required during the degradation phase (i.e. after ended lag phase) in order to estimate a reliable rate constant, unless it can be justified that three sampling points are sufficient for rapidly degradable substances. For substances that are not rapidly degraded more measurements during the degradation phase can easily be made and, therefore, more data points should be used for the estimation of k. No fixed time schedule for sampling can be stated, as the rate of biodegradation varies; however the recommendation is to sample once a week if degradation is slow. If the test substance is rapidly degradable, sampling should take place once a day during the first three days and then every second or third day. Under certain circumstances, such as with very rapidly hydrolysing substances, it may be necessary to sample at hourly intervals. It is recommended that a

preliminary study is conducted prior to the test in order to determine the appropriate sampling intervals. If samples have to be available for further specific analysis, it is advisable to take more samples and then select those to be analysed at the end of the experiment following a backwards strategy, i.e. the last samples are analysed first (see paragraph 41 for guidance on stability of samples during storage).

Number of flasks and samples

31. Set up a sufficient number of test flasks to have:

- test flasks; at least duplicate flasks for each concentration of test substance (preferably a minimum of 3) or multiple test flask for each concentration, if whole flasks are harvested at each sampling time (symbolised F_T);
- test flasks for mass balance calculation; at least duplicate flasks for each test concentration (symbolised F_M);
- blank control, no test substance; at least one blank test flask containing only the test water (symbolised F_B);
- reference control; duplicate flasks with reference substance (e.g. aniline or sodium benzoate, at 10 µg/l) (symbolised F_C). The purpose of the reference control is to confirm a minimum of microbial activity. If convenient, a radiolabelled reference substance may be used, also when the degradation of the test substance is monitored by chemical analyses;
- sterile control; one or two flasks containing sterilised test water for examining possible abiotic degradation or other non-biological removal of the test substance (symbolised F_S). The biological activity can be stopped by autoclaving (121°C; 20 min) the test water or by adding a toxicant (e.g. sodium azide (NaN_3) at 10-20 g/l, mercuric chloride (HgCl_2) at 100 mg/l or formalin at 100 mg/l) or by gamma irradiation. If HgCl_2 is used, it should be disposed of as toxic waste. For water with sediment added in large amount, sterile conditions are not easy to obtain; in this case repeated autoclaving (e.g., three times) is recommended. It should be considered that the sorption characteristics of the sediment may be altered by autoclaving.
- solvent controls, containing test water and test water with reference substance; duplicate flasks treated with the same amount of solvent and by use of the same procedure as that used for application of the test substance. The purpose is to examine possible adverse effects of the solvent by determining the degradation of the reference substance.

32. In the design of the test, the investigator should consider the relative importance of increased experimental replication versus increased number of sampling times. The exact number of flasks required will depend on the method used for measuring the degradation (see also paragraphs 30 and 35-39 and Annex 4).

33. Two subsamples (e.g. 5-ml aliquots) should be withdrawn from each test flask at each sampling time. If multiple flasks are used to allow harvesting of whole flasks, a minimum of two flasks should be sacrificed at each sampling time (see paragraph 28).

Preparation of flasks for suspended sediment test [optional]

34. Add the necessary volumes of test water and sediment, if required, to the test vessels (see paragraph 20). The preparation of flasks for suspended sediment test is the same as for the pelagic test (see paragraphs 28-33). Preferably use serum bottles or similar shaped flasks. Place the closed flasks horizontally on a shaker. Obviously, open flasks for non- ^{14}C -labelled, non-volatile substances should be placed in upright position; in this case magnetic stirring and the use of magnetic bars coated with glass are recommended. If necessary, aerate the bottles to maintain proper aerobic conditions.

Radiochemical determinations

35. The evolved $^{14}\text{CO}_2$ is measured indirectly and directly (see Annex 4). The $^{14}\text{CO}_2$ is determined indirectly by the difference between the initial ^{14}C activity in the test water or suspension and the total residual activity at the sampling time as measured after acidifying the sample to pH 2-3 and stripping off CO_2 . Inorganic carbon is thus removed and the residual activity measured derives from organic material. The indirect $^{14}\text{CO}_2$ determination should not be used, if major volatile transformation products are formed during the transformation of the test substance (see Annex 4). If possible, the $^{14}\text{CO}_2$ evolution should be measured directly (see Annex 4) at each sampling time in at least one test flask; this procedure enables both the mass balance and biodegradation process to be checked, but it is restricted to tests conducted with closed flasks.

