OECD/OCDE TG 310

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Ready Biodegradability – CO_2 in sealed vessels (Headspace Test)

INTRODUCTION

1. This Guideline is a screening method for the evaluation of ready biodegradability of chemical substances and provides similar information to the six test methods described in OECD Test Guideline 301 A to F. Therefore, a chemical substance that shows positive results in this Guideline can be considered readily biodegradable and consequently rapidly degradable in the environment.

2. The well established OECD Carbon dioxide (CO_2) method (1), based on Sturm's original test (2) for assessing biodegradability of organic chemicals, by the measurement of the carbon dioxide produced by microbial action, has normally been the first choice for testing poorly soluble chemicals and those which strongly adsorb. It is also chosen for soluble, (but not volatile chemicals), since the evolution of carbon dioxide is considered by many to be the only unequivocal proof of microbial activity. Removal of dissolved organic carbon can be effected by physico-chemical processes - adsorption, volatilisation, precipitation, hydrolysis - as well as by microbial action and many non-biological reactions consume oxygen; rarely is CO_2 produced from organic chemicals abiotically. In the original and modified Sturm test (1)(2) CO_2 is removed from the liquid phase to the absorbing vessels by sparging (i.e. bubbling air treated to remove CO_2 through the liquid medium), while in the version of Larson (3)(4) CO_2 is transferred from the reaction vessel to the absorbers by passing CO_2-free air through the headspace and, additionally, by shaking the test vessel continuously. Only in the Larson modification is the reaction vessel shaken; stirring is specified only for insoluble substances in the ISO version (5) and in the original US version (6), both of which specify sparging rather than headspace replacement. In another official US EPA method (7) based on Gledhill's method (8), the shaken reaction vessel is closed to the atmosphere and CO_2 produced is collected in an internal alkaline trap directly from the gaseous phase, as in classical Warburg/Barcroft respirometer flasks.

3. However, inorganic carbon (IC) has been shown to accumulate in the medium during the application of the standard, modified Sturm test to a number of chemicals (9). A concentration of IC as high as 8 mg/L was found during the degradation of 20 mg C/L of aniline. Thus, the collection of CO_2 in the alkaline traps did not give a true reflection of the amount of CO_2 produced microbiologically at intermediate times during the degradation. As a result, the specification that >60% theoretical maximum CO_2 production ($ThCO_2$) must be collected within a "10-d window" (the 10 days immediately following the attainment of 10% biodegradation) for a test substance to be classified as readily biodegraded will not be met for some substances which would be so classified using dissolved organic carbon (DOC) removal.

4. When the percentage degradation is a lower value than expected, IC is possibly accumulated in the test solution. Then, the degradability may be assessed with the other OECD ready biodegradability tests.

5. Other drawbacks of the Sturm methodology (cumbersome, time-consuming, more prone to experimental error and not applicable to volatile substances) had earlier prompted a search for a sealed vessel technique, other than Gledhill's, rather than gas flow-through (10)(11). Boatman et al (12) reviewed the earlier methods and adopted an enclosed headspace system in which the CO_2 was released into the headspace at the end of incubation by acidifying the medium. CO_2 was measured by gas chromatography (GC)/IC analysis in automatically taken samples of the headspace but dissolved inorganic carbon (DIC) in the liquid phase was not taken into account. Also, the vessels used were very small (20 ml) containing only 10 ml of medium, which caused problems e.g. when adding the necessarily very small amounts of insoluble test substances, and/or there may be insufficient or no microorganisms present in the inoculated medium that are competent to degrade the test substances.

6. These difficulties have been overcome by the independent studies of Struijs and Stoltenkamp (13) and of Birch and Fletcher (14), the latter being inspired by their experience with apparatus used in the anaerobic biodegradation test (15). In the former method (13) CO_2 is measured in the headspace after acidification and equilibration, while in the latter (14) DIC in both the gaseous and liquid phases was measured, without treatment; over 90% of the IC formed was present in the liquid phase. Both methods had advantages over the Sturm test in that the test system was more compact and manageable, volatile chemicals can be tested and the possibility of delay in measuring CO_2 produced is avoided.

7. The two approaches were combined in the ISO Headspace CO_2 Standard (16), which was ring-tested (17) and it is this Standard which forms the basis of the present Guideline. Similarly, the two approaches have been used in the US EPA method (18). Two methods of measuring CO_2 have been recommended namely, CO_2 in headspace after acidification (13) and IC in the liquid phase after the addition of excess alkali. The latter method was introduced by Peterson during the CONCAWE ring test (19) of this headspace method modified to measure inherent biodegradability. The changes made in the 1992 (20) revision of OECD Guidelines for Ready Biodegradability have been incorporated into this Guideline, so that the conditions (medium, duration etc) are otherwise the same as those in the revised Sturm test (20). Birch and Fletcher (14) have shown that very similar results were obtained with this headspace test as were obtained with the same chemicals in the OECD Ring Test (21) of the revised Guidelines.

