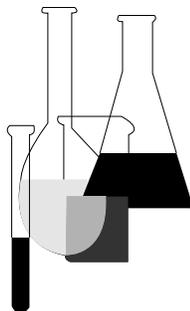




Fate, Transport and Transformation Test Guidelines

OPPTS 835.3300 Soil Biodegradation



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202-512-1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202-512-0132 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from EPA's World Wide Web site (<http://www.epa.gov/epahome/research.htm>) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines."

OPPTS 835.3300 Soil biodegradation.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are the OPPT guideline under 40 CFR 796.3400 and OECD 304 A Inherent Biodegradability in Soil.

(b) **Introductory information**—(1) **Prerequisites.** ¹⁴C-labeled material is required.

(2) **Guidance information.** Information on the toxicity of the test compound is useful for the interpretation of the data obtained. The concentration of the test compound can then be adapted to this information.

(3) **Qualifying statements.** The test is applicable to volatile or non-volatile, soluble or insoluble compounds which are not inhibitory to microorganisms. The mineralization rate refers to the labeled carbonation only. Therefore, the location of the labelling within the structure and the specificity of the label need careful consideration.

(4) **Recommendations.** (i) The results obtained using the basic mineralization test may be supported by determination of the evaporation rate of the parent compound and some of possible volatile metabolites and by determination of soil extractable and nonextractable residues. Both optional tests are described in this test guideline.

(ii) Sometimes it is recommended that information about chemical degradation under anaerobic conditions be obtained. Therefore, in accordance with the description below, the biometer flask filled with the soil sample (preconditioning is not necessary), is flooded with water (2–3 cm layer) to protect against leakage, evacuated and flushed with nitrogen several times. Degradation may be evaluated by means of measurements of methane gas and analysis of both water and soil for ¹⁴C-content.

(5) **Standard documents.** This test guideline is based on the method cited in paragraph (e)(1) of this guideline.

(c) **Method**—(1) **Introduction, purpose, scope, relevance, application and limits of test.** (i) The method described in this test guideline is designed for the evaluation of the mineralization rate of a ¹⁴C-labeled compound in soil. The method is applicable to volatile or nonvolatile, soluble or insoluble compounds which are not inhibitory to microorganisms.

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

it has grown with time, in characteristic layers of a variety of soil types, or disturbed, as it is usually sampled by digging and used in the test described here.

Mineralization (in the context of this guideline) means extensive degradation of a molecule during which a labelled carbon atom is oxidized quantitatively with release of the appropriate amount of $^{14}\text{CO}_2$.

(iii) **Reference substances.** In some cases when investigating a new substance reference substances may be useful; however, reference substances cannot yet be recommended. Reference substances need not be employed in all cases when investigating a new substance. They may primarily be used so that calibration of the method may be performed from time to time and to permit comparison of results when another method is employed.

(iv) **Principle of the test method—(A) Basic test.** (1) A small sample of soil is treated with the ^{14}C -labeled test chemical in a biometer flask apparatus. Release of $^{14}\text{CO}_2$ from the test chemical is measured by means of alkali absorption and liquid scintillation counting.

(2) Optional experiments include the following tests.

(B) **Evaporation test.** When testing chemicals of a vapor pressure higher than 0.0133 Pa, a polyurethane foam plug is placed into the biometer flask apparatus to absorb the labelled volatile part of the parent compound and volatile metabolites for liquid scintillation counting.

(C) **Residue test.** At the point of 50 percent mineralization the test soil may be extracted. The extractable portion of the compound, and its metabolites remaining in the soil, may be determined by liquid scintillation counting. Furthermore, data on the bound residue part may be obtained by measuring the $^{14}\text{CO}_2$ released after combustion of the soil.

(v) **Quality criteria—(A) Reproducibility.** Reproducibility is good if standard conditions, especially preconditioning of the soil, are strictly observed.

(B) **Sensitivity.** The evaluation of sensitivity is not relevant because a moderate amount as 37–185 kBq (\cong 1–5 μCi) of ^{14}C -labeled test chemicals is used for each experiment.

(C) **Specificity.** The method is only applicable if ^{14}C -labeled test chemicals are available. The specificity is very good.

(D) **Possibility of standardization.** This procedure is standardized to a limited extent. The limitation is related to the difficulty of standardization of soil samples between laboratories.

(2) Description of the test procedure—(i) Preparations—(A) Equipment. (1) Liquid scintillation counter.

(2) Oxidizer for combustion of radioactive material.

(3) Ultrasonic bath, 500 mL.

