(B) Shake the containers throughout the equilibration period at a rate that suspends all solids in the solution phase.

(ii) *Centrifugation.* When the equilibration time has expired, centrifuge the containers for t_c minutes.

(iii) *Chemical extraction.* (A) After centrifugation, remove the supernatant aqueous phase from the solid-solution mixture.

(B) Extract the chemical adsorbed on the sediment or soil colloid surfaces with solvent.

(iv) *Chemical analysis.* Determine the amount of parent test chemical in the aqueous equilibrating solution and organic solvent extractions. Use any method or combination of methods suitable for the identification and quantitative detection of the parent test chemical.

(c) *Reporting.* Report the following information:

(1) Temperature at which the test was conducted.

(2) Detailed description of the analytical technique(s) used in the chemical extraction, recovery, and quantitative analysis of the parent chemical.

(3) Amount of parent test chemical applied, the amount recovered, and the percent recovered.

(4) Extent of adsorption by containers and the approach used to correct the data for adsorption by containers.

(5) The individual observations, the mean values, and graphical plots of x/m as a function of C_e for each sediment or soil for (i) the equilibration time determination and (ii) the isotherm determination.

(6) The quantities K, n, and l/n.

(7) Soil information: Soil Order, series, texture, sampling location, horizon, general clay fraction mineralogy.

(8) Sediment information: sampling location, general clay fraction mineralogy.

(9) Sediment and soil physical-chemical properties: percent sand, silt, and clay (particle size analysis); percent organic matter; pH (1/1 solids/H_2O); and cation exchange capacity.

(10) The procedures used to determine the physical/chemical properties listed under paragraphs (c) (7) through (9) of this section.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Aharonson, N., Kafkafi, U. ''Adsorption, mobility and persistence of thiabendazole and methyl 2-benzimidasole carbamate in soils,'' *Journal of Agricultural and Food Chemistry,* 23:720–724 (1975).

(2) Goring, C.A.I., Hamaker, J.W., (eds). *Organic Chemicals in the Soil Environment.* Vol. I & II (New York: Marcel Dekker, Inc., 1972).

(3) Harvey, R.G. et al. ''Soil adsorption and volatility of dinitroaniline herbicides,'' *Weed Science,* 22:120–124 (1974).

(4) Murray, D.S. et al. ''Comparative adsorption, desorption, and mobility of dipropetryn and prometryn in soil,'' *Journal of Agricultural and Food Chemistry,* 23:578–581 (1973).

(5) Saltzman, S.L. et al. ''Adsorption, desorption of parathion as affected by soil organic matter,'' *Journal of Agricultural and Food Chemistry,* 20:1224–1226 (1972).

(6) Weber, J.B. ''Model soil system, herbicide leaching, and sorption,'' *Weed Science,* 19:145–160 (1971).

(7) Wu, C.H., et al. ''Napropamide adsorption, desorption, and movement in soils,'' *Weed Science,* 23:454–457 (1975).

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987; 54 FR 29715, July 14, 1989]

Subpart D—Transformation Processes

§ 796.3100 Aerobic aquatic biodegradation.

(a) *Introduction*—(1) *Purpose.* (i) This Guideline is designed to develop data on the rate and extent of aerobic biodegradation that might occur when chemical substances are released to aquatic environments. A high biodegradability result in this test provides evidence that the test substance will be biodegradable in natural aerobic freshwater environments.

(ii) On the contrary, a low biodegradation result may have other causes than poor biodegradability of the test substance. Inhibition of the microbial inoculum by the test substance at the test concentration may be observed. In such cases, further work is needed to assess the aerobic aquatic biodegradability and to determine the concentrations at which toxic effects are evident. An estimate of the expected environmental concentration will help to put toxic effects into perspective.

(2) *Definitions.* (i) ''Adaptation'' is the process by which a substance induces the synthesis of any degradative enzymes necessary to catalyze the transformation of that substance.

(ii) ''Ultimate Biodegradability'' is the breakdown of an organic compound to CO_2, water, the oxides or mineral salts of other elements and/or to products associated with normal metabolic processes of microorganisms.

