



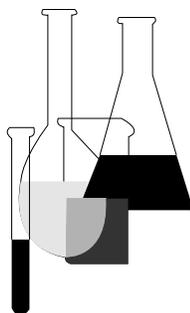
Fate, Transport and Transformation Test Guidelines

OPPTS 835.3180

Sediment/Water

Microcosm

Biodegradation 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 835.3180 Sediment/water microcosm biodegradation 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 (e)(3) through (e)(9) and (e)(11) through (e)(16) of this guideline.

(b) **Introductory information—(1) Prerequisites.** Sediment and water containing a representative sample of the natural microbial community from a test site of interest are required.

(2) **Guidance information.** A preliminary study such as a shake-flask test with site water and sediment (under paragraph (e)(1) of this guideline) is recommended to provide preliminary information about the fate of a test compound. For example, a preliminary study may help identify those fate processes such as volatilization that should receive close attention during a microcosm study, and can provide guidance on sampling frequency and dosing patterns. In addition, an activated sludge respiration inhibition test (under paragraph (e)(10) of this guideline) is recommended to evaluate potential adverse effects of a test substance on the natural microbial community in the receiving environment. This test measures respiration rate under controlled conditions, and can help to obtain information on microbial toxicity that may be important to microcosm design and set up.

(3) **Qualifying statements.** (i) This guideline establishes criteria of minimum acceptability for the development of sediment/water microcosms for use in biodegradation studies. If the nature of a receiving environment requires testing under strictly anaerobic conditions, other test methods may be more appropriate (e.g., the test guideline for anaerobic biodegradability referenced under paragraph (e)(17) of this guideline).

(ii) This test guideline does not require any specific microcosm design because design and operation are compound and site specific.

(iii) Performance of procedures discussed in this guideline may involve contact with hazardous materials and operation of potentially hazardous equipment. This guideline does not purport to address all of the safety concerns associated with its use. It is the responsibility of the user of this guideline to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to using this guideline.

(4) **Recommendations.** (i) The use of ¹⁴C-labeled test substances is recommended.

(ii) The use of a mass-balance approach is recommended.

(c) **Method**—(1) **Introduction, purpose, scope, relevance, application, and limits of test**—(i) **Background.** (A) The fate of chemicals released to the environment may be evaluated in the field or in laboratory studies. Field studies can be used to obtain important information about the fate of chemicals in a particular ecosystem, but have many disadvantages. In field studies, environmental variables generally cannot be controlled and the study may be subject to wide fluctuations in variables such as temperature, rainfall, or sunlight. It is also difficult in many cases to distinguish whether an observed effect is a result of one fate process vs. another, or a result of the interaction of more than one fate process. Microcosms may be used to replicate many of the processes affecting the fate of a chemical in complex ecosystems. These model systems provide an opportunity to manipulate various test conditions and to observe the effects of these alterations and their interactions on fate processes. Because microcosms are more easily replicated, effects of environmental variability are more easily studied than under field conditions. Finally, microcosms can be used to examine the significance of various fate processes (e.g. hydrolysis and biodegradation). This makes it possible to focus on critical processes and consider site-specific environmental situations.

(B) This guideline provides guidance on the development, use and evaluation of microcosms containing intact benthic sediment and overlying water for laboratory evaluations of the fate of chemical substances in the aerobic aquatic environment.

(C) Several examples of information on the fate of chemicals that might be obtained from microcosm studies are:

(1) The ability of a chemical substance to persist in its original form in a particular aquatic environment.

(2) Relative importance of abiotic and biotic processes in determining the fate of a chemical.

(3) The effect that partitioning of a chemical substance into benthic sediment has on the ultimate fate of the compound.

(ii) **Definitions.** (A) Within this guideline, a *microcosm* is defined as an intact, minimally disturbed portion of an ecosystem brought into the laboratory for study under controlled experimental conditions.

(B) *Ultimate biodegradation* is any biologically mediated conversion of an organic compound to inorganic compounds (e.g. CO₂, H₂O, etc.), other products associated with normal metabolic processes, and microbial biomass.

(C) *Primary biodegradation* is any biologically mediated transformation that changes the molecular structure of the parent chemical compound.

(D) Within this guideline, a *mass balance approach* is one in which the test substance's transport to or from and appearance in all applicable media of the microcosm, including sediment, overlying water, interstitial water, off-gases, and test equipment washings is determined, and formation of ¹⁴C-labeled CO₂ is determined if radiolabeled parent compound is used.