36. If the evolved $^{14}\text{CO}_2$ is measured directly during the test, more flasks should be set up for this purpose at the start of the test. Direct $^{14}\text{CO}_2$ determination is recommended, if major volatile transformation products are formed during the transformation of the test substance. At each measuring point the additional test flasks are acidified to pH 2-3 and the $^{14}\text{CO}_2$ is collected in an internal or external absorber (see Annex 4).

37. Optionally the concentrations of ^{14}C -labelled test substance and major transformation products may be determined by use of radiochromatography (e.g. thin layer chromatography, RAD-TLC) or HPLC with radiochemical detection.

38. Optionally the phase distribution of the remaining radioactivity (see Annex 2) and residual test substance and transformation products may be determined.

39. At the end of the test the mass balance should be determined by direct $^{14}\text{CO}_2$ measurement using separate test flasks from which no samples are taken in the course of the test (see Annex 4).

Specific chemical analysis

40. If a sensitive specific analytical method is available, primary biodegradation can be assessed by measuring the total residual concentration of test substance instead of using radiolabelling techniques. If a radiolabelled test substance is used (to measure total mineralisation), specific chemical analyses can be made in parallel to provide useful additional information and check the procedure. Specific chemical analyses may also be used to measure transformation products formed during the degradation of the test substance, and this is recommended for substances that are mineralised with half-lives exceeding 60 days. The concentration of the test substance and the transformation products at every sampling time should be measured and reported (as a concentration and as percentage of applied). In general, transformation products detected at $\geq 10\%$ of the applied concentration at any sampling time should be identified unless reasonably justified otherwise. Transformation products for which concentrations are continuously increasing during the study should also be considered for identification, even if their concentrations do not exceed the limit given above, as this may indicate persistence. Analyses of transformation products in sterile controls should be considered, if rapid abiotic transformation of the test substance (e.g. hydrolysis) is thought possible. The need for quantification and identification of transformation products should be considered on a case by case basis, with justifications being provided in the report. Extraction techniques with organic solvent should be applied according to directions given in the respective analytical procedure.

41. All samples should be stored at 2 to 4°C and air-tight if analysis is carried out within 24 hours (preferred). For longer storage, the samples should be frozen below -18°C or chemically preserved. Acidification is not a recommended method to preserve the samples, because acidified samples may be unstable. If the samples are not analysed within 24 hours and are subject to longer storage, a storage

stability study should be conducted to demonstrate the stability of chemicals of interest under -18°C storage or preserved conditions. If the analytical method involves either solvent extraction or solid phase extraction (SPE), the extraction should be performed immediately after sampling or after storing the sample refrigerated for a maximum of 24 hours.

42. Depending on the sensitivity of the analytical method larger sample volumes than those indicated in paragraph 16 may be necessary. The test can easily be carried out with test volumes of one litre in flasks of 2-3-litre volume, which makes it possible to collect samples of approx. 100 ml.

DATA AND REPORTING

Treatment of results

Plot of data

43. Round off sampling times to a whole number of hours (unless the substance degrades substantially in a matter of minutes to hours) but not to a whole number of days. Plot the estimates of the residual activity of test substance (for ^{14}C -labelled substances) or the residual concentration (for non-labelled substances), against time both in a linear and in a semi-logarithmic plot (see Figures 1a, 1b). If degradation has taken place, compare the results from flasks F_T with those from flasks F_S . If the means of the results from the flasks with test substance (F_T) and the sterile flasks (F_S) deviate by less than 10%, it can be assumed that the degradation observed is predominantly abiotic. If the degradation in flasks F_S is lower, the figures may be used to correct those obtained with flasks F_T (by subtraction) in order to estimate the extent of biodegradation. When optional analyses are performed for major transformation products, plots of their formation and decline should be provided in addition to a plot of the decline of the test substance.

