



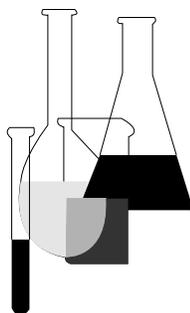
# Fate, Transport and Transformation Test Guidelines

## OPPTS 835.5154

### Anaerobic

### Biodegradation in the

### Subsurface



## 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 835.5154 Anaerobic biodegradation in the subsurface**

(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 is the OPPT guideline under 40 CFR 795.54 Anaerobic Microbiological Transformation Rate Data for Chemicals in the Subsurface Environment.

(b) **Introduction.** (1) This guideline describes laboratory methods for developing anaerobic microbiological transformation rate data for organic chemicals in subsurface materials. The method is based on a time-tiered approach. For chemicals that are degraded rapidly, only a portion (for example, the 0-, 4-, and 8-week sampling periods) of the test will have to be completed; however, for slowly degrading chemicals, the entire test may have to be performed (64 weeks). The data will be used to calculate degradation rate constants for each tested chemical over a range of environmental conditions. The rate constants obtained from testing will be integrated into algorithms to assess the fate of organic chemicals leaching into ground water from waste management facilities.

(2) Anaerobic transformations are evaluated under methanogenic and sulfur-reducing conditions. Aerobic biodegradation was not included in the modeling analysis for two reasons:

(i) Aerobic biodegradation would be limited by the concentration of oxygen in ground water. In the laboratory, oxygen would probably not be limiting, and the resulting degradation rates obtained would possibly be overestimations of actual subsurface degradation rates.

(ii) Aerobic degradation would only occur at the leading edge of a contaminant plume where dispersion and other processes dilute the plume with oxygenated water, as stated in Wilson et al. (1985), in paragraph (d)(24) of this guideline.

(3) The anaerobic transformation of chemicals in selected subsurface samples should be estimated from subsurface microcosm studies using methods adapted from procedures recently reported by Wilson et al. (1986), in paragraph (e)(25) of this guideline. These procedures should be used to determine the length of the adaptation period (time interval before detectable degradation of the chemical can be observed) and the half-life of the chemical following the adaptation period. Supporting laboratory methods should be used to measure the levels of residual test chemical, intermediate degradation products, biomass, and other physical-chemical parameters.

**(c) Laboratory procedures—(1) Identification of subsurface sampling sites, collection of subsurface materials, and transportation and storage of subsurface materials.**

(i) A minimum of six subsurface sampling sites should be identified on the basis of two temperatures and three pH values. Three of the sites should have annual average temperatures near 10 °C, and three of the sites should have temperatures near 20 °C. These values are chosen to represent the high and low temperatures commonly observed in aquifers and are one standard deviation on either side of the mean temperature of 15 °C. Generally, low temperature sites are located in northern latitude areas of the United States, and high temperatures correspond to southern latitude areas.

(ii) Acidic (pH 4.5 to 6.0), neutral (pH 6.5 to 7.5), and alkaline (pH 8.0 to 9.5) sites should be selected for each temperature range. These ranges of pH values for ground waters are selected to estimate the effect of pH on microbial degradation capacity and to examine the effect of chemical form on the degradation of chemicals having dissociable hydrogen (i.e., degradation of the protonated and unprotonated forms of the chemical). Ground waters at all sites should have dissolved-O<sub>2</sub> levels at <0.1 mg/L and sulfate concentrations at <10 mg/L.

(iii) Samples of subsurface materials should be collected in a manner that protects them from contamination from surface materials and maintains anaerobic conditions. An appropriate procedure has been reported by Wilson et al. (1983), in paragraph (e)(26) of this guideline. First, a bore hole is drilled to the desired depth with an auger. Then the auger is removed and the sample taken with a wireline piston core barrel, as reported by Zapico et al. (1987), in paragraph (e)(14) of this guideline. The core barrel is immediately transferred to an anaerobic chamber, which is filled and continually purged with nitrogen gas, and all further manipulations are performed in the chamber. Using aseptic procedures, up to 5 cm of the core is extruded, then broken off to produce an uncontaminated face. A sterile paring device is then installed, and the middle 30 to 35 cm of the core is extruded, paring away the outer 1.0 cm of core material. As a result, the material that had been in contact with the core barrel, and thus might be contaminated with surface microorganisms, is discarded. Modifications of this technique can be used for samples obtained from deep coring devices when auger equipment is insufficient because of the depth of the aquifer. Subsurface material should be stored under nitrogen gas and on ice and should be used in microcosm studies within 7 days of collection.