(iii) ''Ready Biodegradability'' is an expression used to describe those substances which, in certain biodegradation test procedures, produce positive results that are unequivocal and which lead to the reasonable assumption that the substance will undergo rapid and ultimate biodegradation in aerobic aquatic environments.

(3) *Principle of the test method.* This Guideline method is based on the method described by William Gledhill (1975) under paragraph (d)(1) of this section. The method consists of a 2-week inoculum buildup period during which soil and sewage microorganisms are provided the opportunity to adapt to the test compound. This inoculum is added to a specially equipped Erlenmeyer flask containing a defined medium with test substance. A reservoir holding barium hydroxide solution is suspended in the test flask. After inoculation, the test flasks are sparged with CO_2-free air, sealed, and incubated, with shaking in the dark. Periodically, samples of the test mixture containing water-soluble test substances are analyzed for dissolved organic carbon (DOC) and the $Ba(OH)_2$ from the reservoirs is titrated to measure the amount of CO_2 evolved. Differences in the extent of DOC disappearance and CO_2 evolution between control flasks containing no test substance, and flasks containing test substance are used to estimate the degree of ultimate biodegradation.

(4) *Prerequisites.* The total organic carbon (TOC) content of the test substance shall be calculated or, if this is not possible, analyzed, to enable the percent of theoretical yield of carbon dioxide and percent of DOC loss to be calculated.

(5) *Guideline information.* (i) Information on the relative proportions of the major components of the test substance will be useful in interpreting the results obtained, particularly in those cases where the result lies close to a ''pass level.''

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(6) *Reference substances.* Where investigating a chemical substance, reference compounds may be useful and an inventory of suitable reference compounds needs to be identified. In order to check the activity of the inoculum the use of a reference compound is desirable. Aniline, sodium citrate, dextrose, phthalic acid and trimellitic acid will exhibit ultimate biodegradation under the conditions of this Test Guideline method. These reference substances must yield 60 percent of theoretical maximum CO_2 and show a removal of 70 percent DOC within 28 days. Otherwise the test is regarded as invalid and shall be repeated using an inoculum from a different source.

(7) *Reproducibility.* The reproducibility of the method has not yet been determined; however it is believed to be appropriate for a screening test which has solely an acceptance but no rejective function.

(8) *Sensitivity.* The sensitivity of the method is determined by the ability to measure the endogenous CO_2 production of the inoculum in the blank flask and by the sensitivity limit of the dissolved organic carbon analysis. If the test is adapted to handle ^{14}C-labeled test substances, test substance concentrations can be much lower.

(9) *Possibility of standardization.* This possibility exists. The major difficulty is to standardize the inoculum in such a way that interlaboratory reproducibility is ensured.

(10) *Possibility of automation.* None at present, although parts of the analyses may be automated.

(b) *Test procedures*—(1) *Preparations*—(i) *Apparatus.* The shake flask apparatus under the following Figure 1 contains 10 mL of 0.2N $Ba(OH)_2$ in an open container suspended over 1 liter of culture medium in a 2-liter Erlenmeyer flask.

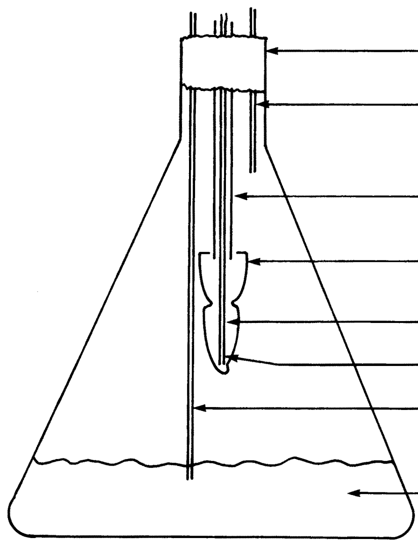


FIGURE 1—SHAKE-FLASK SYSTEM FOR CARBON DIOXIDE EVOLUTION

The $Ba(OH)_2$ container is made by placing a constriction just above the 10 mL mark of a 50 mL heavy-duty centrifuge tube and attaching the centrifuge tube to a 2 mm I.D.×9 mm O.D. glass tube by means of 3 glass support rods. The centrifuge tube opening is large enough to permit CO_2 to diffuse into the $Ba(OH)_2$, while the constriction permits transferal of the flask to and from the shaker without $Ba(OH)_2$ spillage into the medium. For periodic removal and addition of base from the center well, a polypropylene capillary tube, attached at one end to a 10 ml disposable syringe, is inserted through the 9 mm O.D. glass tube into the $Ba(OH)_2$ reservoir. The reservoir access port is easily sealed during incubation with a serum bottle stopper. Two glass tubes are added for sparging, venting, and medium sampling. The tops of these tubes are connected with a short section of flexible tubing during incubation.