PRINCIPLE OF THE TEST

8. The test substance, normally at 20 mgC/L, as the sole source of carbon and energy, is incubated in a buffer-mineral salts medium which has been inoculated with a mixed population of micro-organisms. The test is performed in sealed bottles with a headspace of air, which provides a reservoir of oxygen for aerobic biodegradation. The CO_2 evolution resulting from the ultimate aerobic biodegradation of the test substance is determined by measuring the IC produced in the test bottles in excess of that produced in blank vessels containing inoculated medium only. The extent of biodegradation is expressed as a percentage of the theoretical maximum IC production (ThIC), based on the quantity of test substance (as organic carbon) added initially.

9. The DOC removal and/or the extent of primary biodegradation of the test substance can also be measured (20).

INFORMATION ON THE TEST SUBSTANCE

10. The organic carbon content (% w/w) of the test substance needs to be known, either from its chemical structure or by measurement, so that the percentage degradation may be calculated. For volatile test substances, a measured or calculated Henry's law constant is helpful for determining a suitable headspace to liquid volume ratio. Information on the toxicity of the test substance to micro-organisms is useful in selecting an appropriate test concentration and for interpreting results showing poor biodegradability: it is recommended to include the inhibition control unless it is known that the test substance is not inhibitory to microbial activities (see paragraph 24).

APPLICABILITY OF THE METHOD

11. The test is applicable to water-soluble and insoluble test substances, though good dispersion of the substance should be ensured. Using the recommended headspace to liquid volume ratio of 1:2, volatile substances with a Henry's law constant of up to 50 $Pa.m^3.mol^{-1}$ can be tested as the proportion of test substance in the headspace will not exceed 1% (13). A smaller headspace volume may be used when testing substances, which are more volatile, but their bioavailability may be limiting especially if they are poorly soluble in water. However, users must ensure that the headspace to liquid volume ratio and the test substance concentration are such that sufficient oxygen is available to allow complete aerobic biodegradation to occur (e.g. avoid using a high substrate concentration and a small headspace volume). Guidance on this matter can be found in (13)(23).

REFERENCE SUBSTANCES

12. In order to check the test procedure, a reference substance of known biodegradability should be tested in parallel. For this purpose, aniline, sodium benzoate or ethylene glycol may be used when testing water-soluble test substances and 1-octanol for poorly soluble test substances (13). Biodegradation of these substances must reach >60% ThIC within 14 days.

REPRODUCIBILITY

13. In the ISO ring test of the method (17), the following results were obtained using the recommended conditions, including 20 mgC test substance/L.

Test Substance	Mean Percentage Biodegradation (28d)	Coefficient of variation (%)	Number of Laboratories
Aniline	90	16	17
1-Octanol	85	12	14

Within-test variability (replicability), using aniline, was low with coefficients of variability not greater than 5% in nearly all test runs. In the two cases in which the replicability was worse, the greater variability was probably due to high IC production in the blanks. Replicability was worse with 1-octanol but was still less than 10% for 79% of test runs. This greater within-test variability may have been due to dosing errors, as a small volume (3 to 4 µl) of 1-octanol had to be injected into sealed test bottles. Higher coefficients of variation would result when lower concentrations of test substance are used, especially at concentrations lower than 10 mgC/L. This could be partially overcome by reducing the concentration of total inorganic carbon (TIC) in the inoculum.

14. In an EU ring-test (24) of five surfactants added at 10 mgC/L, the following results were obtained:

Test Substance	Mean Percentage biodegradation (28d)	Coefficient of variation (%)	Number of laboratories
Tetrapropylene Benzene sulphonate	17	45	10
Di-iso-octylsulpho-Succinate (anionic)	72	22	9
Hexadecyl-trimethyl* Ammonium chloride (cationic)	75	13	10
Iso-Nonylphenol - $(ethoxylate)_9$ (non-ionic)	41	32	10
Coco-amide-propyl Dimethylhydroxy Sulphobetaine (amphoteric)	60	23	11

* SiO_2 was added to neutralize toxicity.
The results show that generally, the variability was higher for the less well-degraded surfactants. Within-test variability was less than 15% for over 90% of cases, the highest reaching 30-40%.

NOTE: Most surfactants are not single molecular species but are mixtures of isomers, homologues, etc. which degrade after different characteristic lag periods and at different kinetic rates resulting in "blurred", extenuated curves, so that the 60% pass value may not be reached within "the 10-d window", even though each individual molecular species would reach >60% within 10 days if tested alone. This may be observed with other complex mixtures as well.

