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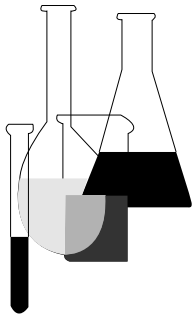
Fate, Transport and Transformation Test Guidelines

OPPTS 835.3170
Shake Flask Die-Away Test



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.*).

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OPPTS 835.3170 Shake flask die-away test.

(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 material used in developing this harmonized OPPTS test guideline are the articles referenced under paragraphs (j)(2) through (j)(9) of this guideline.

(b) **General.** (1) This guideline describes procedures for assessing the biodegradation of chemicals in natural surface water samples, and provides an opportunity to evaluate rates of biodegradation in the presence and absence of natural sediment materials. It also may provide limited information on the abiotic degradation rate, and sorption to sediment and vessel walls. The method allows for the development of a first-order rate constant, based on the disappearance of the test compound with time, and a second-order rate constant, normalized for changes in microbial biomass.

(2) A compound-specific analytical method is required and the concentrations of test compound employed depend on the sensitivity of the analytical method. The test method is designed to be applicable to compounds that are not inhibitory to bacteria at the concentrations used in the test, that do not rapidly volatilize from water, that are soluble at the initial test concentration, and that do not degrade rapidly by abiotic processes, such as hydrolysis.

(3) This guideline may involve hazardous materials, operations, and equipment, but does not purport to address all of the safety problems associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

(c) **Summary of test method.** The shake-flask die-away biodegradation method is similar to river water die-away tests described by Degens et al., Eichelberger and Lichtenberg, Paris et al., and Saeger and Tucker under paragraphs (j)(6) through (j)(9) of this guideline. It is based on the Chemical/Toxicity Abatement Test (CTA Test) described by Cripe et al. under paragraph (j)(5) of this guideline, and is essentially identical to ASTM Standard Test Method E 1279–89 under paragraph (j)(3) of this guideline. It differs from most die-away methods by providing for an evaluation of the effects of natural sediments on the transformation of the test compound and by the use of shaking to ensure a dissolved oxygen supply. Each test compound is dissolved in water collected from a field site, with and without added natural sediment, and with and without sterilization. Initial test compound concentrations typically are relatively low (micrograms per liter), analytical capabilities permitting. Loss of test compound with time is followed by an appropriate compound-specific ana-

lytical technique. Changes in microbial biomass may also be followed by the use of an appropriate technique such as bacterial plate counts. Data obtained from this test are used to provide the abiotic degradation rate in the presence and absence of sediment, and the combined biotic and abiotic degradation rate in the presence and absence of sediment.

(d) **Significance and use.** (1) Most of the simpler methods used to screen chemicals for biodegradation potential employ measures of biodegradation that are not specific to the test compound, such as loss of dissolved organic carbon (DOC), evolution of respiratory CO_2, or uptake of dissolved oxygen (biochemical oxygen demand (BOD)). Such methods are used to evaluate ultimate biodegradability. They require the use of relatively high initial concentrations of the test compound, generally 10 mg/L or higher, unless the tests are conducted using radiolabeled (^{14}C-labeled) test compounds. Biodegradation tests measuring ^{14}C-CO_2 evolution, for example, can be conducted using initial concentrations of test compound in parts per billion. However, these tests require specialized equipment, and the custom preparation of appropriately labeled compounds is often very expensive.

(2) Die-away biodegradation methods are simple simulation methods that employ water collected from natural water sources and follow the disappearance of an added amount of the test compound resulting from the activity of microorganisms in the water sample. The compound-specific analytical techniques used to follow the disappearance of the test compound typically are employed using relatively low initial concentrations of the test compound. Most environmental pollutants are present in the environment at relatively low concentrations (less than 1 mg/L), and it has been observed that biodegradation rates obtained using high test compound concentrations may be quite different from those observed at lower concentrations (refer to paragraph (j)(4) of this guideline).

(3) The transformation of the test compound to an extent sufficient to remove some characteristic property of the molecule, resulting in the loss of detection by the compound-specific analytical technique, is referred to as primary biodegradation. For many purposes, evidence of primary biodegradation is sufficient, especially when it is known or can be shown that toxicity or some other undesirable feature associated with the test compound is removed or significantly reduced as a result of the primary biodegradation. A determination of ultimate biodegradation, on the other hand, is usually required only when treatability or organic loading are issues of concern, or when assurance is needed that potentially toxic or bioaccumulative degradation products are not formed. Nonspecific measures of biodegradation such as DOC and BOD (see paragraph (d)(1) of this guideline) do not directly reflect primary degradation.

(4) Because the use of low test compound concentrations enhances the probability of observing first-order or pseudo first-order kinetics, a rate

constant for the primary biodegradation reaction and a half-life for the test compound can be derived under defined incubation conditions. Rate constants are required in many environmental fate mathematical models.