(ii) *Reagents and stock solutions.* (A) Stock solutions, I, II, and III under the following Table 1.

(B) Yeast extract.

(C) Vitamin-free casamino acids.

(D) 70 percent O_2 in nitrogen or CO_2-free air.

(E) 0.2N $Ba(OH)_2$.

(F) 0.1 N HCl.

(G) 20 percent H_2SO_4.

(H) Phenolphthalein.

(I) Dilution water—distilled, deionized water (DIW).

(iii) *Soil inoculum.* A fresh sample of an organically rich soil is used as the inoculum in the ultimate biodegradation test. Soil is collected, prepared, and stored according to the recommendations of Pramer and Bartha (1972) under paragraph (d)(2) of this section. The soil surface is cleared of litter and a soil sample is obtained 10 to 20 cm below the surface. The sample is screened through a sieve with 2 to 5 mm openings and stored in a polyethylene bag at 2 to 4 °C for not more than 30 days prior to use. The soil is never allowed to air-dry, and shall not be frozen during storage.

TABLE 1—MEDIUM EMPLOYED FOR ASSAY OF CO_2 EVOLUTION

Solution [1]	Compound	Stock Solution Conc. (g/L)
I	NH_4Cl	35
	KNO_3	15
	$K_2HPO_4 \cdot 3H_2O$	750
	$NaH_2PO_4 \cdot H_2O$	25
II [2]	KCl	10
	$MgSO_4$	20
	$FeSO_4 \cdot 7H_2O$	1
III	$CaCl_2$	5
	$ZnCl_2$	0.05
	$MnCl_2 \cdot 4H_2O$	0.5
	$CuCl_2$	0.05
	$CoCl_2$	0.001
	H_3BO_3	0.001
	MoO_3	0.0004

[1]= Each liter of test medium contains 1 mL of each solution.
[2]= Final pH is adjusted to 3.0 with 0.10 N HCl.

(iv) *Acclimation Medium.* Acclimation medium is prepared by adding, for each liter of distilled, deionized water (DIW): 1 mL each of solutions I, II, and III in Table 1 in paragraph (b)(1)(iii) of this section, 1.0 gm of soil inoculum (prepared according to paragraph (b)(1)(iii) of this section), 2.0 mL of aerated mixed liquor (obtained from an activated sludge treatment plant not more than 2 days prior to commencing the acclimation phase, and stored in the interim at 4 °C) and 50 mL raw domestic influent sewage. This medium is mixed for 15 minutes and filtered through a glass wool plug in a glass funnel. The filtrate is permitted to stand for 1 hour, refiltered through glass wool, and supplemented with 25 mg/L each of Difco vitamin-free casamino acids and yeast extract. Appropriate volumes are added to 2-liter Erlenmeyer flasks. Test compounds are added incrementally during the acclimation period at concentrations equivalent to 4, 8, and 8 mg/L carbon on days 0, 7, and 11, respectively. On day 14, the medium is refiltered through glass wool prior to use in the test. For evaluating the biodegradability of a series of functionally or structurally related chemicals, media from all inoculum flasks may be combined before final filtration.