DESCRIPTION OF THE METHOD

Apparatus

15. Normal laboratory apparatus and:

(a) Glass serum bottles, sealed with butyl rubber stoppers and crimp-on aluminium seals. The recommended size is '125 ml' which have a total volume of around 160 ml (In this case the volume of each bottle should be known to be 160 ± 1 ml). A smaller size of vessel may be used when the results fulfil the conditions described in paragraph 66 and 67;
(b) Carbon analyser or other instrument (e.g. gas chromatograph) for measuring inorganic carbon;
(c) Syringes of high precision for gaseous and liquid samples;
(d) Orbital shaker in a temperature-controlled environment;
(e) A supply of CO_2 free air - this can be prepared by passing air through soda lime granules or by using an 80% N_2 / 20% 0_2 gas mixture (optional) (see paragraph 28);
(f) Membrane-filtration device of 0.20 - 0.45 µm porosity (optional);
(g) Organic carbon analyser (optional).

Reagents

16. Use analytical grade reagents throughout.

Water

17. Distilled or de-ionised water should be used containing ≤1 mg/L as total organic carbon. This represents ≤ 5% of the initial organic carbon content introduced by the recommended dose of the test substance.

Stock solutions for the mineral salts medium

18. The stock solutions and the mineral salts medium are similar to those in ISO 14593 (16) and OECD 301 "ready biodegradability" tests (20). The use of a higher concentration of ammonium chloride (2.0 g/L instead of 0.5 g/L) should only be necessary in very exceptional cases, e.g. when the test substance concentration is > 40 mg C/L. Stock solutions should be stored under refrigeration and disposed of after six months, or earlier if there is evidence of precipitation or microbial growth. Prepare the following stock solutions:

(a) Potassium dihydrogen phosphate (KH_2PO_4) 8.50g
Dipotassium hydrogen phosphate (K_2HPO_4) 21.75g
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) 33.40g
Ammonium chloride (NH_4Cl) 0.50g

Dissolve in water and make up to 1 litre. The pH of this solution should be 7.4 (± 0.2). If this is not the case, then prepare a new solution.

(b) Calcium chloride dihydrate ($CaCl_2.2H_2O$) 36.40g

Dissolve in water and make up to 1 litre.

(c) Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 22.50g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate ($FeCl_3.6H_20$) 0.25g

Dissolve in water and make up to 1 litre and add one drop of concentrated.

Preparation of mineral medium

19. Mix 10 ml of solution (a) with approximately 800 ml water (paragraph 17), then add 1 ml of solutions (b), (c) and (d) and make up to 1 litre with water (paragraph 17).

Other reagents

20. Concentrated ortho-phosphoric acid (H_3PO_4) (>85% mass per volume).

Sodium hydroxide solution 7M

21. Dissolve 280 g of sodium hydroxide (NaOH) in 1 litre of water (paragraph 17). Determine the concentration of DIC of this solution and consider this value when calculating the test result (see paragraphs 55 and 61), especially in the light of the validity criterion in paragraph 66 (b). Prepare a fresh solution if the concentration of DIC is too high.

Test substance

22. Prepare a stock solution of a sufficiently water-soluble test substance in water (paragraph 17) or in the test medium (paragraph 19) at a concentration preferably 100-fold greater than the final concentration to be used in the test; it may be necessary to adjust the pH of the stock solution. The stock solution should be added to the mineral medium to give a final organic carbon concentration of between 2 and 40 mgC/L, preferably 20mgC/L. If concentrations lower than these are used, the precision obtained may be impaired. Soluble and insoluble liquid substances may be added to the vessels directly using high precision syringes. Poorly soluble and insoluble test substances may require special treatment (25). The choices are:

(a) direct addition of known weighed amounts;
(b) ultrasonic dispersion before addition;
(c) dispersion with the aid of emulsifying agents to be required to establish whether they have any inhibitory or stimulatory effects on microbial activity before addition;
(d) adsorption of liquid test substances, or a solution in a suitable volatile solvent, on to an inert medium or support (e.g. glass fibre filter), followed by evaporation of the solvent, if used, and direct addition of known amounts;
(e) addition of known volume of a solution of the test substance in an easily volatile solvent to an empty test vessel, followed by evaporation of the solvent.

Agents or solvents used in (c), (d) and (e) have to be tested for any stimulatory or inhibitory effect on microbial activity (see paragraph 42(b).)

Reference substance

23. Prepare a stock solution of the (soluble) reference substance in water (paragraph 17) at a concentration preferably 100-fold greater than the final concentration to be used (20 mg C/L) in the test.

Inhibition check

24. Test compounds frequently show no significant degradation under the conditions used in ready biodegradation assessments. One possible cause is that the test substance is inhibitory to the inoculum at the concentration at which it is applied in the test. An inhibition check may be included in the test design to facilitate identification (in retrospect) of inhibition as a possible cause or contributory factor. Alternatively, the inhibition check may rule out such interferences and show that zero or slight degradation is attributable solely to non-amenability to microbial attack under the conditions of the test. In order to obtain information on the toxicity of the test substance to (aerobic) micro-organisms, prepare a solution in the test medium containing the test substance and the reference substance (paragraph 19), each at the same concentrations as added, respectively (see paragraph 22 and 23).