(4) Glassware: 250 mL Erlenmeyer flasks fused to 50 mL round bottom tubes (biometer flasks, see Figure 1); 25 mL syringes (e.g. Luer-Lok); 15 gauge syringe needle, 15 cm in length; 100 μ L syringes (e.g. Hamilton); 25 mL graduated cylinders with stopper; 1 mL pipets; Soxhlet extraction apparatus; scintillation vials; polyurethane plugs, 30 mm diameter, 30 mm length, density 16 kg/m³.

(B) Reagents—(1) Test substance: ¹⁴C-labeled compounds are dissolved in water or acetone to give radioactivity of 37–185 KBq (\cong 1–5 μ Ci)/100 μ L. Using unlabeled material this solution is made up to the required concentration (e.g. 0.5 mg/100 μ L \cong 10 mg/kg soil, or depending on the toxicity of the substance).

(2) **Chemicals.** (i) KOH, analytical grade, 0.1 N solution.

(ii) Acetone, analytical grade.

(iii) Methanol, analytical grade (for optional tests).

(iv) *n*-Hexane, analytical grade (for optional test).

(v) Ascarite (A.H. Thomas Co. Philadelphia or equivalent).

(vi) Scintillation cocktail.

(3) **Soil.** (i) Alfisol: pH between 5.5 and 6.5 organic carbon content between 1 and 1.5 percent clay content (i.e. particles <0.002 mm in diameter) between 10 and 20 percent cation exchange capacity between 10 and 15 mval.

(ii) Spodosol: pH between 4.0 and 5.0 organic carbon content between 1.5 and 3.5 percent clay content \leq 10 percent cation exchange capacity <10 mval.

(iii) Entisol: pH between 6.6 and 8.0 organic carbon content between 1 and 4 percent clay content between 11 and 25 percent cation exchange capacity >10 mval.

In special cases it is recommended that two additional soils be used: One with high silt-fraction (diameter between 0.002 and 0.063 mm) content, the other with a high clay content (30 percent). Air dried test soil stored at +4 °C is remoisturized to 40 per cent maximum water capacity. After incubation for 2 weeks at 22 \pm 2 °C in the dark it is ready for the experiments.

(ii) **Test conditions**—(A) **Test temperature.** During the whole test period the flasks are incubated in the dark at 22 ± 2 °C.

(B) **Soil characterization data.** (1) for determination of the pH value of the soil for selecting the soil type, 10 g air-dried soil is suspended in 25 mL 0.01 M CaCl₂.

(2) After standing overnight, the sample is disturbed once more and measured in a potentiometric apparatus with a 0.1 M KCl electrode. Immediately before the measurement the instrument must be calibrated with two standard solutions within the measuring range of the sample values expected.

(3) For determination of the organic carbon content of the soil for selecting the soil type, 1.0 g air-dried soil is heated with 15 mL 2M K₂K₁Cr₂O₇ and 20 ml H₂SO₄ (analytical reagent, ρ_c = 1.84 g/cm³) at 145–155 ° C for 15 min. After cooling to room temperature sample volume is made up to 150 mL with distilled water. A 20 mL aliquot is measured spectrophotometrically, after centrifuging, in a 1 cm cuvet at 590 nm compared to distilled water. The self-destroying property of the K₂Cr₂O₇ reagent must be determined by two blank samples. Calculation is conducted using the following equation:

$$C = \frac{1,000 \times v \times E_2(E_x - \alpha_2 \times c)}{e \times E_1 \times (\alpha_1 - \alpha_2 \times F)}$$

where

C = carbon content (percent)

V = gross volume (mL)

E₁ = equivalent weight of Cr₂O₃ (25.332)

E₂ = equivalent weight of carbon (3.0028)

E_x = extinction at 590 nm and 1 cm layer thickness

F = factor for calculating K₂Cr₂O₇ from Cr₂O₃

c = concentration of Cr (g) per 100 mL (= 1.9356)

e = sample weight (mg)

α₁ = extinction coefficient of Cr(III); α₁ is an average value from five single determinations for the calibration curve, each obtained by division of E_x by the amount of Cr₂O₃ (g)

α₂ = extinction coefficient of Cr(VI); α₂ is an average value from two single estimations, each obtained by division of E_x by the respective amounts of K₂Cr₂O₇.