(iii) **Reference substances.** If required, methyl parathion or linear alkylbenzene sulfonate (LAS) may be used as an appropriate reference standard with the shake flask and microcosm tests.

(iv) **Principle of the test method.** Microcosms are laboratory test systems designed to study fate processes such as biodegradation of chemical substances by natural microbial communities. Microcosms contain sediment and water that have been collected from test sites in a manner that maintains the physical and biological integrity of the ecosystem under study. Physical parameters such as lighting, mixing, and temperature may be controlled to simulate the environmental conditions of the site from which water and sediment samples are collected. Test compounds are introduced into the system either as a single dose or by continuous dosing over the duration of testing. The microcosms are sampled on a periodic basis, and the water, sediment and off-gases (latter for volatile test compounds) are analyzed for disappearance of the parent compound (i.e. primary biodegradation) and, if feasible, appearance of metabolites. Measurements of ultimate biodegradation may also be made.

(v) **Quality criteria—(A) Reproducibility.** Microcosms containing sediment and water samples collected simultaneously from the same site should provide for adequate reproducibility between replicate microcosms, provided that standard testing conditions are strictly observed. Variability may be greater when sediment and water samples are collected at different test sites or at the same sites over different time intervals. The presence of organisms other than microorganisms (e.g., amphipods) in sediment/water samples may also cause significant changes to occur in the sediments, and therefore complicate test results.

(B) **Sensitivity.** The sensitivity of the test is dependent upon the dosing protocol, adequate control of specific testing conditions, and the analytical methods used.

(C) **Specificity.** This method is applicable to various classes of inorganic and organic compounds. The specific type of test compound used should be considered in selecting an appropriate microcosm design, testing protocol, and analytical technique.

(D) **Possibility of standardization.** Standardization of all aspects of microcosm testing is difficult, but minimum criteria for acceptability are provided by this guideline. Specific microcosm designs and testing protocols should be selected based on the type of test compound used and the environmental conditions at the test site.

(E) **Possibility of automation.** Not foreseen.

(2) **Description of test procedure—**(i) **Physical/chemical site characterization—**(A) **Sediment/water.** To ensure that aerobic, sediment/water microcosms adequately replicate the environmental habitats from which they are derived and to identify important environmental factors that could potentially affect the rate or extent of biodegradation of a chemical substance, physical/chemical characteristics of the site of collection should be determined. These include:

(1) **Physical characteristics.** Light intensity and photoperiod, temperature, total suspended solids (TSS), etc.

(2) **Chemical characteristics.** Dissolved oxygen (DO), total organic carbon (TOC), dissolved organic carbon (DOC), alkalinity, conductivity, pH, redox gradient, etc.

(B) **Site contamination.** Because certain contaminants can adversely affect the fate (e.g. rate or extent of biodegradation) of a chemical substance, it is recommended that to the extent possible collection sites be screened for the presence, identity, and extent of contamination by the test chemical or close chemical analogs and the following chemical substances: Pesticides, PCBs and other hazardous substances, heavy metals.

(ii) **Design features—**(A) **Size.** Microcosms vary in size from a fraction of a liter to several hundred liters. A microcosm should be sufficiently large to permit removal of water and sediment samples without significantly affecting surface area to volume ratios over the course of an experiment, and to readily accommodate monitoring probes and mixing apparatus where necessary. Alternatively, smaller microcosms may be used in sufficient numbers to allow for destructive sampling. Small microcosms (i.e. containing several hundred milliliters or less of site water and sediment) may be the most appropriate for studies of chemical fate processes such as biodegradation and sorption.

(B) **Alternative methods for establishing microcosms.** (1) One method of establishing a sediment/water microcosm is to use a sediment coring device which becomes the microcosm vessel. A glass tube of suitable size (e.g. approximately 3.5 cm in diameter and 40 cm in length) is inserted into the sediment to a depth equal to or greater than the depth of biological activity. The top of the tube is closed with a silicone stopper. The tube is removed from the sediment and closed with another silicone stopper. After the cores are transported back to the laboratory, additional site water (i.e. collected separately) may be added to the microcosm as necessary to achieve the desired sediment surface area to water volume ratio.