Inoculum

25. The inoculum may be derived from a variety of sources: activated sludge; sewage effluent (non-chlorinated); surface waters and soils; or from a mixture of these (20). The biodegradative activity of the source should be checked by using a reference substance. Whatever the source, micro-organisms previously exposed to the test substance should not be used if the procedure is to be used as a test for ready biodegradability.

Warning: Activated sludge, sewage and sewage effluent contain pathogenic organisms and must be handled with caution.

26. Based on experience, the optimal volume for the inoculum is that which:

- is sufficient to give adequate biodegradative activity;
- degrades the reference substance by the stipulated percentage (see paragraph 66);
- gives 10^2 to 10^5 colony-forming units per millilitre in the final mixture;
- normally gives a concentration of 4 mg/L suspended solids in the final mixture when activated sludge is used, concentrations up to 30 mg/L may be used but may significantly increase CO_2 production of the blanks (26);
- contributes less than 10% of the initial concentration of organic carbon introduced by the test substance;
- is generally 1-10 ml of inoculum for 1 litre of test solution.

Activated sludge

27. Activated sludge is freshly collected from the aeration tank of a sewage treatment plant or laboratory-scale unit treating predominantly domestic sewage. If necessary, coarse particles should be removed by sieving (e.g. using a 1 mm^2 mesh sieve) and the sludge should be kept aerobic until used.

28. Alternatively, after removal of any coarse particles, settle or centrifuge (e.g. 1100 x g for 10 minutes). Discard the supernatant liquid. The sludge may be washed in the mineral solution. Suspend the concentrated sludge in mineral medium to yield a concentration of 3-5 g suspended solids/L. Thereafter aerate until required.

29. Sludge should be taken from a properly working conventional treatment plant. If sludge has to be taken from a high rate treatment plant, or is thought to contain inhibitors, it should be washed. Settle or centrifuge the re-suspended sludge after thorough mixing, discard the supernatant liquid and again suspend the washed sludge in a further volume of mineral medium. Repeat this procedure until the sludge is considered to be free from excess substrate or inhibitor.

30. After complete re-suspension is achieved, or with untreated sludge, withdraw a sample just before use for the determination of the dry weight of the suspended solids.

31. A further alternative is to homogenise activated sludge (3-5 g suspended solids/L). Treat the sludge in a Waring blender for 2 minutes at medium speed. Settle the blended sludge for 30 minutes or longer if required and decant liquid for use as inoculum at the rate of about 10 mg/L of mineral medium.

32. Still further reduction of the blank CO_2 evolution can be achieved by aerating the sludge overnight with CO_2-free air. Use 4 mg/L activated sludge solids as the concentration of the inoculum in this test (13).

Secondary sewage effluent

33. Alternatively, the inoculum can be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Maintain the sample under aerobic conditions and use on the day of collection, or pre-condition if necessary. The effluent should be filtered through a coarse filter to remove gross particulate matter and the pH value is measured.

34. To reduce its IC content, the filtrate is sparged with CO_2-free air (paragraph 15-e) for 1 h while maintaining the pH at 6.5 using orthophosphoric acid (paragraph 20). The pH value is restored to its original value with sodium hydroxide (paragraph 21) and after settling for about 1 h a suitable volume of the supernatant is taken for inoculation. This sparging procedure reduces the IC content of the inoculum. For example, when the maximum recommended volume of filtered sparged effluent (100 ml) per litre was used as inoculum, the amount of IC present in blank control vessels was in the range 0.4 to 1.3 mg/L (14), representing 2-6.5% of test substance C at 20 mgC/L and 4-13% at 10 mgC/L.

Surface waters

35. A sample is taken of an appropriate surface water. It should be kept under aerobic conditions and used on the day of collection. The sample should be concentrated, if necessary, by filtration or centrifugation. The volume of inoculum to be used in each test vessel should meet the criteria given in paragraph 26.

Soils

36. A sample is taken of an appropriate soil, collected to a depth of up to 20 cm below the soil surface. Stones, plant remains and invertebrates should be removed from the sample of soil before it is sieved through a 2 mm mesh (if the sample is too wet to sieve immediately, then partially air dry to facilitate sieving). It should be kept under aerobic conditions and used on the day of collection (If the sample is transported in a loosely-tied black polythene bag, it can be stored at 2 to 4 ^{o}C in the bag for up to one month).

Preconditioning of inoculum

37. Inoculum may be pre-conditioned to the experimental conditions, but not pre-adapted to the test substance. Pre-conditioning can reduce the blank CO_2 evolution. Pre-conditioning consists of aerating activated sludge after diluting in test medium to 30 mg/L with moist CO_2-free air for up to 5-7 days at the test temperature.

TEST PROCEDURE

Number of bottles

38. The number of bottles (paragraph 15-a) needed for a test will depend on the frequency of analysis and the test duration.