44. Estimate the lag phase duration t_L from the degradation curve (semi-logarithmic plot) by extrapolating its linear part to zero degradation or alternatively by determining the time for approximately 10% degradation (see Figures 1a and 1b). From the semi-logarithmic plot, estimate the first order rate constant, k , and its standard error by linear regression of \ln (residual ^{14}C activity or test substance concentration) versus time. With ^{14}C measurements in particular, use only data belonging to the initial linear part of the curve after the ended lag phase, and give preference to selecting few and representative data rather than selecting a greater number of more uncertain data. Uncertainty includes here errors inherent in the recommended direct use of measured residual ^{14}C activities (see below). It may sometimes be relevant to calculate two different rate constants, if the degradation follows a biphasic pattern. For this purpose two different phases of the degradation curve are defined. Calculations of the rate constant, k , and the half-life $t_{1/2} = \ln 2/k$, should be carried out for each of the individual replicate flasks, when sub-samples are withdrawn from the same flask, or by using the average values, when whole flasks are harvested at each sampling time (see paragraph 33). When the first-mentioned procedure is used, the rate constant and half-life should be reported for each of the individual replicate flasks and as an average value with a standard error. If high concentrations of test substance have been used, the degradation curve may deviate considerably from a straight line (semi-logarithmic plot) and first order kinetics may not be valid. Defining a half-life has therefore no meaning. However, for a limited data range, pseudo first order kinetics can be applied and the degradation half-time DT_{50} (time to reach 50% degradation) estimated. It must be borne in mind, however, that the time course of degradation beyond the selected data range cannot be predicted using the DT_{50} which is merely a descriptor of a given set of data. Analytical tools to facilitate statistical calculations and curve fitting are easily available and the use of this kind of software is recommended.

45. If specific chemical analyses are made, estimate rate constants and half-lives for primary degradation as above for total mineralisation. If the primary degradation is the limiting process data points from the entire course of degradation may sometimes be used. This is because measurements are direct by contrast to measurements of ^{14}C activity.

46. If ^{14}C -labelled substances are used, a mass balance should be expressed in percentage of the applied initial concentration, at least at the end of the test.

Residual activity

47. When the ^{14}C -labelled part of an organic substance is biodegraded, the major part of the ^{14}C is converted to $^{14}\text{CO}_2$, while another part is used for growth of biomass and/or synthesis of extra-cellular metabolites. Therefore, complete "ultimate" biodegradation of a substance does not result in a 100% conversion of its carbon into $^{14}\text{CO}_2$. The ^{14}C built into products formed by biosynthesis is subsequently released slowly as $^{14}\text{CO}_2$ due to "secondary mineralisation". For these reasons plots of residual organic ^{14}C activity (measured after stripping off CO_2) or of $^{14}\text{CO}_2$ produced versus time will show a "tailing" after degradation has been completed. This complicates a kinetic interpretation of the data and for this purpose, only the initial part of the curve (after ended lag phase and before approx. 50% degradation is reached) should normally be used for the estimation of a degradation rate constant. If the test substance is degraded, the total residual organic ^{14}C activity is always higher than the ^{14}C activity associated with the remaining intact test substance. If the test substance is degraded by a first order reaction and a constant fraction α is mineralised into CO_2 , the initial slope of the ^{14}C disappearance curve (total organic ^{14}C versus time) will be α times the slope of the corresponding curve for the concentration of test substance (or, to be precise, the part of the test substance labelled with ^{14}C). Using measurements of the total organic ^{14}C activity uncorrected, the calculated degradation rate constant will therefore be conservative. Procedures for estimating the concentrations of the test substance from the measured radiochemical activities based on various simplifying assumptions have been described in the literature (1)(8)(9)(10). Such procedures are most easily applied for rapidly degradable substances.

Interpretation of results

48. If k is found to be independent of the added concentration (i.e. if the calculated k is approximately the same at the different concentrations of test substance), it can be assumed that the first order rate constant is representative of the testing conditions used, i.e. the test substance, the water sample and the test temperature. To what extent the results can be generalised or extrapolated to other systems must be evaluated by expert judgement. If a high concentration of test substance is used, and the degradation therefore does not follow first order kinetics, the data cannot be used for direct estimation of a first order rate constant or a corresponding half-life. However, data derived from a test using a high concentration of test substance may still be usable for estimating the degree of total mineralisation and/or detection and quantification of transformation products.

49. If the rates of other loss processes than biodegradation are known (e.g. hydrolysis or volatilisation), they may be subtracted from the net loss rate observed during the test to give an approximated estimate of the biodegradation rate. Data for hydrolysis may, for example, be obtained from the sterile control or from parallel test using a higher concentration of the test substance.