(iv) Ground waters will be collected from the bore hole used to collect subsurface materials. Ground waters will be pumped to the surface. The bore hole should be purged with argon before pumping begins. The pumping mechanism should be flushed with enough ground water to ensure that a representative ground water sample is obtained. This flushing process generally requires a volume equal to 3 to 10 times the volume of

water in the bore hole. Once flushing is complete, ground water samples should be collected, and stored under nitrogen and on ice for transport back to the laboratory. Ground waters should be sterilized by filtration through 0.22  $\mu\text{m}$  membranes on-site in a portable anaerobic chamber which has been filled and continually purged with nitrogen gas. The sterile water should be stored under nitrogen and on ice, and should be used in microcosm studies within 7 days of collection.

(v) Two samples should be collected from each of the six sites. Each core sample should be assayed for test chemical degradation and analyzed for biomass (heterotrophic, sulfate-reducing, and methanogenic) and physical-chemical parameters (pH, cation exchange capacity, total organic carbon, percent base saturation, percent silt, percent sand, percent clay, redox potential, percent ash-free dry weight). Each corresponding ground water sample will be analyzed for pH, dissolved oxygen, dissolved organic carbon, nutrients (sulfate, phosphate, nitrate), conductivity, and temperature.

(2) **Anaerobic microcosm assay.** (i) Microcosms should consist of 160-mL serum bottles which have been filled completely with a slurry of subsurface material and ground water (20 gm equivalent dry weight (dried at 103 °C) solid to 80 mL ground water). One series of serum bottles should be amended to a level of 200 mg/L sulfate added as sodium sulfate to stimulate sulfate-reducing conditions. If the level of soluble sulfate falls below 50 mg/L at any sampling time, additional sulfate (200 mg/L) should be added to all remaining sulfate-amended microcosms. Soluble sulfate levels should be measured by the method of Watwood et al. (1986), in paragraph (e)(23) of this guideline. A second series should be left unamended to simulate methanogenic conditions. All manipulations in preparing the microcosms should be performed aseptically under strict anaerobic conditions, as described in Kaspar and Tiedje (1982) in paragraph (e)(10) of this guideline, or other equivalent methods, and all equipment in contact with the subsurface samples should be sterilized. Sterile controls should be prepared by autoclaving the samples for a minimum of 1 h on each of 3 consecutive days. Test chemical amendments should be prepared in sterile nitrogen-purged ground water. Sparingly soluble and volatile chemicals should be added to sterile, nitrogen-purged ground water and then stirred overnight without a head space.

(ii) The active and control microcosms should be dosed with the test chemical and 0.0002 percent (W/V) Resazurin as a redox indicator, and then each unit should be immediately sealed with a Teflon-coated gray butyl rubber septum and crimp seal. As stated previously, all manipulations should be performed under strict anaerobic conditions, as described in Kaspar and Tiedje (1982) in paragraph (e)(10) of this guideline, or other equivalent methods. The microcosms should be stored in the dark at the original in situ temperature. Active microcosms and control microcosms, randomly selected from the sulfate-amended series and the unamended series, should be sacrificed and analyzed at 0, 4, 8, 16, 32, and 64 weeks

for residual test chemical and the formation of degradation intermediates. Once the residual level of the chemical drops below 5 percent of the initial concentration, analysis of microcosms at subsequent time periods is not required. The active microcosms and control microcosms from both series, at weeks 0, 16, and 64 (or randomly selected from the remaining samples the week following 95 percent degradation of the chemical, if less than week 64) should also be analyzed for heterotrophic, sulfate-reducing, and methanogenic bacteria.

(iii) Three concentrations of each chemical tested should be used. The test chemical concentrations should range between a low level of 30 times the health-based level and a level that equates to the chemical's solubility (or to a level that causes inhibition of the test chemical's degradation).