(e) **Materials and apparatus.** The following materials and apparatus are needed to perform the test.

(1) Carefully cleaned glass or plastic carboys, required for the collection and transport of field water samples.

(2) Field sediment samples, obtained using scoop, beaker, or box sampler, as appropriate.

(3) A rotary shaker, capable of holding 2–L Erlenmeyer flasks and shaking at 140 to 150 r/min is required for the incubation of test flasks. Temperature control (± 2 °C) may be incorporated in an incubator/shaker unit or may be attained by placing the shaker in a temperature-controlled space. The flasks should be constructed of material that minimizes sorption of test or reference compound to the walls of the flasks. In general, glass is the best choice.

(4) A gas chromatograph, or other suitable instrument equipped with a detector sensitive to the test and reference compounds, is required for compound-specific analysis.

(5) The use of reference compounds is desirable to evaluate the biodegradation potential of the microbial population. A suitable reference compound will be biodegradable under the test conditions but not so readily biodegradable that it is completely degraded within a small fraction of the normal test period.

(f) **Procedure**—(1) **Field sampling.** (i) Collect water and sediment from a selected field site (for example, river, lake, or estuary) the day before test initiation. Measure the salinity (when appropriate), water temperature, and pH at the time of sampling. Collect water from approximately 60 mm below the air/water surface in clean glass or plastic carboys. Remove floating or suspended particulates, preferably by filtering the water through a 3–μm pore-size membrane filter. Collect the upper 5 to 10 mm of underlying sediment by skimming with a beaker, scoop, or box sampler. Screen the sediment through a sieve with 2 mm-openings to remove larger particles and biota. Remove sand by resuspending detritus and fine particles and decanting the suspension from the sand. (This is necessary because sand cannot be quantitatively transferred from a slurry with a pipet.) Adjust the volume of field water by adding it to or decanting it from the sieved sediment until there is approximately a 1:1 ratio between sediment and water volumes. Transport the water sample and the sediment slurry to the laboratory in closed containers.

(ii) If there is no sediment layer at the field site (for example, the stream or lake bed is all rock), omit the sediment collection and use procedures.

(2) **Handling of field samples.** (i) Stir the sediment slurry and site water continuously at room temperature until they are used in the test.

(ii) Measure the concentration of sediment in the slurry by filtering 5–mL samples of well-mixed slurry through predried (at 105 °C for 1 h) 0.45–μm pore-size membrane filters. The slurry must be stirred vigorously during sampling to ensure homogeneity. Rinse the slurry sampling pipet, sediment, and filter with 2 to 3 mL of deionized water, and dry the filter and sediment at 105 °C for 1 h. Weigh the sediment after the dried filter and sediment have cooled to room temperature in a desiccator. Use the weight of sediment per milliliter of slurry to calculate the volume of slurry to be used in test flasks.

(3) **Preparation of flasks**—(i) **Test compound concentration.** Initial test compound concentration in the method typically is 200 μg/L. This concentration is generally high enough for analytical sensitivity yet low enough to be environmentally realistic. Choose other concentrations as appropriate.

(ii) **Control water (CW) flasks.** Add 1 L of site water to each of two 2–L Erlenmeyer flasks.

(iii) **Control sediment (CS) flasks.** Add 900 to 950 mL of site water to each of two 2–L Erlenmeyer flasks, then add sufficient sediment slurry to each flask to achieve a final (following a second addition of site water) suspended sediment concentration of 500 mg/L based on dry weight of sediment. Add additional site water to achieve a final volume of site water plus sediment equal to 1 L.

(iv) **Amended site water.** Add sufficient test or reference compound to 9 to 10 L of site water to produce the desired initial concentration. Generally, analytical sensitivity permitting, the desired initial concentration is 200 μg/L, so that 2.0 mg of test compound is added to 10 L of site water. Test compound may be added as a stock solution in a volatile solvent (for example, acetone). Add the solution to a clean, empty vessel, remove the volatile solvent by flushing with a stream of clean air or nitrogen, and add 10 L of site water. Analyze the final solution to determine the concentration of test compound. To compensate for the volume of sediment slurry and formalin added later (see paragraphs (f)(vi) through (f)(viii) of this guideline), an excess of test compound may be added to yield a concentration greater than 200 μg test compound per liter. The amount of amended site water added to the active water, active sediment, sterile water, and sterile sediment flasks is adjusted to yield a final concentration of 200 μg test compound per liter. Unamended site water is used, as necessary, to produce a final volume of 1 L in each flask.

(v) **Active water flasks.** Add 1 L of amended water to each of two 2–L Erlenmeyer flasks.