(2) Alternatively, an intact core may be obtained in the field and extruded into a microcosm vessel in the laboratory. A simple and effective

coring device can be made from clear, acrylic pipe. The coring device is inserted into the sediment to a depth equal to or greater than the depth of biological activity. The coring device is closed with stoppers as noted above and the core is returned to the laboratory. The bottom stopper is removed and the core is inserted into the microcosm vessel. The top stopper is removed and the coring device is lifted out of the microcosm leaving the sediment core intact. For large water volume to sediment surface area ratios, the coring device may be placed in a glass dish (e.g. a crystallization dish) having a diameter slightly larger than the corer, and the entire assembly (core, corer, and dish) placed into the microcosms.

(C) **Diagrams of representative microcosms.** Figures 1, 2 and 3 present a representative selection of microcosm designs that are appropriate for conducting sediment/water microcosm biodegradation tests.

Figure 1. Ecocore Microcosm (under paragraph (e)(12) of this guideline)

Figure 2. Sediment/Water System (under paragraph (e)(13) of this guideline)

Figure 3. Flow-Through Microcosm (under paragraph (e)(14) of this guideline)

(iii) **Preparation**—(A) **Reagents**—(1) **Water.** Water for the microcosm shall be collected from above or nearly above the site of sediment core collection. Water may be collected by hand bucketing, grab sample, or pumping. Water samples shall be transported to the test facility with minimum delay. If water must be held in the laboratory overnight, it may be kept at room temperature and gently stirred. Where necessary (e.g. flow-through microcosm test), larger quantities of site water may be held for longer periods of time at 4 °C. Prior to use in an aerobic, sediment/water microcosm test, water should be brought to the test temperature ± 2 °C and gently stirred.

(2) **Sediment cores.** Sediment cores shall be collected in such a manner as to preserve to the extent possible the structural integrity of the sample, including the redox gradient and the benthic community.

(B) **Materials**—(1) **Sampling containers.** In order to minimize leaching of plasticizers and other contaminants into the water, sampling containers shall be composed of materials such as glass or fluorocarbon plastics (e.g. Teflon®).

(2) **Microcosm construction materials.** Microcosms shall be composed of inert fluorocarbon plastics (e.g. Teflon®) and/or glass. If rubber stoppers are used, they shall be composed of silicone rubber.

(iv) **Procedure for setting up and maintaining microcosms**—(A) **Control microcosms**—(1) **Sterile control microcosms.** Use of sterile

control microcosms permits determination of the relative importance of biotic and abiotic processes in the fate of a test compound.

(2) **Other control microcosms.** If necessary, solvent control microcosms, glucose-amended controls, and controls containing other standard reference compounds such as aniline should also be used.

(B) **Dosing microcosms.** (1) To the extent possible, the method and pattern of applying a test substance to a microcosm should reflect the release pattern expected in the natural environment.

(2) Microcosms may be maintained in either flow-through or static-renewal modes. For the latter, a fixed percentage of microcosm water is replaced with fresh site water at appropriate time intervals. A single (i.e. pulse) dose of test compound may be applied in conjunction with either of these modes. For a pulse dose in a flowing system, the relationship between molecular turnover (partial replacement) time and the flow rate and volume of the microcosm chamber has been described by Sprague (under paragraph (e)(15) of this guideline)

(3) For flow-through systems employing relatively large concentrations of test compounds, a pump or headbox/siphon arrangement is recommended. All parts of the pump and delivery tubing that come into contact with either the test compound or diluent water should be composed of inert materials to minimize sorption of the test substance.

(4) The test substance may be dissolved in a carrier and the resulting stock solution metered into flowing diluent water. Ideally, the carrier should be dissolved in sterile diluent water. Peristaltic pumps using silicone rubber tubing may be used for adding diluent water. However, it is not recommended that these be used to deliver stock solutions because sorption of test compound to the tubing may be significant. A syringe pump with glass syringes and inert plastic (fluorocarbon) plungers and tubing is more desirable for introducing a test compound and carrier into flowing diluent water.

(5) If the test compound is insoluble in water, but soluble in a nontoxic, water-miscible solvent, it should be dissolved in the minimum volume of carrier or solvent required to form a homogeneous stock solution of known concentration. Carriers other than water that are acceptable in aquatic toxicity testing may be used, including acetone, ethanol, methanol, diethyl sulfoxide, ethylene glycol monomethyl ether, dimethylformamide, and triethylene glycol (under paragraph (e)(2) of this guideline). Care should be taken that an increased organic carbon load due to the addition of a carrier does not significantly affect the test results. In addition, if a carrier or solvent is used, a control microcosm should be established to assess the effect of the solvent on microbial activity.