50. The indirect and direct determination of $^{14}\text{CO}_2$ (paragraphs 35-39 and Annex 4) can only be used to measure the extent of mineralisation of the test substance to CO_2 . Radiochromatography (RAD-TLC) or HPLC may be used to analyse the concentrations of ^{14}C -labelled test substance and the formation of major transformation products (paragraph 37). To enable a direct estimation of the half-life, it is necessary that no major transformation products (defined as $\geq 10\%$ of the applied amount of test substance) be present. If

major transformation products as defined here are present, a detailed evaluation of the data is required. This may include repeated testing and/or identification of the transformation products (see paragraph 40) unless the fate of the transformation products can be reasonably assessed by use of experience (e.g. information on degradation pathway). As the proportion of test substance carbon converted to CO₂ varies (depending largely on the concentration of test substance and other substrates available, the test conditions and the microbial community), this test does not allow a straightforward estimation of ultimate biodegradation as in a DOC die-away test; but the result is similar to that obtained with a respirometric test. The degree of mineralisation will thus be less than or equal to the minimum level of ultimate biodegradation. To obtain a more complete picture of the ultimate biodegradation (mineralisation and incorporation into biomass), the analysis of the phase distribution of ¹⁴C should be performed at the end of the test (see Annex 2). The ¹⁴C in the particulate pool will consist of ¹⁴C incorporated into bacterial biomass and ¹⁴C sorbed to organic particles.

Validity of the test

51. If the reference substance is not degraded within the expected time interval (for aniline and sodium benzoate, usually less than two weeks), the validity of the test is suspected and must be further verified, or alternatively the test should be repeated with a new water sample. In an ISO ring-test of the method where seven laboratories located around Europe participated, adapted degradation rate constants for aniline ranged from 0.3 to 1.7 day⁻¹ with an average of 0.8 d⁻¹ at 20°C and a standard error of ± 0.4 d⁻¹ (t_{1/2} = 0.9 days). Typical lag times were 1 to 7 days. The waters examined were reported to have a bacterial biomass corresponding to 10³ to 10⁴ colony forming units (CFU) per ml. Degradation rates in nutrient-rich Mid-European waters were greater than in Nordic oligotrophic waters, which may be due to the different trophic status or previous exposure to chemical substances.

52. The total recovery (mass balance) at the end of the experiment should be between 90% and 110% for radiolabelled substances, whereas the initial recovery at the beginning of the experiment should be between 70% and 110% for non-labelled substances. However, the indicated ranges should only be interpreted as targets and should not be used as criteria for acceptance of the test.

Test report

53. The type of study, i.e. pelagic or suspended sediment test, must be clearly stated in the test report, which shall also contain at least the following information:

Test substance and reference substance(s):

- common names, chemical names (recommend IUPAC and/or CAS names), CAS numbers, structural formulas (indicating position of ¹⁴C if radiolabelled substance is used) and relevant physico-chemical properties of test and reference substance (see paragraphs 9-12);
- chemical names, CAS numbers, structural formulas (indicating position of ¹⁴C if radiolabelled substance is used) and relevant physico-chemical properties of substances used as standards for identification and quantification of transformation products;
- purity (impurities) of test and reference substances;
- radiochemical purity of labelled chemical and specific activity (where appropriate).

Surface water:

The following minimum information for the water sample taken must be provided:

- location and description of sampling site including, if possible, contamination history;

- date and time of sample collection;
nutrients (total N, ammonium, nitrite, nitrate, total P, dissolved orthophosphate);
- depth of collection;
- appearance of sample (e.g. colour and turbidity);
- DOC and TOC;
BOD;
- temperature and pH at the place and time of collection;
- oxygen or redox potential (mandatory only if aerobic conditions are not obvious);
- salinity or conductivity (in the case of sea water and brackish water);
- suspended solids (in case of a turbid sample);
- possibly other relevant information about the sampling location at the time of sampling (e.g. actual or historical data on flow rate of rivers or marine currents, nearby major discharges and type of discharges, weather conditions preceding the sampling time);

and optionally:

- microbial biomass (e.g. acridine orange direct count or colony forming units);
- inorganic carbon;
- chlorophyll-a concentration as a specific estimate for algal biomass.

In addition, the following information of the sediment should be provided if the suspended sediment test is conducted:

- depth of sediment collection;
- appearance of the sediment (such as coloured, muddy, silty, or sandy);
- texture (e.g. % coarse sand, fine sand, silt and clay)
- dry weight in g/l of the suspended solids, TOC concentration or weight loss on ignition as a measure of the content of organic matter;
- pH
- oxygen or redox potential (mandatory only if aerobic conditions are not obvious).