39. It is recommended that triplicate bottles be analysed after a sufficient number of time intervals such that the 10-d window may be identified. Also at least five test bottles (paragraph 15-a) from sets (a), (b) and (c) (see paragraph 42) are analysed at the end of the test, to enable 95% confidence intervals to be calculated for the mean percentage biodegradation value.

Inoculated medium

40. The inoculum is used at a concentration of 4 mg/L activated sludge dry solids. Prepare immediately before use sufficient inoculated medium by adding, for example, 2 ml suitably treated activated sludge (paragraphs 27 to 32) at 2000 mg/L to 1 litre of mineral salts medium (paragraph 19). When secondary sewage effluent is to be used add up to 100 ml effluent (paragraph 33) to 900 ml mineral salts medium (paragraph 19) and dilute to 1 litre with medium.

Preparation of bottles

41. Aliquots of inoculated medium are dispensed into replicate bottles to give a headspace to liquid ratio of 1:2 (e.g. add 107 ml to 160 ml-capacity bottles). Other ratios may be used, but see the warning given in paragraph 11. When using either type of inoculum, care must be taken to ensure that the inoculated medium is adequately mixed to ensure that it is uniformly distributed to the test bottles.

42. Sets of bottles (paragraph 15a) are prepared to contain the following:

(a) Test vessels (denoted F_T) containing the test substance;
(b) Blank controls (denoted F_B) containing only the test medium plus inoculum; any chemicals, solvents, agents or glass fibre filters used to introduce the test substance into the test vessels must also be added;
(c) Vessels (denoted F_C) for checking the procedure containing the reference substance;
(d) If needed, vessels (denoted F_I) for checking a possible inhibitory effect of the test substance containing both the test substance and reference substance at the same concentrations (paragraph 24) as in bottles F_T and F_C, respectively;
(e) Vessels (denoted F_S) for checking a possible abiotic degradation as (a) plus 50 mg/L $HgCl_2$ or sterilised by some other means (e.g. by autoclaving).

43. Water-soluble test substances and reference substances are added as aqueous stock solutions (paragraphs 22, 23 and 24) to give a concentration of 10 to 20 mg C/L.

44. Insoluble test and insoluble reference substances are added to bottles in a variety of ways (see paragraph 22a-e) according to the nature of the substance, either before or after addition of the inoculated medium, depending on the method of treatment of the substance. If one of the procedures given in paragraph 22a-e is used, then the blank bottles F_B (paragraph 42b) should be treated in a similar fashion but excluding the test or reference substance.

45. Volatile test substances should be injected into sealed bottles (paragraph 47) using a micro syringe. The dose is calculated from the volume injected and the density of the substance.

46. Water should be added to vessels, where necessary, to give the same liquid volume in each vessel. It must be ensured that the headspace to liquid ratio (usually 1:2) and concentration of the test substance are such that sufficient oxygen is available in the headspace to allow for complete biodegradation.

47. All bottles are then sealed for example, with butyl rubber septa and aluminium caps. Volatile tests substances should be added at this stage (paragraph 45). If the decrease in DOC concentration of the test solution is to be monitored and for time zero analyses to be performed for initial IC concentration

(sterile controls, paragraph 42e) or other determinands, remove an appropriate sample from the test vessel. The test vessel and its contents are then discarded.

48. The sealed bottles are placed on a rotary shaker (paragraph 15d), with a shaking rate sufficient to keep the bottle contents well mixed and in suspension (e.g. 150 to 200 rpm), and incubated in the dark at 20°C, to be kept within ± 1°C.

Sampling

49. The pattern of sampling will depend on the lag period and kinetic rate of biodegradation of the test substance. Bottles are sacrificed for analysis on the day of sampling, which should be at least weekly or more frequently (e.g. twice per week) if a complete degradation curve is required. The requisite number of replicate bottles is taken from the shaker, representing F_T, F_B and F_C and, if used F_I and F_S (see paragraph 42). The test normally runs for 28d. If the biodegradation curve indicates that a plateau has been attained before 28d, the test may be concluded earlier than 28d. Take samples from the five bottles reserved for the 28th day of the test for analysis and use the results to calculate the confidence limits or coefficient of variation of percentage biodegradation. Bottles representing the checks for inhibition and for abiotic degradation need not be sampled as frequently as the other bottles; day 1 and day 28 would be sufficient.

Inorganic carbon (IC) analysis

50. CO_2 production in the bottles is determined by measuring the increase in the concentration of inorganic carbon (IC) during incubation. There are two recommended methods available for measuring the amount of IC produced in the test, and these are described immediately below. Since the methods can give slightly different results only one should be used in a test run.