(2) *Procedures.* (i) Inoculum (100 mL of acclimation medium) is added to 900 mL DIW containing 1 mL each of solutions I, II, and III in Table 1 under paragraph (b)(1)(iii) of this section in a 2-liter Erlenmeyer flask. Test compound equivalent to 10 mg/liter carbon is added to each of the replicate flasks containing the test medium. Ten mL of 0.2 N Ba $(OH)_2$ are added to the suspended reservoir in each flask and duplicate 10 mL samples of $Ba(OH)_2$ are also saved as titration blanks for analysis with test samples. Flasks are sparged with CO_2-free air (for volatile test materials, sparging is done prior to addition of the chemical), sealed, and placed on a gyrotary shaker (approximately 125 rpm) at 20 to 25 °C in the dark. For each set of experiments, each test, reference, inhibited, and control system should be analyzed at time zero and at a minimum of four other times from time zero through day 28. Sampling must be made with sufficient frequency to allow for a smooth plot of biodegradation with time. Sampling times should be varied by the investigator as deemed appropriate to match the rate of degradation of the test substance. Tests may be terminated when biodegradation reaches a plateau and is consistent (±10 percent) over 3 consecutive days or on day 28, whichever occurs first. For chemicals which are water soluble at the test concentration, an adequate volume (5 to 10 mL) of medium is removed for DOC analysis. Each sample for DOC analysis should be filtered through a membrane filter of 0.45 micrometer pore diameter before DOC analysis. For all test and reference compounds, $Ba(OH)_2$ from the center well is removed for analysis. The center well is rinsed with 10 mL CO_2-free DIW and is refilled with fresh base. Rinse water is combined with the $Ba(OH)_2$ sample to be analyzed. Flasks are resealed and placed on the shaker. On the day prior to terminating the test, 3 mL of 20 percent H_2SO_4 are added to the medium to release carbonate bound CO_2.

(ii) For each set of experiments, each test substance shall be tested in triplicate.

(iii) For each set of experiments, one or two reference compounds are included to assess the microbial activity of the test medium. Duplicate reference flasks are prepared by adding reference compound equivalent to 10 mg/liter carbon to each of two flasks containing the test medium. Reference compounds which are positive for ultimate biodegradability include: sodium citrate, dextrose, phthalic acid, trimellitic acid, and aniline.

(iv) For each test set, triplicate controls receiving inoculated medium and no test compound, plus all test and reference flasks, are analyzed for CO_2 evolution and DOC removal. Results from analysis of the control flasks (DOC, CO_2 evolution, etc.) are subtracted from corresponding experimental flasks containing test compound in order to arrive at the net effect due to the test compound.

(v) A test system containing a growth inhibitor should be established as a control for each substance tested for biodegradation by this method. That inhibited system must contain the same amount of water, mineral nutrients, inoculum, and test substance used in the uninhibited test systems, plus 50 mg/L mercuric chloride ($HgCl_2$) to inhibit microbial activity.

(vi) Flasks shall be incubated in the dark to minimize both photochemical reactions and algal growth. Appropriate sterile controls or controls containing a metabolic inhibitor, such as 50 mg/1 $HgCl_2$, are needed to correct for interferences due to nonbiological degradation. With volatile organic materials, sparging with CO_2-free air is performed only once, just prior to addition of the test chemical. Analyses for CO_2 evolution and DOC removal are conducted within 2 to 3 hours of sampling to minimize interferences which may occur in storage. All glassware should be free of organic carbon contaminants.

(3) *Analytical measurements.* The quantity of CO_2 evolved is measured by titration of the entire $Ba(OH)_2$ sample (10 mL $Ba(OH)_2$+10 mL rinse water) with 0.1 N HCl to the phenolphthalein end point. $Ba(OH)_2$ blanks are also supplemented with 10 mL CO_2-free DIW and titrated in a similar manner. Samples (5 mL) for DOC are centrifuged and/or filtered and supernatant or filtrate analyzed by a suitable total organic carbon method.

(c) *Data and reporting*—(1) *Treatment of results.* (i) Test compound (10 mg carbon) is theoretically converted to 0.833 mmol CO_2. Absorbed CO_2 precipitates as $BaCO_3$ from $Ba(OH)_2$, causing a reduction in alkalinity by the equivalent of 16.67 mL of 0.1 N HCl for complete conversion of the test compound carbon to CO_2. Therefore, the percent theoretical CO_2 evolved from the test compound is calculated at any sampling time from the formula:

Percent CO_2 evolution=[(TF − CF)/16.67] 100 (for 10 mg/L test compound carbon)

where:

TF= mL 0.1 N HCl required to titrate $Ba(OH)_2$ samples from the test flask

CF= mL 0.1 N HCl required to titrate $Ba(OH)_2$ samples from the control flask.