(6) If the test compound is added continuously, the stock solution and delivery lines should be kept free of microbial contamination to avoid degradation of the test compound before it reaches the microcosm.

(C) **Test compound concentration.** The test compound concentration(s) shall approximate the expected ambient environmental concentration. Solubility in water of the test compound, analytical detection limit, and toxic effects on microbiota in the microcosm should be considered in the selection of the test compound concentration. A preliminary shake-flask study (under paragraph (e)(1) of this guideline) and/or a microbial respiration inhibition test (under paragraph (e)(10) of this guideline) may aid in identifying an appropriate concentration.

(D) **Temperature.** It is recommended that microcosms be maintained at field temperature ± 2 °C. However, specific circumstances may suggest that another temperature may be more appropriate. The microcosms should be placed in a water bath to maintain a constant temperature.

(E) **Lighting.** (1) It may be desirable to control the quantity and quality of light entering the microcosms. Light intensity may be adjusted to a level that is equivalent to the average light intensity on the sediment surface in the natural system from which the core was obtained. The preferred source of artificial light is a xenon lamp since its spectrum is closest to that of sunlight.

(2) Photoperiod may be controlled by the use of simple timers. Photoperiod is usually fixed at some arbitrary ratio (e.g. 12 h of light and 12 h of dark), or is maintained at ambient field conditions.

(F) **Mixing.** Mixing of the microcosm water shall be adequate to uniformly distribute the test chemical in the water column but not so great as to resuspended sediment. Mixing can be accomplished with pumps, aeration, or stirrers. Use of glass or fluorocarbon plastic (e.g. Teflon®) stirrers attached to small motors is recommended. Simple aeration of microcosm water is often unsatisfactory because it may cause significant losses of volatile test substance and may result in uneven mixing.

(v) **Microcosm replication techniques.** A detailed discussion of techniques for determining adequate microcosm replication and appropriate statistical treatment of the relevant test data is beyond the scope of this guideline. However, treatment microcosms must be established in triplicate for each identified dose (e.g. 1 ppm, 10 ppm, 1,000 ppm), and control microcosms in duplicate for each identified type of control (e.g. sterile control, solvent control, glucose-amended control), to be minimally acceptable for this test guideline.

(vi) **Microcosm sampling techniques—(A) Monitoring physical/chemical characteristics.** To ensure the functional capability of the sediment/water microcosms and to maintain environmental conditions that ade-

quately represent those of the receiving environment, it is recommended that to the extent that they are applicable, certain physical/chemical characteristics (listed in paragraph (c)(2)(i)(A) of this guideline) be monitored over the course of the test.

(B) Water samples. (1) Two replicate water samples shall be collected from each microcosm at dosing time (after an appropriate mixing period) and periodically thereafter. Sampling regimes for both static and flow-through systems should be designed according to the expected disappearance rate of the parent compound.

(2) To characterize sorption and/or volatilization of the chemical substance during the start-up and initial phase of the microcosm test, it may be appropriate to collect water samples at frequent intervals initially (e.g. at 0, 1, 3, 6, 12, and 24 h) and at less frequent intervals (e.g. 1, 4, 7, 14, 24, 36 days) thereafter.

(3) The duration of microcosm testing should be limited to 60 days or less, unless specific circumstances and microcosm function warrant longer operation. During this time, the microcosm should be monitored to ensure its viability and stability (under paragraph (e)(11) of this guideline).

(4) A preliminary study such as a shake-flask test (under paragraph (e)(1) of this guideline), using site water and sediment, is recommended to help identify a sampling protocol that is appropriate for the specific site and test parameters.