51. Method (a) is recommended if the medium is likely to contain remnants of, for example, a glass-filter paper and/or insoluble test substance. This analysis can be performed using a gas chromatograph if a carbon analyser is not available. It is important that the bottles should be at or close to the test temperature when the headspace gas is analysed. Method (b) can be easier for laboratories using carbon analysers to measure IC. It is important that the sodium hydroxide solution (paragraph 21) used to convert CO_2 to carbonate is either freshly prepared or its IC content is known, so that this can be taken into account when calculating the test results (see paragraph 66-b.)

Method (a): acidification to pH <3

52. Before each batch of analyses, the IC analyser is calibrated using an appropriate IC standard (e.g. 1% w/w CO_2 in N_2). Concentrated orthophosphoric acid (paragraph 20) is injected through the septum of each bottle sampled to lower the pH of the medium to <3 (e.g. add 1 ml to 107 ml test medium). The bottles are placed back on the shaker. After shaking for one hour at the test temperature the bottles are removed from the shaker, aliquots (e.g. 1 ml) of gas are withdrawn from the headspace of each bottle and injected into the IC analyser. The measured IC concentrations are recorded as mg C/L.

53. The principle of this method is that after acidification to pH <3 and equilibration at 20°C, the equilibrium constant for the distribution of CO_2 between the liquid and gaseous phases in the test bottles is 1.0 when measured as a concentration (13). This should be demonstrated for the test system at least once as follows:

Set up bottles containing 5 and 10 mg/L as IC using a solution of anhydrous sodium carbonate (Na_2 CO_3) in CO_2-free water prepared by acidifying water to pH 6.5 with concentrated ortho-phosphoric acid (paragraph 20), sparging overnight with CO_2-free air and raising the pH to neutrality with alkali. Ensure that the ratio of the headspace volume to the liquid volume is the same as in the tests (e.g. 1:2). Acidify and equilibrate as described in paragraph 52, and measure the IC concentrations of both the headspace and liquid phases. Check that the two concentrations are the same within experimental error. If they are not, the operator should review the procedures.

This check on the distribution of IC between liquid and gaseous phases need not be made every time the test is performed; it could presumably be made while performing the calibration.

54. If DOC removal is to be measured (water-soluble test substances only), samples should be taken of the liquid phase from separate (non-acidified) bottles, membrane -filtered and injected into the DOC analyser. These bottles can be used for other analyses as necessary, to measure primary biodegradation.

Method (b): conversion of CO_2 to carbonate

55. Before each batch of analyses, the IC analyser is calibrated using an appropriate standard - for example, a solution of sodium bicarbonate ($NaHCO_3$) in CO_2 free water (see paragraph 53) in the range 0 to 20 mg/L as IC. Sodium hydroxide solution (7M, paragraph 21) (e.g. 1 ml to 107 ml medium) is injected through the septum of each bottle sampled and the bottles are shaken for 1 h at the test temperature. Use the same NaOH solution on all bottles sacrificed on a particular day, but not necessarily on all sampling occasions throughout a test. If absolute blank IC values are required at all sampling occasions, IC determinations of the NaOH solution will be required each time it is used. The bottles are removed from the shaker and allowed to settle. Suitable volumes (e.g. 50 to 1000 µl) of the liquid phase in each vessel are withdrawn by syringe. The samples are injected into the IC analyser and the concentrations of IC are recorded. It should be ensured that the analyser used is equipped properly to deal with the alkaline samples produced in this method.

56. The principle of this method is that after the addition of alkali and shaking, the concentration of IC in the headspace is negligible. This should be checked for the test system at least once by using IC standards, adding alkali and equilibrating, and measuring the concentration of IC in both the headspace and liquid phases (see paragraph 53). The concentration in the headspace should approach zero. This check on the virtually complete absorption of CO_2 need not be made every time the test is performed.

57. If DOC removal is to be measured (water-soluble test substances only), samples should be taken of the liquid phase from separate bottles (containing no added alkali), membrane filtered and injected into the DOC analyser. These bottles can be used for other analyses, as necessary, to measure primary biodegradability.

DATA AND REPORTING

Calculating of results

58. Assuming 100% mineralisation of the test substance to CO_2, the ThIC in excess of that produced in the blank controls equals the TOC added to each test bottle at the start of the test, that is:

ThIC = TOC.

The total mass (mg) of inorganic carbon (TIC) in each bottle is:

$$TIC = (\text{mg C in the liquid} + \text{mg C in the headspace}) = (V_L \times C_L) + (V_H \times C_H) \quad \text{Equation [1]}$$

where :

V_L = volume of liquid in the bottle (litre);
C_L = concentration of IC in the liquid (mg/L as carbon);
V_H = volume of the headspace (litre);
C_H = concentration of IC in the headspace (mg/L as carbon).

The calculations of TIC for the two analytical methods used for measuring IC in this test are described below in paragraphs 60 and 61. Percentage biodegradation (*% D*) in each case is given by:

$$\%D = \frac{(TIC_t - TIC_b)}{TOC} \times 100 \quad \text{Equation [2]}$$

where :

TIC_t = mg TIC in test bottle at time t;
TIC_b = mean mg TIC in blank bottles at time t;
TOC = mg TOC added initially to the test vessel.