Test conditions:

- delay between collection and use in the laboratory test, sample storage and pre-treatment of the sample dates of performance of the studies;
- amount of test substance applied, test concentration and reference substance;
- method of application of the test substance including any use of solvents;
- volume of surface water used and sediment (if used) and volume sampled at each interval for analysis;
- description of the test system used;
if dark conditions are not to be maintained, information on the “diffuse light” conditions;
- information on the method(s) used for establishing sterile controls (e.g. temperature, time and number of autoclavings);
- incubation temperature;
- information on analytical techniques and the method(s) used for radiochemical measurements and for mass balance check and measurements of phase distribution (if conducted).
- number of replicates;

Results:

- percentages of recovery (see paragraph 13);
- repeatability and sensitivity of the analytical methods used including the limit of detection (LOD) and the limit of quantification (LOQ) (see paragraphs 14 and 15);
- all measured data (including sampling time points) and calculated values in tabular form and the degradation curves; for each test concentration and for each replicate flask, report the linear correlation coefficient for the slope of the logarithmic plot, the estimated lag phase and a first-order or pseudo-first order rate constant (if possible), and the corresponding degradation half-life (or the half-life period, $t_{1/2}$);
- report relevant values as the averages of the results observed in individual replicates, for example length of lag phase, degradation rate constant and degradation half-life (or $t_{1/2}$);
- categorise the system as either non-adapted or adapted as judged from the appearance of the degradation curve and from the possible influence of the test concentration;
- the results of the final mass balance check and results on phase distribution measurements (if any);
- the fraction of ^{14}C mineralised and, if specific analyses are used, the final level of primary degradation;
- the identification, molar concentration and percentage of applied of major transformation products (see paragraph 40), where appropriate;
- a proposed pathway of transformation, where appropriate;
- discussion of results.

LITERATURE

- (1) ISO/DIS 14592-1 (1999) 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.
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- (5) ISO 8245 (1999). Water quality – Guidelines on the determination of total organic carbon (TOC) and dissolved organic carbon (DOC).
- (6) ISO 10634 (1995). Water quality – Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium.
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- (9) Ingerslev, F. and N. Nyholm. (2000). Shake-flask test for determination of biodegradation rates of ^{14}C -labeled chemicals at low concentrations in surface water systems. *Ecotoxicol. Environ. Saf.* 45, 274-283.
- (10) ISO/CD 14592-1 (1999). Ring test report: Water Quality – Evaluation of the aerobic biodegradability of organic compounds at low concentrations part 1 – report of 1998/1999 ring-test. Shake flask batch test with surface water or surface water/sediment suspensions.

ANNEX 1DEFINITIONS AND UNITS

Primary biodegradation: The structural change (transformation) of a chemical substance by microorganisms resulting in the loss of chemical identity.

Functional biodegradation: The structural change (transformation) of a chemical substance by microorganisms resulting in the loss of a specific property.

Ultimate aerobic biodegradation: The breakdown of a chemical substance by microorganisms in the presence of oxygen to carbon dioxide, water and mineral salts of any other elements present (mineralisation) and the production of new biomass and organic microbial biosynthesis products.

Mineralisation: The breakdown of a chemical substance or organic matter by microorganisms in the presence of oxygen to carbon dioxide, water and mineral salts of any other elements present.

Lag phase: The time from the start of a test until adaptation of the degrading micro-organisms is achieved and the biodegradation degree of a chemical substance or organic matter has increased to a detectable level (e.g. 10 % of the maximum theoretical biodegradation, or lower, dependent on the accuracy of the measuring technique).

Maximum level of biodegradation: The degree of biodegradation of a chemical substance or organic matter in a test, recorded in per cent, above which no further biodegradation takes place during the test.

Primary substrate: A collection of natural carbon and energy sources that provide growth and maintenance of the microbial biomass.

Secondary substrate: A substrate component present in a such low concentration, that by its degradation, only insignificant amounts of carbon and energy are supplied to the competent micro-organisms, as compared to the carbon and energy supplied by their degradation of main substrate components (primary substrates).

Degradation rate constant: A first order or pseudo first order kinetic rate constant, k (d^{-1}), which indicates the rate of degradation processes. For a batch experiment k is estimated from the initial part of the degradation curve obtained after the end of the lag phase.

Half-life, $t_{1/2}$ (d): Term used to characterise the rate of a first order reaction. It is the time interval that corresponds to a concentration decrease by a factor 2. The half-life and the degradation rate constant are related by the equation $t_{1/2} = \ln 2/k$.

Degradation half time, DT_{50} (d): Term used to quantify the outcome of biodegradation tests. It is the time interval, including the lag phase, needed to reach a value of 50% biodegradation.

Limit of detection (LOD) and limit of quantification (LOQ): The limit of detection (LOD) is the concentration of a substance below which the identity of the substance cannot be distinguished from analytical artefacts. The limit of quantification (LOQ) is the concentration of a substance below which the concentration cannot be determined with an acceptable accuracy.