(4) To determine particle size of the soil for selecting the soil type, 10.0 g air-dried soil is reacted with 100 mL H₂O₂ (15 percent W/V) for 15 h, heated until CO₂ evolution is complete. The suspension is left to stand overnight with 25 mL 0.4 N Na₄P₂O₇, after which water is added

to make it up to 250 mL and the solution is sieved through a mesh of 0.2 mm width. The portion <0.2 mm is fractionated further by sieving. The smaller particles (silty fractions) are fractionated by homogenous partitioning of the particles in the aqueous medium, which is made up to 1,000 mL with water in an elutriating cylinder.

(5) Portions (10 mL) are removed by pipet from various heights of the cylinder after different sedimentation times; measurement of the dry weights of the suspended material in these portions yields the particle composition according to the following scheme:

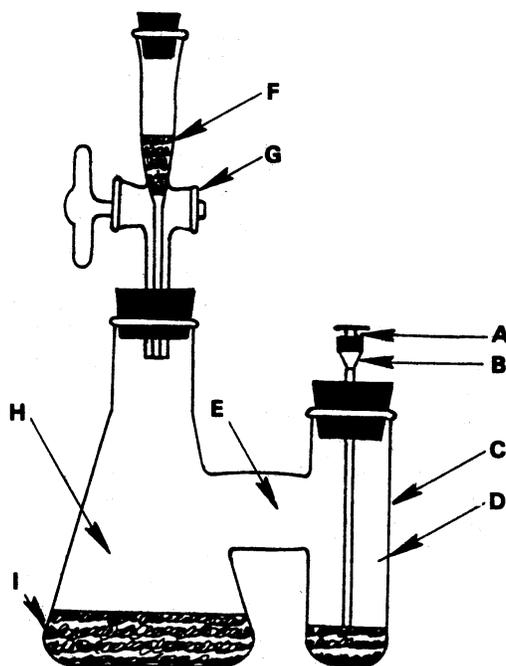
Particle fraction diameter (mm)	Dipping depth (cm)			
	20	15	10	5
<0.002		7h 45min	3h 52min
<0.0063	1h 33min 49sec	1h 10min 52sec	46min 55sec	23min 27sec
<0.02	9min 19sec	6min 59sec	4min 39sec	2min 20sec
<0.063	59sec	42sec	

(6) For determination of the cation exchange capacity of the soil, in order to select the soil type, a glass column 15 cm in length and 30 mm i. d. is reduced in diameter at one end like a funnel. This side is stuffed with glass wool. About 1 cm quartz sand is spread on the wool, followed by 10.0 g air-dried test soil, which is in turn covered by about 1 cm of quartz sand. Forty milliliters of a mixed solution consisting of 100 g triethanolamine in 2 L water (adjusted to pH 8.1 with HCl) plus 100 g BaCl₂·2H₂O in 2 L) is poured over these layers. After 1 h the solution is collected in a 250 mL Erlenmeyer flask. The procedure is repeated. In addition, 40 mL of a solution of 25 g BaCl₂·2H₂O in 1 L is poured onto the column.

(7) This solution is collected after standing overnight and the column is washed with 100 mL water. The combined eluates are titrated against HCl (bromocresol green plus methyl red as indicators) to measure H⁺, Ca²⁺, K⁺, Na⁺. The column is leached in a similar manner with 200 mL of 20 g MgCl₂·6H₂O in 1 L water for the determination of Ba²⁺. This cation is determined by flame absorption spectrophotometry. The cation exchange capacity is expressed as the sum of all the cation equivalents sorbed by 100 g soil.

(iii) **Performance of test**—(A) **Basic test.** (1) Fifty grams of soil (dry weight basis) is placed into each Erlenmeyer part (H) of the biometer flask (see the following Figure 1). The radioactive test solution (100 μL) is added in 50 drops over the whole soil surface (I) of each flask. The soil is carefully mixed with a Pasteur pipet (from which the lower part is cut off) and left in the flask.

FIGURE 1—TEST FLASK



(2) In addition, an equivalent volume of test solution is placed in a 100 mL volumetric flask for direct determination of the added radioactivity.

(3) The biometer flask is closed with a Teflon-coated silicon rubber stopper through which an Ascarite filter (F) is inserted. The filter (F) is provided with a stopper and stopcock (G). The side tube (C) is sealed with a teflon stopper pierced by a 15-gauge needle (B), 15 cm long. The needle (B) is capped by a silicone rubber stopper (A), and its tip at (D) is covered with a short length of silicone tubing that remains in contact with the base of the side tube (C).