The percentage biodegradation % D is calculated for the test (F_T), reference (F_C) and, if included inhibition monitoring control (F_I) bottles from the respective amounts of TIC produced up to each sampling time.

59. If there has been a significant increase in the TIC content of the sterile controls (F_S) over the test period, then it may be concluded that abiotic degradation of the test substance has occurred and this must be taken into account in the calculation of D in Equation [2].

Acidification to pH <3

60. Since acidification to pH <3 and equilibration results in the equalisation of the concentration of TIC in the liquid and gaseous phases, only the concentration of IC in the gas phase needs to be measured. Thus, from Equation [1] TIC = $(V_L + V_H) \times C_H = V_B \times C_H$, where V_B = volume of the serum bottle.

Conversion of CO_2 to carbonate

61. In this method calculations are performed as in Equation [1], but the negligible amount of IC in the gaseous phase is ignored, that is $V_H \times C_H = 0$, and TIC = $V_L \times C_L$.

Expression of Results

62. A biodegradation curve is obtained by plotting percentage biodegradation, D, against time of incubation and if possible, the lag phase, biodegradation phase, 10-d window and plateau phase, that is the phase in which the maximal degradation has been reached and the biodegradation curve has levelled out, are indicated. If comparable results are obtained for parallel test vessels F_T (<20% difference), a mean curve is plotted (see Annex 2, Fig.1); if not, curves are plotted for each vessel. The mean value of the percentage biodegradation in the plateau phase is determined or the highest value is assessed (e.g. when the curve decreases in the plateau phase), but it is important to assess that in the latter case the value is not an outlier. Indicate this maximum level of biodegradation as "degree of biodegradation of the test substance"

in the test report. If the number of test vessels was insufficient to indicate a plateau phase, the measured data of the last day of the test are used to calculate a mean value. This last value, the mean of five replicates, serves to indicate the precision with which the percentage biodegradation was determined. Also report the value obtained at the end of the 10-d window.

63. In the same way, a curve for the reference substance, F_C, is plotted and, if included, for the abiotic elimination check, F_S and the inhibition control, F_I.

64. The amounts of TIC present in the blank controls (F_B) are recorded as are those in flasks F_S (abiotic check), if these vessels were included in the test.

65. Calculate D for the F_I vessels, based on the theoretical IC yield anticipated from only the reference component of the mixture. If, at day 28, $[(D_{FC}^{1} - D_{FI}^{2})/D_{FC}]$ x 100 > 25%, it may be assumed that the test substance inhibited the activity of the inoculum, and this may account for low values of D_{FT} obtained under the conditions of the test. In this case the test could be repeated using a lower test concentration and preferably reducing the DIC in the inoculum and TIC formed in the blank controls, since the lower concentration will otherwise reduce the precision of the method. Alternatively, another inoculum may be used. If in flask F_S (abiotic) a significant increase (>10%) in the amount of TIC is observed, abiotic degradation processes may have occurred.

Validity of results

66. A test is considered valid if:
   (a) the mean percentage degradation in vessels F_C containing the reference substance is >60% by the 14th day of incubation; and
   (b) the mean amount of TIC present in the blank controls F_B at the end of the test is <3mg C/L.

If these limits are not met, the test should be repeated with an inoculum from another source and/or the procedures used should be reviewed. For example, if high blank IC production is a problem the procedure given in paragraphs 27 to 32 should be followed.

67. If the test substance does not reach 60% ThIC and was shown not to be inhibitory (paragraph 65), the test could be repeated with increased concentration of inoculum (up to 30 mg/L activated sludge and 100 ml effluent/L) or inocula from other sources, especially if degradation had been in the range 20 to 60%.

Interpretation of results

68. Biodegradation >60% ThIC within the 10-d window in this test demonstrates that the test substance is readily biodegradable under aerobic conditions.

69. If the pass value of 60% ThIC is not attained, determine the pH value in media in bottles which have not been made acid or alkaline; a value of less than 6.5 could indicate that nitrification had occurred. In such a case repeat the test with a buffer solution of higher concentration.

[1] The percentage degradation in Vessels F_C containing the reference substance

[2] The percentage degradation in Vessels F_I

Test Report

70. Compile a table of % D for each test (F_T), reference (F_C) and, if included, inhibition control bottle (F_I) for each day sampled. If comparable results are obtained for replicate bottles, plot a curve of mean % D against time. Record the amount of TIC in the blanks (F_B) and in the sterile controls (F_S) DOC and/or other determinands, and their percentage removal.

71. Determine the mean value of % D in the plateau phase, or use the highest value if the biodegradation curve decreases in the plateau phase, and report this as the 'degree of biodegradation of the test substance'. It is important to ensure that in the latter case the highest value is not an outlier.