Dissolved organic carbon (DOC): That part of the organic carbon in a sample of water which cannot be removed by specified phase separation, for example by centrifugation at 40000 ms^{-2} for 15 min or by membrane filtration using membranes with pores of $0.2 \text{ }\mu\text{m}$ - $0.45 \text{ }\mu\text{m}$ diameter.

Total organic ^{14}C activity (TOA): The total ^{14}C activity associated with organic carbon.

Dissolved organic ^{14}C activity (DOA): The total ^{14}C activity associated with dissolved organic carbon.

Particulate organic ^{14}C activity (POA): The total ^{14}C activity associated with particulate organic carbon.

ANNEX 2PHASE DISTRIBUTION OF ¹⁴C

In order to check the procedure, the routine measurements of residual total organic ¹⁴C activity (TOA) should be supplemented by mass balance measurements involving a direct determination of the evolved ¹⁴CO₂ after trapping in an absorber (see Annex 4). In itself, a positive ¹⁴CO₂ formation is a direct evidence of biodegradation as opposed to abiotic degradation or other loss mechanisms, such as volatilisation and sorption. Additional useful information characterising the biodegradability behaviour can be obtained from measurements of the distribution of TOA between the dissolved state (dissolved organic ¹⁴C activity, DOA) and the particulate state (particulate organic ¹⁴C activity, POA) after separation of particulate by membrane filtration or centrifugation. POA consists of test substance sorbed onto the microbial biomass and onto other particles in addition to the test substance carbon that has been used for synthesis of new cellular material and thereby incorporated into the particulate biomass fraction. The formation of dissolved ¹⁴C organic material can be estimated as the DOA at the end of biodegradation (plateau on the degradation versus time curve).

Estimate the phase distribution of residual ¹⁴C 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 substance (polycarbonate filters may be suitable). If sorption of test substance onto the filter is too large to be ignored (to be checked prior to the experiment) high-speed centrifugation (2,000 g; 10 min) can be used instead of filtration.

Proceed with the filtrate or centrifugate as described in Annex 4 for unfiltered samples. Dissolve membrane filters in a suitable scintillation fluid and count as usually, normally using only the external standard ratio method to correct for quenching, or use a sample oxidiser. If centrifugation has been used, re-suspend the pellet formed of the particulate fraction in 1-2 ml of distilled water and transfer to a scintillation vial. Wash subsequently twice with 1 ml distilled water and transfer the washing water to the vial. If necessary, the suspension can be embedded in a gel for liquid scintillation counting.

ANNEX 3SEMI-CONTINUOUS PROCEDURE

Prolonged incubation for up to several months may be required in order to achieve a sufficient degradation of recalcitrant substances. The duration of the test should normally not exceed 60 days unless the characteristics of the original water sample are maintained by renewal of the test suspension. However, the test period may be extended to a maximum of 90 days without renewal of the test suspension, if the degradation of the test substance has started within the first 60 days.

During incubation for long periods, the diversity of the microbial community may be reduced due to various loss mechanisms and due to possible depletion of the water sample of essential nutrients and primary carbon substrates. It is therefore recommended that a semi-continuous test is used to adequately determine the degradation rate of slowly degrading substances. The test should be initiated by use of the semi-continuous procedure if, based on previous experience, an incubation period of three months is expected to be necessary to achieve 20% degradation of the substance. Alternatively, the normal batch test may be changed into a semi-continuous test, if no degradation of the test substance has been achieved during approximately 60 days of testing using the batch procedure. The semi-continuous procedure may be stopped and the test continued as a batch experiment, when a substantial degradation has been recorded (e.g. >20%).

In the semi-continuous test, every two weeks, about one third of the volume of the test suspension is replaced by freshly collected water with the test substance added to the initial concentration. Sediment is likewise added to the replacement water to the initial concentration (between 0.01 and 1 g/l), if the optional suspended sediment test is performed. Carrying out the test with suspended sediment solids, it is important that a fully suspended system is maintained also during water renewal, and that the residence time is identical for solids and water, as otherwise the intended similarity to a homogenous aqueous system with no fixed phases can be lost. For these reasons, an initial concentration of suspended sediments in the lower range of the specified interval is preferred, when the semi-continuous procedure is used.