(vi) **Active sediment flasks.** Add 900 to 950 mL of amended water to each of two 2–L Erlenmeyer flasks and then add sufficient sediment slurry to each flask to achieve a final (following a second addition of amended site water) suspended sediment concentration of 500 mg/L. Add additional amended site water to achieve a final volume of water plus sediment equal to 1 L.

(vii) **Sterile water flasks.** Add 900 to 950 mL of amended water to each of two 2–L Erlenmeyer flasks. Add 20 mL of 37 percent formaldehyde solution (formalin) to each flask to act as a sterilizing agent. Add additional amended site water to each flask to achieve a volume of 1 L. If any interaction between formalin and the test or reference compound is likely or suspected, another sterilization procedure (for example, use of phenylmercuric acetate or autoclaving) may be required.

(viii) **Sterile sediment flasks.** Add 900 to 950 mL of amended water to each of two 2–L Erlenmeyer flasks and then add sufficient sediment slurry to each flask to achieve a final (following a second addition of amended water) suspended sediment concentration of 500 mg/L, then add 20 mL of formalin to each flask to act as a sterilizing agent. Add additional amended site water to each flask to achieve a final volume of site water, sediment, and formalin equal to 1 L.

(ix) **Flask incubation.** Close the flasks with polyurethane foam plugs and place them on a rotary shaker at 140 to 150 r/min and 25 ± 2 °C. If a closer simulation of site conditions is desired, the incubation may be held at a temperature representative of the collection site ±2 °C. Determine the pH of the water in each flask on day–0 and at least every other day for the remainder of the test. Maintain the pH at the value observed at the time of collection (± 0.2 pH units) throughout the test by adding a few drops of 1 N HCl or 1 N NaOH as required.

(4) **Preliminary check.** This test method is not suitable for biodegradation rate determinations of compounds that are rapidly lost (50 percent or greater decrease in 24 h) from solution due to chemical instability, volatility, or photolysis. To determine suitability, a preliminary test may be set up using a 2–L flask containing 1.0 L of reagent water, with a purity equal to or better than Type II of Specification D 1193 under paragraphs (j)(1) and (j)(2) of this guideline, to which 20 mL of formalin is added. Amend the flask with test compound to a concentration of about 200 μg/L. Close the amended flask with a stopper and provide with laboratory lighting of the same type and intensity provided to the test shaker flasks. This flask serves as a check for abiotic losses (for example, by photolysis, hydrolysis, or volatilization). Sample the flask for test compound concentration at time zero (t_0) and after 24 h. If one-half or less of the test

compound is present at 24 h, no further work is carried out and the method is considered unsuitable for testing the compound.

(5) **Total organic carbon (TOC) analysis.** Analyze well-mixed samples from the CS and CW flasks for TOC content using a suitable method, such as that described in ASTM Test Method D 4129 under paragraph (j)(2) of this guideline. This value is used in calculating the equilibrium sorption coefficient.

(6) **Equilibrium sorption coefficient.** Sample the sterile sediment flasks at half-hour intervals until the test compound concentrations are relatively constant at each of two sequential sampling times, indicating no more sorption to sediment and vessel walls. This generally occurs within the first 6 h. Place duplicate 25–mL samples from each flask in centrifuge tubes and centrifuge to remove suspended particles before analysis to determine test or reference compound concentration. Concentration at t_0 (C_0) is the concentration observed in samples obtained immediately following the preparation of the sterile sediment flasks.

(g) **Sampling.** (1) Collect samples from each flask according to a schedule appropriate to the rate of biodegradation of the test and reference compounds. Sampling should be sufficiently frequent to establish plots of degradation versus time and to permit the determination of rate constants. Collect a minimum of six samples from t_0 until completion of the test. A nominal test duration of 28 days allows a reasonable period for observations with slowly degraded compounds. The test period may be extended beyond 28 d if necessary to calculate a half-life. Tests may be terminated prior to 28 days when more than 50 percent of the starting material has disappeared from solution due to biodegradation.

(2) Remove duplicate samples of a sufficient size from each flask at appropriate intervals from day 1 (t = 24 h) until completion of the test. Centrifuge each sample to remove suspended particles. Analyze the supernatant (or a suitable extract of the supernatant) to determine the concentration of test or reference compound. Maintain a record of compound concentration versus time for each flask. If sorption to sediment solids is a significant factor, extract the sediment plug and analyze the extract to account for untransformed test compound.

(3) If microbial adaptation (a lag phase with little or no loss of test compound followed by relatively rapid loss) is suspected, add additional test compound to that flask and the corresponding control flask, at or near the normal end of the test period. Adaptation is indicated if the microorganisms in the test flask degrade the added compound without a lag period and the control flask, to which test compound has just been added for the first time, exhibits a lag prior to degradation. Do not use the lag period in the calculation of the biodegradation rate. If there is a lag period due to adaptation, use the end of the lag period as t_0 when calculating the

first-order rate constant (see paragraph (h)(2)(i) of this guideline). For example, see Cripe et al. under paragraph (j)(5) of this guideline.