(iv) Biomass measurements should be made for heterotrophic, sulfate-reducing, and methanogenic bacteria. Biomass measurements have been included to ensure comparability of results between samples of subsurface materials. Degradation rates derived from sediment samples having significantly high or low (student "t" test, 90 percent level) bacterial populations would not be considered in subsequent modeling efforts. Also, the ratio of sulfate-reducing organisms to methanogenic organisms would be used to determine if the dominant redox conditions were sulfate-reducing or methanogenic. Anaerobic techniques described by Kaspar and Tiedje (1982), cited in paragraph (e)(10) of this guideline, or other equivalent methods, should be used.

(v) Heterotrophic bacterial concentrations should be measured by a modification of the procedure developed by Molongoski and Klug (1976) and Clark (1965), cited in paragraphs (e)(13) and (e)(6) of this guideline, respectively. A 10-mL sample taken from the center of the appropriate microcosm, which has been well mixed, should be aseptically transferred to 100 mL of sterile dilution medium and agitated to suspend the organisms. Samples (10 mL volume) should then be transferred immediately from the center of the suspension to a 90-mL sterile dilution medium blank to give a  $10^{-2}$  dilution; 10 mL should be similarly transferred to another 90-mL of sterile dilution medium to obtain a dilution of  $10^{-3}$ . This process should be repeated to give a dilution series through at least  $10^{-7}$ . Only the  $10^{-1}$  dilution need be prepared from control samples. The dilution series can be modified to include dilutions of greater than  $10^{-7}$ , if necessary, and if sufficient sample is available. From the highest dilution, 0.1-mL portions should be transferred to the surface of each of three dilute tryptone glucose extract agar plates. The sample should be spread immediately over the surface of the plates and the process should be repeated for lower dilutions. Dilute tryptone glucose agar plates should be prepared by combining 24.0 g tryptone glucose extract agar in 1 L of distilled water. The mixture should be autoclaved, and 25 mL of the molten agar should be transferred to Petri plates. Agar plates should be stored in an anaerobic chamber for a minimum of 24 hours before use. The inoculated plates

should be incubated in plastic bags in the glove box, or, if necessary, removed and kept in anaerobic jars. After 14 days of incubation, the plates should be examined and the total count per gram of dry sediment material should be determined. If the plates from the most dilute sample show more than 300 colonies, the dilution series was inadequate. In this case, all of the plates should be discarded, and the process should be repeated with greater dilutions, as appropriate.

(vi) Sulfate-reducing species should be enumerated by the MPN (most probable number) technique as described in Pankhurst (1971) in paragraph (e)(15) of this guideline, or other equivalent method. The dilution series should be prepared as described for heterotrophic bacteria.

(vii) Methanogenic bacteria should be enumerated by the MPN technique as described by Jones et al. (1982) in paragraph (e)(9) of this guideline, or by another equivalent method. The dilution series should be prepared as described for heterotrophic bacteria.

**(3) Analytical measures of the loss of test chemical and intermediate degradation products.** (i) The loss of test chemical should be quantified by measuring the residual test chemical. The formation of degradation intermediates should be quantified in microcosm assays for test chemicals that can potentially be transformed. Analysis for degradation intermediates should be required when the level of test chemical has been reduced by more than 25 percent. Concentrations of the potential degradation products 1,2-, 1,3-, and 1,4-dichlorobenzene, and 1,2,4,5-tetrachlorobenzene should be measured in the appropriate microcosms used to analyze the degradation of pentachlorobenzene. The concentration of the potential degradation product dibromomethane should be measured in the appropriate microcosms used to analyze the degradation of bromoform. The potential degradation products methanethiol and chloromethane (methyl chloride) should be measured in the appropriate microcosm used to analyze the degradation of trichloromethanethiol. The potential intermediate products 1,2-, 1,3-, and 1,4-dichlorobenzene should be measured in the appropriate microcosm used to analyze the degradation of 1,2,4,5-tetrachlorobenzene.

(ii) Measurements of test chemical and intermediate degradation products will require organic analytical techniques tailored to the specific test chemical and subsurface material being investigated. Several extraction and purge-trap techniques are available for the recovery of residual test chemicals and degradative intermediates from subsurface materials. Unique analytical procedures would have to be developed or modified for each test chemical and sediment. The following represent examples of such techniques:

(A) Soxhlet extraction as described in Anderson et al. (1985), Bossart et al. (1984), Eiceman et al. (1986), Grimalt et al. (1984), and Kjolholt

(1985) under paragraphs (e)(2), (e)(3), (e)(7), (e)(8), and (e)(11) of this guideline, respectively.