The prescribed addition of test substance implies that the initial concentration of test substance is not exceeded by the partial renewal of the test suspension and, hence, the adaptation, which is frequently seen with high concentrations of a test substance, is avoided. As the procedure comprises both a re-inoculation and a compensation of depleted nutrients and primary substrates, the original microbial diversity is restored, and the duration of the test can be extended to infinity in principle. When the semi-continuous procedure is used, it is important to note that the residual concentration of the test substance must be corrected for the amounts of test substance added and removed at each renewal procedure. The total and the dissolved test substance concentration can be used interchangeably for compounds that sorb little. Sorption is insignificant (< 5%) under the specified conditions (0.1-1 g solids/l) for substances of $\log K_{ow} < 3$ (valid for neutral, lipophilic compounds). This is illustrated by the following calculation example. 0.1 g/l of solids roughly corresponds to 10 mg of carbon per litre (fraction of carbon, $f_C = 0.01$). Assuming that;

$\log K_{ow}$ (of the test substance) = 3

$K_{oc} = 0.42 \times K_{ow}$

Partition coefficient, $K_d = f_C \times K_{oc}$

then, the dissolved fraction of the total concentration (C-water (C_w)/C-total (C_t)) is:

$$C_w/C_t = 1/(1 + K_d \times SS) = 1/(1 + K_{oc} \times f_C \times SS) = 1/(1 + 0.42 \times 10^3 \times 0.01 \times 0.1 \times 10^{-3}) = 0.999$$

ANNEX 4DETERMINATION OF $^{14}\text{CO}_2$ Indirect $^{14}\text{CO}_2$ determination

For routine measurements, the indirect method is normally the least time-consuming and most precise method if the test substance is non-volatile and is not transformed into volatile transformation products. Simply transfer unfiltered samples, for example 5-ml size to scintillation vials. A suitable activity in samples is 5,000 dpm - 10,000 dpm (80-170 Bq) initially, and a minimum initial activity is about 1000 dpm. The CO_2 should be stripped off after acidifying to pH 2-3 with 1-2 drops of concentrated H_3PO_4 or HCl. The CO_2 stripping can be performed by bubbling with air for about ½-1 hour. Alternatively, vials can be shaken vigorously for 1-2 hours (for instance on a microplate shaker) or with more gentle shaking be left overnight. The efficiency of the CO_2 stripping procedure must be checked (by prolonging the aeration or shaking period). A scintillation liquid, suitable for counting aqueous samples should then be added, the sample homogenised on a whirling mixer and the radioactivity determined by liquid scintillation counting, subtracting the background activity found in the test blanks (F_B). Unless the test water is very coloured or contains a high concentration of particles, the samples will normally show uniform quenching and it will be sufficient to perform quench corrections using an external standard. If the test water 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 samples may be large. In that case the counting method described below for test slurries can be used. If the test is carried out as a suspended sediment test, the $^{14}\text{CO}_2$ measurement could be done indirectly by taking a homogeneous 10-ml sample of the test water/suspension and separating the phases by centrifugation at a suitable speed (e.g. at 40,000 m/s^2 for 15 min). The aqueous phase should then be then treated as described above. The ^{14}C activity in the particulate phase (POA) should be determined by re-suspending the sediment into a small volume of distilled water, transferring to scintillation vials, and adding scintillation liquid to form a gel (special scintillation liquids are available for that purpose). Depending on the nature of particles (e.g. their content of organic material), it may be feasible to digest the sample overnight with a tissue solubiliser and then homogenise on a whirling mixer prior to the addition of scintillation liquid. Alternatively, the POA can be determined by combustion in excess of oxygen by use of a sample oxidiser. When counting, internal standards should always be included, and it may be necessary to perform quench corrections using internal standard addition for each individual sample.

Direct $^{14}\text{CO}_2$ determination

If the evolved $^{14}\text{CO}_2$ is measured directly, it should be done by setting up more flasks at the start of the test, harvesting the test flasks at each measuring point by acidifying the test flasks to pH 2-3 and collecting the $^{14}\text{CO}_2$ in an internal (placed in each test flask at the start of the test) or external absorber. As absorbing medium either alkali (e.g. 1 N NaOH solution, or a NaOH pellet), ethanolamine or an ethanolamine-based, and commercially available absorbers can be used. For direct measurement of the $^{14}\text{CO}_2$, the flasks should be closed with e.g. butyl rubber septa.