(4) The unit is charged by injecting 10 mL of alkali solution into the side tube (C) in the following manner: the small stopper (A) is replaced by a calibrated Luer Lok syringe containing 0.1 N KOH; the filter stopper on (F) is removed and the stopcock (G) is opened; the alkali solution is introduced through the needle (B) into the side tube (C); the stopcock is closed; the syringe is removed; the small stopper (A) and filter stopper on (F) are returned to their initial positions. The ^{14}C -carbon dioxide produced is absorbed by the alkali.

(5) To recover the $^{14}\text{CO}_2$ -loaded alkali for liquid scintillation analysis, the procedure for charging each parallel unit at increasing time intervals after start of the experiment is performed in reverse order. Thereafter the side tube (C) is rinsed with 5 mL alkali. Before recharging the side tube (C) fresh alkali, 3 volumes of 25 mL air are sucked through the system with the empty syringe to maintain the soil in an aerobic condition. A 1 mL aliquot of the alkali solution is taken for liquid scintillation counting.

(6) Experiment duration times of 1, 2, 4, 8, 16, 32 and—if necessary—64 days should be chosen for measurement. The test requires parallel determinations. The $^{14}\text{CO}_2$ radioactivity recovered is plotted versus time. This graph shows when to terminate the experiment. Incubation time is sufficient, when a total of 50 per cent CO_2 expressed as ^{14}C originally applied can be measured. Incubation should be stopped after reaching 64 days, whether or not this value is obtained.

(B) Optional tests—(1) Estimation of evaporation. If the volatility of a chemical is higher than 10^{-5} torr at 20°C , it is recommended that a 3 cm diameter polyurethane foam plug be introduced into the arm E of the biometer flask. This plug absorbs the volatile parent compound as well as volatile organic degradation products but does not absorb $^{14}\text{CO}_2$. The plugs are extracted in a Soxhlet extraction apparatus with an *n*-hexane/methanol mixture (1:4), and aliquots are taken for liquid scintillation counting.

(2) Determination of soil-extractable and non-extractable residues. (i) In cases of relatively persistent chemicals (50 percent mineralization in 10 days), further information concerning the soil-extractable radioactivity (parent compound plus degradation products) and soil bound residues is recommended.

(ii) For this purpose two further biometer flasks in addition to the four others must be prepared. At the point of 50 (or x-) percent mineralization in the basic test, the soil in the two separate biometer flasks is extracted with 100 mL acetone (5 min ultrasonic treatment) followed by an extraction with methanol in the same manner. Aliquots of the combined extracts are taken for liquid scintillation counting. Other extract portions may be used—if necessary—for further identification studies.

(iii) Aliquots of the air dried soil are combusted to $^{14}\text{CO}_2$ and measured by liquid scintillation counting to determine the soil bound residues.

(d) Data and reporting—(1) Treatment of results—(i) Basic test. Radioactivity values for $^{14}\text{CO}_2$ (average of 4 parallel experiments) obtained from the alkali solution after 1, 2, 4, 8, 16, 32 and 64 days are expressed as the percentage of test chemical (radioactivity) initially applied and are plotted in a graph versus time. The time at which 50 percent of the radioactivity is recovered as $^{14}\text{CO}_2$ is considered to be the “50 percent

mineralization'' level. If this level has not been reached by the 64th day, the data at this time are taken and expressed as X-percent-mineralization.

(ii) **Evaporation test.** The radioactivity of vaporized (and trapped) original compound plus degradation products at the point of 50– (or X–) percent mineralization is extracted, measured and interpreted as the percentage of volatilization at the point of 50– (or X–) percent-mineralization.

(iii) **Residue test.** Radioactivity values for extractable and non-extractable residues of the parent compound plus degradation products obtained after the extraction procedure of the soil at the point of 50– (or X–) percent–mineralization are given.

(2) **Test report.** The report of the degradability of a test chemical must include:

- (i) Name of the test chemical, formula.
- (ii) Amount applied, if not standard.
- (iii) Exact characteristic data of the soil used.
- (iv) Dates of the performance of the measurements.

(3) **Interpretation and evaluation of results.** The results are artificial because they are obtained with disturbed soil. However, since standardized soils are used, the test data are intercomparable and enable the experimenter to group relatively the chemicals tested within one scale for this property.

(e) **References.** The following references should be consulted for additional background information on this test guideline.

- (1) Bartha, R. and Pramer D. *Soil Science* 100:68–70 (1965).
- (2) Soil Survey Staff. U.S. Department of Agriculture. *Soil Taxonomy Handbook* No. 436. Washington, DC (1975).
- (3) Butler, B.E. *Soil Classification for Soil Survey* Oxford (1980) p. 129.