(B) Shake flask method as described in Brunner et al. (1985), and Russel and McDuffie (1983) in paragraphs (e) (4) and (16) of this guideline, respectively.

(C) Sonification as described in Schellenberg et al. (1984) in paragraph (e)(17) of this guideline.

(D) Homogenization as described in Fowlie and Sulman (1986), Lopez-Avila et al. (1983), Sims et al. (1982), Stott and Tabatabai (1985), and U.S. EPA (1982) in paragraphs (e)(5), (e)(12), (e)(18), (e)(19), and (e)(22) of this guideline, respectively.

(E) Purge-trap techniques have been described by Wilson et al. (1986) in paragraph (e)(24) of this guideline.

(iii) These procedures can be readily coupled to gas chromatography (GC) and high-pressure liquid chromatography (HPLC) procedures to quantify the chemicals of interest. Whatever analytical procedure is selected should follow Good Laboratory Practice Standards of 40 CFR part 792.

**(4) Characterization of subsurface materials and ground waters.**

(i) Subsurface materials should be classified, described, and characterized as to soil type and physical and chemical properties using standard procedures as described by the Soil Conservation Service (U.S. Department of Agriculture, 1972 and 1975) under paragraphs (e)(20) and (e)(21) of this guideline, or other equivalent methods. Ten parameters will be measured as follows:

(A) Total organic carbon (TOC).

(B) pH.

(C) Cation exchange capacity.

(D) Percent base saturation.

(E) Percent silt.

(F) Percent sand.

(G) Percent clay.

(H) Redox potential.

(I) Percent ash-free dry weight.

(J) Texture.

(ii) Ground water should be characterized for the following, by standard water and wastewater methods described by the American Public Health Association (1985) in paragraph (e)(1) of this guideline, or other equivalent methods:

- (A) pH.
- (B) Dissolved oxygen.
- (C) Dissolved organic carbon.
- (D) Nutrients including sulfate, phosphate, and nitrate.
- (E) Conductivity.
- (F) Temperature.

(iii) The properties of pH, dissolved oxygen, and temperature should be measured at the site of collection. All other properties should be measured in the laboratory.

(d) **Data to be reported to the Agency.** Data should be reported for the two subsurface samples and corresponding ground waters taken from the six different sampling sites.

(1) The following should be reported for subsurface sediment samples:

(i) Levels of residual test chemicals (milligrams per gram of dry weight) quantified in each of the randomly selected replicate microcosm and sterile controls at the specific time periods identified under the anaerobic microcosm assay.

(ii) Numbers of heterotrophic, sulfate-reducing, and methanogenic bacteria (colony forming units (CFU) or most probable number units (MPNU) per gram dry weight) enumerated in each replicate microcosm and sterile controls at the specific time periods identified under the anaerobic microcosm assay.

(iii) Levels of persistent degradation intermediates identified in microcosm and sterile controls at the specific time periods identified under the anaerobic microcosm assay.

(iv) Measured values for pH, cation exchange capacity (meq/100 gm dry wt), percent base saturation, percent silt (percent dry weight), percent sand (percent dry weight), percent clay (percent dry weight), redox potential (Eh, Standard Hydrogen Electrode), percent ash free dry weight (percent dry weight), and a description of texture.

(2) For ground water samples, the analysis report should provide measured values (in units specified) for:

- (i) pH.
- (ii) Dissolved oxygen (in milligrams per liter).
- (iii) Dissolved organic carbon (in milligrams per liter).
- (iv) Nutrients including sulfate, phosphate, and nitrate (in milligrams per liter).
- (v) Conductivity (S, 25 °C).
- (vi) Temperature (expressed as degrees celsius).

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

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- (14) Zapico, M.M. et al. A wireline piston core barrel for sampling cohesionless sand and gravel below the water table. *Ground Water Monitoring Review*. Summer, Vol. 7, No. 3:74–82 (1987).
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- (17) Schellenberg, K. et al. Sorption of chlorinated phenols by natural sediments and aquifer materials. *Environmental Science and Technology*. 18:652–657 (1984).
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