(C) Sediment samples. Sediment samples shall be collected periodically during the test. To determine the importance of partitioning to sediment, it is recommended that sediment samples be collected initially at frequent intervals (e.g. 0, 12 h), and at less frequent intervals (e.g. 1, 4, 7, 14, 24, 36 days) thereafter. Sediment samples shall be collected in triplicate from each treatment microcosm, and in duplicate from each control microcosm, for each time interval and test concentration. If the diameter of the sediment core is sufficiently large for repeated sampling without disturbing the sediment/water interface, this may be accomplished at a minimum by collecting one sample from each of the triplicate treatment microcosms for each concentration of test substance. However, it should be noted that such a sampling design may not be statistically optimal, since the extent of sample variability for each treatment microcosm is not known. If the nature of the sediment or the diameter of the core is such that disturbance of the sediment is a likely consequence of sampling, it is recommended that the study design include three times as many treatment microcosms as there are sampling times, such that treatment microcosms are destructively sampled in triplicate.

(D) Additional sampling. All of the test substance added to the microcosm during the study should be accounted for by mass balance. The

use of radiolabeled test compound is therefore recommended. Potential losses of test substance and transformation products to the atmosphere may be evaluated by trapping and sampling off-gases using sampling techniques described by Bourquin et al. (under paragraph (e)(3) of this guideline), and in the U.S. Environmental Protection Agency Test Guideline for a site-specific microcosm test (under paragraph (e)(16) of this guideline). The significance of volatilization and sorption can also be evaluated based on preliminary tests and sterile controls, and should be accounted for in the design of the microcosm.

(vii) **Analytical methods.** Detailed discussion of compound-specific analytical methods is beyond the scope of this guideline. Gas chromatography (GC) and high performance liquid chromatography (HPLC) are suitable for the quantification of many test compounds. Use of appropriately radiolabeled test substances is recommended, especially when quantifying mineralization or identifying degradation products that need further characterization by conventional analysis.

(d) **Data and reporting.** (1) A mass balance shall be determined for the test substance describing its fate, including its transport to or from and appearance in all applicable media of the microcosm. Where appropriate, the media should include at a minimum the following: sediment, overlying water, sediment core washings, resin traps for volatile compounds, KOH or other standard solvent used as a trap for ^{14}C -labeled CO_2 from parent compound, and test equipment washings. Analysis of these components should account for >80 percent of the initial added concentration of ^{14}C -labeled substrate or parent compound (under paragraph (e)(4), (5), (6), (7), (8), (9), (12), and (13) of this guideline). A spreadsheet format may be useful for reporting data.

(2) The rate constant for the loss of the test compound from the water column can be determined, assuming first-order kinetics, from a plot of $\ln C$ vs. t :

$$\ln C = k_1 t + a$$

where C is the test compound concentration, a is the Y-axis intercept, and t is time. The rate constant is k_1 , which is determined as the best-fit slope of a liner regression of $\ln C$ vs. t . The half-life ($t_{1/2}$) can then be determined by use of the following equation:

$$t_{1/2} = 0.693/k_1$$

(3) Reports should account for any unusual observations as well as include where applicable the following information:

- (i) Date the study began and ended.
- (ii) Name and address of testing laboratory.

- (iii) Principal investigator(s).
- (iv) Staff members actually conducting the test.
- (v) Full description of the experimental design and procedures, including a description of the test equipment.
- (vi) Identity of the test substance, percentage of active ingredient, molecular structure, and radiolabel placement.
- (vii) Manufacture and lot number of the test substance.
- (viii) Physical and chemical properties of the site.
- (ix) Results of all plate counts.
- (x) Principal mathematic equations.
- (xi) Residue data or contamination profile of sediment and water.
- (xii) Results of trapped volatiles and CO₂ analyses.
- (xiii) Residue decline curves (i.e. loss of parent compound).
- (xiv) Metabolite characteristics as determined by TLC, HPLC or other analytical technique suitable for identifying metabolites; identity of each metabolite with >10 percent yield.
- (xv) Incubation temperature; photoperiod and lighting conditions.
- (xvi) Materials balance, rate constants, half-lives.
- (xvii) Concentration of dissolved ¹⁴C-labeled CO₂ in water.
- (xviii) Dates and results of QA/QC audits.
- (xix) Location of raw data.
- (xx) Complete description of any deviation from the established test guideline.
- (xxi) Experimental test system monitoring data including TOC, pH, lighting, temperature, etc.

(e) **References.** (1) American Society for Testing and Materials (ASTM), ASTM Method E1279–89: Standard test method for biodegradation by a shake-flask die-away method. Philadelphia, PA (1989).

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(17) U.S. Environmental Protection Agency. Anaerobic biodegradability of organic chemicals. 40 CFR 796.3140 (1992).