(ii) The cumulative percent CO_2 evolution at any sample time is calculated as the summation of the percent CO_2 evolved at all sample points of the test.

(iii) The percent DOC disappearance from the test compound is calculated from the following equation:

Percent DOC Removal=[1 − (DTF_x − DCF_x)/(DTF_o − DCF_o)] 100

where:

DTF= Dissolved organic carbon from test flask

DCF= Dissolved organic carbon from control flask

o= Day zero measurements

x= Day of measurements during test.

(iv) The difference between the amount of 0.1 N HCl used for the $Ba(OH)_2$ titration blank samples and the $Ba(OH)_2$ samples from the control units (no test compound) is an indication of the activity of the microorganisms in the test system. In general, this difference is approximately 1 to 3 mL of 0.1 N HCl at each sampling time. A finding of no difference in the titration volumes between these two samples indicates a poor inoculum. In this case, the validity of the test results is questionable and the test set shall be rerun beginning with the acclimation phase.

(v) CO_2 evolution in the reference flasks is also indicative of the activity of the microbial test system. The suggested reference compounds should all yield final CO_2 evolution values of at least 60 percent of theoretical CO_2. If, for any test set, the percent theoretical

CO_2 evolution value for the reference flasks is outside this range, the test results are considered invalid and the test is rerun.

(vi) Inhibition by the test compound is indicated by lower CO_2 evolution in the test flasks than in the control flasks. If inhibition is noted, the study for this compound is rerun beginning with the acclimation phase. During the test phase for inhibitory compounds, the test chemical is added incrementally according to the schedule: Day 0—0.5 mg/liter as organic carbon, Day 2—1 mg/liter C, Day 4—1.5 mg/liter C, Day 7—2 mg/liter C, Day 10—5 mg/liter C. For this case, the $Ba(OH)_2$ is sampled on Day 10, and weekly thereafter. The total test duration remains 28 days.

(vii) The use of ^{14}C-labeled chemicals is not required. If appropriately labeled test substance is readily available and if the investigator chooses to use this procedure with labeled test substance, this is an acceptable alternative. If this option is chosen, the investigator may use lower test substance concentrations if those concentrations are more representative of environmental levels.

(2) *Test report.* (i) For each test and reference compound, the following data shall be reported.

(ii) Information on the inoculum, including source, collection date, handling, storage and adaptation possibilities (i.e., that the inoculum might have been exposed to the test substance either before or after collection and prior to use in the test).

(iii) Results from each test, reference, inhibited (with $HgCl_2$) and control system at each sampling time, including an average result for the triplicate test substance systems and the standard deviation for that average.

(iv) Average cumulative percent theoretical CO_2 evolution over the test duration.

(v) Dissolved organic carbon due to test compound at each sampling time (DTF-DCF).

(vi) Average percent DOC removal at each sampling time.

(vii) Twenty-eight day standard deviation for percent CO_2 evolution and DOC removal.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Gledhill, W.E. ''Screening Test for Assessment of Ultimate Biodegradability: Linear Alkyl Benzene Sulfonate,'' *Applied Microbiology*, 30:922–929 (1975).

(2) Pramer, D., Bartha, R. ''Preparation and Processing of Soil Samples for Biodegradation Testing,'' *Environmental Letters*, 2:217–224 (1972).

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987]

§ 796.3500 Hydrolysis as a function of pH at 25 °C.

(a) *Introduction*—(1) *Background and purpose.* (i) Water is one of the most widely distributed substances in the environment. It covers a large portion of the earth's surface as oceans, rivers, and lakes. The soil also contains water, as does the atmosphere in the form of water vapor. As a result of this ubiquitousness, chemicals introduced into the environment almost always come into contact with aqueous media. Certain classes of these chemicals, upon such contact, can undergo hydrolysis, which is one of the most common reactions controlling chemical stability and is, therefore, one of the main chemical degradation paths of these substances in the environment.