(4) If desired, samples may also be taken for biomass determinations. Sampling times should coincide with the times of sampling for test and reference compound concentration.

(h) **Calculations**—(1) **Equilibrium sorption coefficient.** Calculate the equilibrium sorption coefficient (K_{oc}) using the following equation:

$$(K_{oc}) = 1000\ (C_0 - C_e)/C_e\ (CS - CW)$$

where

C_0 = test compound concentration at t_0 (μg/mL)

C_e = test compound concentration at equilibrium (μg/mL)

CS = TOC in control sediment sample (g/L)

CW = TOC in control water (CW) sample (g/L)

(2) **Biodegradation rates and half-lives**—(i) **First-order rate constants.** (A) First-order rate constants (K_1) are determined using a regression equation of the type

$$C = a + K_1 t$$

where

C = concentration of test compound (μg/L)

a = Y-axis intercept

K_1 = slope (first-order rate constant)

t = time

(B) See paragraph (g)(3) of this guideline for information on calculating K_1 if there is microbial adaptation resulting in a lag period.

(ii) **Half-life.** The half-life of the test compound, based on the first-order rate constant, is given by $t_{1/2} = 0.693/K_1$. Calculate the half-life for the test compound in each flask and calculate an average value for replicate flasks.

(3) **Second-order rate constants.** (i) Second-order rate constants are of interest because some mathematical fate models use a second-order rate expression to describe the biotransformation of chemical compounds in environmental waters. In such models, the disappearance rates for compounds are calculated from the concentration of indigenous bacteria, the concentration of the compound, and the second-order rate constant.

(ii) A second-order rate constant (K_2) can be obtained by dividing K_1 by the average bacterial concentration (B). If plate count methods are used, (B) is expressed in colony forming units per mililiter. Bacterial concentrations normally do not change significantly during these tests, since concentrations of growth substrates are normally too low to support significant microbial growth. Therefore, measured bacterial concentrations are averaged to obtain (B). K_2 is calculated as follows:

$$K_2 = K_1/B$$

(i) **Report.** Report the following data and information:

(1) Test and reference compound identities.

(2) Site, date, and time of field water and sediment collection.

(3) Temperature, pH, and salinity (when appropriate) of site water at the time of collection.

(4) Concentration of sediment (dry weight) per milliliter of slurry.

(5) TOC in the CS and CW samples expressed as grams per liter.

(6) Measured concentrations of test and reference compounds at each sampling time during the preliminary check, sorption coefficient determination, and test sampling steps.

(7) Equilibrium sorption coefficient (K_{oc}) calculations and results.

(8) The average first-order rate constants for each replicate pair of flasks. If microbial adaptation was observed (with a lag period following test startup), describe the lag period and how it was evaluated.

(9) The average half-life for the compound in each replicate pair of flasks.

(10) The plate count or other biomass data, if applicable.

(11) The average second-order rate constants for each replicate pair of flasks, if applicable.

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

(1) American Chemical Society (ACS). *Reagent Chemicals: American Chemical Society Specifications.* ACS, Washington, DC (1994).

(2) American Society for Testing and Materials (ASTM). *Annual Book of Standards* ASTM, Philadelphia, PA (1993).

(3) American Society for Testing and Materials (ASTM). Standard Test Method for Biodegradation by a Shake-flask Die-Away Method, ASTM E 1279–89. ASTM, Philadelphia, PA (1993).

(4) Boethling, R. S. and M. Alexander. Effect of Concentration of Organic Chemicals on Their Biodegradation by Natural Microbial Communities. *Applied and Environmental Microbiology* 37:1211–1216 (1979).

(5) Cripe, C.R. et al. A Shake-Flask Test for the Biodegradability of Toxic Organic Substances in the Aquatic Environment. *Ecotoxicology and Environmental Safety* 14:239–251 (1987).

(6) Degens, P.N., Jr. et al. Influence of Anionic Detergents on the Diffused Air Activated Sludge Process. *Sewage and Industrial Wastes* 27:10–25 (1955).

(7) Eichelberger, J.W. and J.J. Lichtenberg. Persistence of Pesticides in River Water. *Environmental Science and Technology* 5:541–544 (1971).

(8) Paris, D.F. et al. Second-Order Model to Predict Microbial Degradation of Organic Compounds in Natural Waters. *Applied and Environmental Microbiology* 41:603–609 (1981).

(9) Saeger, V.W. and E.S. Tucker. Biodegradation of Phthalic Acid Esters in River Water and Activated Sludge. *Applied and Environmental Microbiology* 31:29–34 (1976).