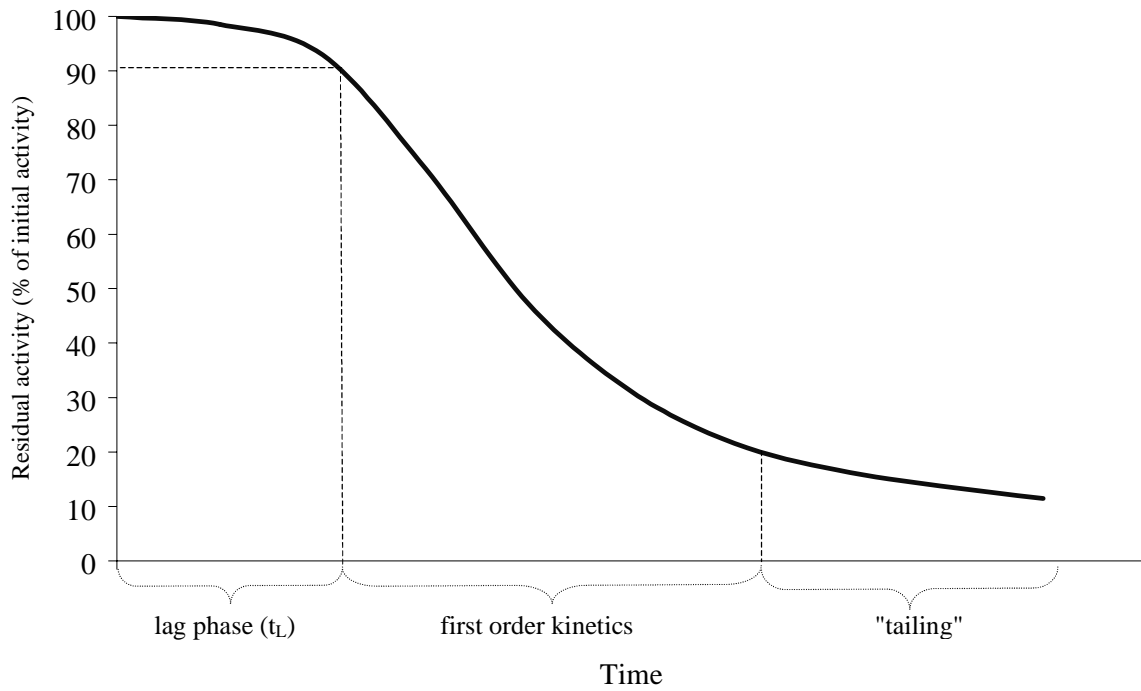


Figure 1a. Example of arithmetic plot of data (residual activity versus time)

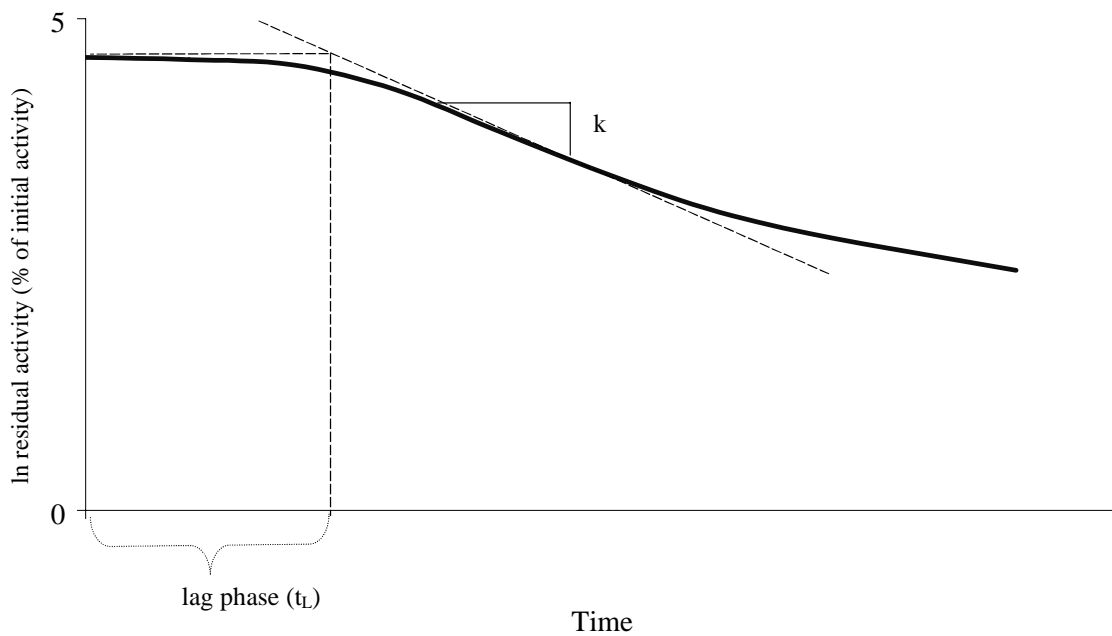


Figure 1b. Example of semi-logarithmic plot of data (ln to residual activity versus time).