(ii) Since hydrolysis can be such an important degradation path for certain classes of chemicals, it is necessary, in assessing the fate of these chemicals in the environment, to know whether, at what rate, and under what conditions a substance will hydrolyze. Some of these reactions can occur so rapidly that there may be greater concern about the products of the transformation than about the parent compounds. In other cases, a substance will be resistant to hydrolysis under typical environmental conditions, while, in still other instances, the substance may have an intermediate stability that can result in the necessity for an assessment of both the original compound and its transformation products. The importance of transformation of chemicals via hydrolysis in aqueous media in the environment can be determined quantitatively from data on hydrolysis rate constants. This hydrolysis Test Guideline represents a test to allow one to determine rates of hydrolysis at any pH of environmental concern at 25°C.

(2) *Definitions and units.* (i) ''Hydrolysis'' is defined as the reaction of an organic chemical with water, such that one or more bonds are broken and the reaction products of the transformation incorporate the elements of water (H_2O).

(ii) ''Elimination'' is defined in this Test Guideline to be a reaction of an organic chemical (RX) in water in which the X group is lost. These reactions generally follow the same type of rate laws that hydrolysis reactions follow and, thus, are also covered in this Test Guideline.

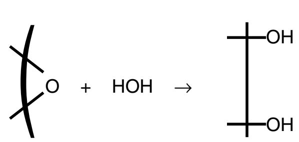
(iii) A ''first-order reaction'' is defined as a reaction in which the rate of disappearance of the chemical substance being tested is directly proportional to the concentration of the chemical substance and is not a function of the concentrations of any other substances present in the reaction mixture.

(iv) The ''half-life'' of a chemical is defined as the time required for the concentration of the chemical substance being tested to be reduced to one-half its initial value.

(v) ''Hydrolysis'' refers to a reaction of an organic chemical with water such that one or more bonds are broken and the reaction products incorporate the elements of water (H_2O). This type of transformation often results in the net exchange of a group X, on an organic chemical RX, for the OH group from water. This can be written as:

$$RX+HOH \rightarrow ROH+HX.$$

(A) Another result of hydrolysis can be the incorporation of both H and OH in a single product. An example of this is the hydrolysis of epoxides, which can be represented by



(B) The hydrolysis reaction can be catalyzed by acidic or basic species, including OH^- and $H_3O^{=}$ ($H^{=}$). The promotion of the reaction by H_3O^- or OH^- is called specific acid or specific base catalysis, respectively, as contrasted with general acid or base catalysis encountered with other cationic or anionic species. Usually, the rate law for chemical RX can be written as:

Equation 1

$$-d[RX]/_{d=} = k_h[RX]=k_A[H^{=}]\,[RX]$$

$$+k_B[OH^-]\,[RX]+k'_N\,[H_2O]\,[RX],$$

where K_A, k_B and k'_N are the second-order rate constants for acid and base catalyzed and neutral water processes, respectively. In dilute solutions, such as are encountered in following this Test Guideline, water is present in great excess and its concentration is, thus, essentially constant during the course of the hydrolysis reaction. At fixed pH, the reaction, therefore, becomes pseudo first-order, and the rate constant (k_h) can be written as:

Equation 2

$$k_h=k_A\,[H^{=}]+k_B\,[OH^-]+k_N,$$

where k_N is the first-order neutral water rate constant. Since this is a pseudo first-order process, the half-life is independent of the concentration and can be written as:

Equation 3

$$t_{1/2}=0.693/k_h.$$

At constant pH, Equation 1 can be integrated to yield the first order rate expression

Equation 4

$$\log_{10}C=-\,(k_h\,t/_{2.303})+\log_{10}C_o,$$

where C is the concentration of the test chemical at time t and C_o is the initial chemical concentration (t=0).