72. The test report must include the following information:

Test substance:

- common name, chemical name, CAS number, structural formula and relevant physical-chemical properties;
- purity (impurities) of test substance.

Test conditions:

- reference to this Test Guideline;
- description of the test system used (e.g. volume of the vessel, head space to liquid ratio, method of stirring, etc);
- application of test substance and reference substance to test system: test concentration used and amount of carbon dosed into each test bottle, any use of solvents;
- details of the inoculum used, any pre-treatment and pre-conditioning;
- incubation temperature;
- validation of the principle of IC analysis;
- main characteristics of the IC analyser employed (and any other analytical methods used);
- number of replicates.

Results:

- raw data and calculated values of biodegradability in tabular form;
- the graph of percentage degradation against time for the test and reference substances, the lag phase, degradation phase, 10-d window and slope;
- percentage removal at plateau, at end of test, and after 10-d window;
- reasons for any rejection of the test results;
- any other facts that are relevant to the procedure followed;
- discussion of results.

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ANNEX 1

ABBREVIATIONS AND DEFINITIONS

IC: Inorganic carbon

$ThCO_2$: Theoretical carbon dioxide (mg) is the quantity of carbon dioxide calculated to be produced from the known or measured carbon content of the test substance when fully mineralised; also expressed as mg carbon dioxide evolved per mg test substance.

DOC: Dissolved organic carbon is the organic carbon present in solution or that which passes through a 0.45 micrometre filter or remains in the supernatant after centrifuging at approx. 4000 g (about 40.000 m sec^{-2}) for 15 min.

DIC: Dissolved inorganic carbon

ThIC: Theoretical inorganic carbon

TIC: Total inorganic carbon

Readily Biodegradable: An arbitrary classification of chemicals which have passed certain specified screening tests for ultimate biodegradability; these tests are so stringent that it is assumed that such substances will rapidly and completely biodegrade in aquatic environments under aerobic conditions.

10-d window: The 10 days immediately following the attainment of 10% biodegradation.

Inherent Biodegradability: A classification of chemicals for which there is unequivocal evidence of biodegradation (primary or ultimate) in any test of biodegradability.

Ultimate Aerobic Biodegradation: The level of degradation achieved when the test substance is totally utilised by micro-organisms resulting in the production of carbon dioxide, water, mineral salts and new microbial cellular constituents (biomass).

Mineralisation: Mineralisation is the complete degradation of an organic compound to CO_2 and H_2O under aerobic conditions, and CH_4, CO_2 and H_2O under anaerobic conditions.

Lag Phase: The time from the start of a test until acclimatization and/or adaptation of the degrading microorganisms 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).

Degradation Phase: The time from the end of the lag period to the time when 90 % of the maximum level of degradation has been reached.

Plateau Phase: Plateau phase is the phase in which the maximal degradation has been reached and the biodegradation curve has levelled out.

ANNEX 2

EXAMPLE OF A BIODEGRADATION CURVE

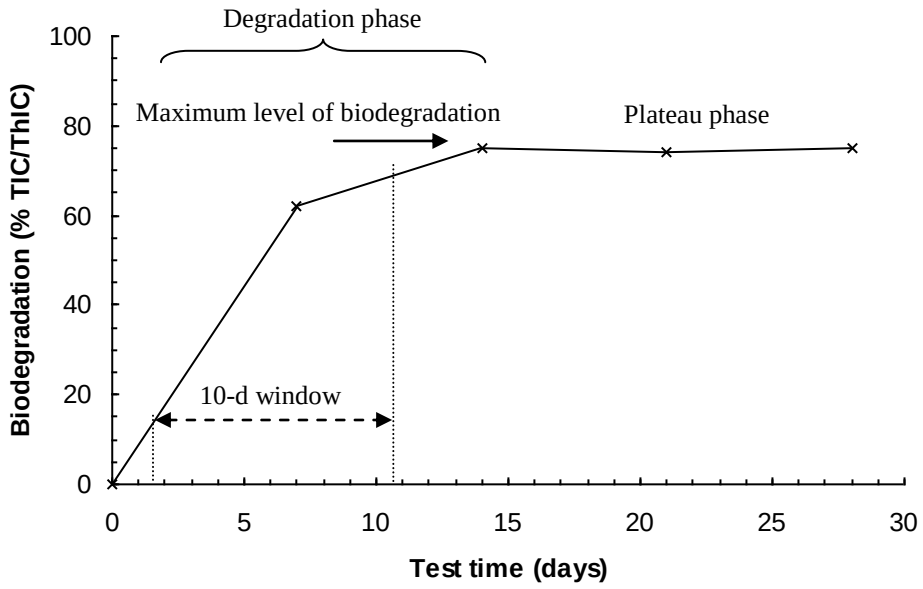

Figure 1: Biodegradation of 1-octanol in the CO_2 headspace test