(C) At a given pH, Equation 2 under paragraph (a)(2)(v)(B) of this section contains three unknowns, k_A, k_B, and k_N. Therefore, three equations (i.e., measurements at three different pH's at a fixed temperature) are required if one wishes to solve for these quantities. Making suitable approximations for quantities that are negligible, the expressions for k_A, k_B, and k_N using values of k_h measured at pH 3, 7, and 11 are:

Equation 5

$$k_A=10^3\,[k_h\,(3)-k_h\,(7)+10^{-4}\,k_h\,(11)]$$

$$k_B=10^3\,[k_h\,(11)-k_h\,(7)+10^{-4}\,k_h\,(3)]$$

$$k_N=k_h\,(7)-10^{-4}\,[k_h\,(3)+k_h\,(11)]$$

The calculated rate constants from equation 5 under this paragraph can be employed in equation 2 under paragraph (a)(2)(v)(B) of this section to calculate the hydrolysis rate of a chemical at any pH of environmental concern.

(D) The equations under paragraph (a)(2) of this section apply whether the test chemical has one or more hydrolyzable groups. In the latter case, the rate may be written as:

Equation 6

$$-d[RX]/_{dt}=[RX]=k_2\,[RX]+\;.\;.\;.\;.\;+k_n\,[RX]=(k_1+k_2+\;.\;.\;.\;.\;.\;k_n)\,[RX]=k_h\,[RX].$$

Equation 6 applies to the hydrolysis rate of a molecule having n hydrolyzable groups, each of which follows first-order reaction kinetics. The measured k_h is now the sum of the individual reaction rates and is the only rate constant required in this section.

(3) *Principle of the test method.* Procedures described in this section enable sponsors to obtain quantitative information on hydrolysis rates through a determination of hydrolysis rate constants and half-lives of chemicals at pH 3.00, 7.00, and 11.00 at 25 °C. The three measured rate constants are used to determine the acidic, basic, and neutral rate constants associated with a hydrolytic reaction. The latter constants can then be employed in determining the hydrolysis rates of chemicals at any pH of environmental concern at 25 °C.

(4) *Applicability and specificity.* There are several different common classes of organic chemicals that are subject to hydrolysis transformation, including esters, amides, lactones, carbamates, organophosphates, and alkyl halides. Processes other than nucleophilic displacement by water can also take place. Among these are elimination reactions that exhibit behavior similar to hydrolysis and, therefore, are also covered in this section.

(b) *Test procedures*—(1) *Test conditions*—(i) *Special laboratory equipment.* (A) A thermostatic bath that can be maintained at a temperature of 25 ±1 °C.

(B) A pH meter that can resolve differences of 0.05 pH units or less.

(C) Stoppered volumetric flasks (no grease) or glass ampoules that can be sealed.

(ii) *Purity of water.* Reagent-grade water (e.g., water meeting ASTM Type IIA standards or an equivalent grade) shall be used to minimize biodegradation. ASTM Type IIA water is described in ASTM D 1193–77 (Reapproved 1983), "Standard Specification for Reagent Water." ASTM D 1193–77 (Reapproved 1983) is available for inspection at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202–741–6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.* This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the Non-Confidential Information Center (NCIC) (7407), Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, Room B–607 NEM, 401 M St., SW., Washington, DC 20460, between the hours of 12 p.m. and 4 p.m. weekdays excluding legal holidays, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(iii) *Sterilization.* All glassware shall be sterilized. Aseptic conditions shall be used in the preparation of all solutions and in carrying out all hydrolysis experiments to eliminate or minimize biodegradation. Glassware can be sterilized in an autoclave or by any other suitable method.

(iv) *Precautions for volatility.* If the chemical is volatile the reaction vessels shall be almost completely filled and sealed.

(v) *Temperature controls.* All hydrolysis reactions shall be carried out at 25 °C (±1 °C) and with the temperature controlled to ±0.1 °C.

(vi) *pH conditions.* It is recommended that all hydrolysis experiments be performed at pH 3.00, 7.00, and 11.00 ±0.05 using the appropriate buffers described in paragraph (b)(2)(i)(A) of this section.

(vii) *Concentration of solutions of chemical substances.* The concentration of the test chemical shall be less than one-half the chemical's solubility in water but not greater than 10^{-3} M.

(viii) *Effect of acidic and basic groups.* Complications can arise upon measuring the rate of hydrolysis of chemicals that reversibly ionize or are protonated in the pH range 3.00 to 11.00. Therefore, for these chemicals, it is recommended that these hydrolysis tests be performed at pH 5.00, 7.00, and 900 ±0.05 using the appropriate buffers described in paragraphs (b)(2)(i) (A) and (B) of this section. If a test chemical reversibly ionizes or protonates in the pH range 5.00 to 9.00, then it is recommended that additional hydrolysis tests should be carried out at pH 6.00 and 8.00 ±0.05 using the buffers described in paragraph (b)(2)(i)(B) of this section.

(ix) *Buffer catalysis.* For certain chemicals, buffers may catalyze the hydrolysis reaction. If this is suspected, hydrolysis rate determination shall be carried out with the appropriate buffers and the same experiments repeated at buffer concentrations lowered by at least a factor of five. If the hydrolysis reaction produces a change of greater than 0.05 pH units in the lower concentration buffers at the end of the measurement time, the test chemical concentrations also shall be lowered by at least a factor of five. Alternatively, test chemical concentrations and buffer concentrations may both be lowered simultaneously by a factor of five. A sufficient criterion for minimization of buffer catalysis is an observed equality in the hydrolysis rate constant for two different solutions differing in buffer or test chemical concentration by a factor of five.

(x) *Photosensitive chemicals.* The solution absorption spectrum can be employed to determine whether a particular chemical is potentially subject to photolytic transformation upon exposure to light. For chemicals that absorb light of wavelengths greater than 290 nm, the hydrolysis experiment shall be carried out in the dark, under amber or red safelights, in amber or red glassware, or employing other suitable methods for preventing photolysis. The absorption spectrum of the chemical in aqueous solution can be measured under § 796.1050.

(xi) *Chemical analysis of solutions.* In determining the concentrations of the test chemicals in solution, any suitable analytical method may be employed, although methods which are specific for the compound to be tested are preferred. Chromatographic methods are recommended because of their compound specificity in analyzing the parent chemical without interferences from impurities. Whenever practicable, the chosen analytical method should have a precision within ±5 percent.

(2) *Preparation*—(i) *Reagents and solutions*—(A) *Buffer solutions.* Prepare buffer solutions using reagent-grade chemicals and reagent-grade water as follows:

(*1*) pH 3.00: use 250 mL of 0.100M potassium hydrogen phthalate; 111 mL of 0.100M hydrochloric acid; and adjust volume to 500 mL with reagent-grade water.

(*2*) pH 7.00: use 250 mL of 0.100M potassium dihydrogen phosphate; 145 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*3*) pH 11.00: use 250 mL of 0.0500M sodium bicarbonate; 113 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(B) *Additional buffer solutions.* For chemicals that ionize or are protonated as discussed in paragraph (b)(1)(viii) of this section, prepare buffers using reagent-grade water and reagent-grade chemicals as follows:

(*1*) pH 5.00: use 250 mL of 0.100M potassium hydrogen phthalate; 113 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*2*) pH 6.00: use 250 mL of 0.100M potassium dihydrogen phosphate; 28 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*3*) pH 8.00: use 250 mL of 0.100M potassium dihydrogen phosphate; 234 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*4*) pH 9.00: use 250 mL of 0.0250M borax ($Na_2 B_4O_7$); 23 mL of 0.100M hydrochloric aid; and adjust volume to 500 mL with reagent-grade water.

(C) *Adjustment of buffer concentrations.* (*1*) The concentrations of all the above buffer solutions are the maximum concentration to be employed in carrying out hydrolysis measurements. If the initial concentration of the test chemical is less than 10^{-3} M, the buffer concentration shall be lowered by a corresponding amount; e.g., if the initial test chemical concentration is 10^{-4} M, the concentration of the above buffers shall be reduced by a factor of 10. In addition, for those reactions in which an acid or base is not a reaction product, the minimum buffer concentration necessary for maintaining the pH within +0.05 units shall be employed.

(*2*) Check the pH of all buffer solutions with a pH meter at 25 °C and adjust the pH to the proper value, if necessary.

(D) *Preparation of test solution.* (*1*) If the test chemical is readily soluble in water, prepare an aqueous solution of the chemical in the appropriate buffer and determine the concentration of the chemical. Alternatively, a solution of the chemical in water may be prepared and added to an appropriate buffer solution and the concentration of the chemical then determined. In the latter case, the aliquot shall be small enough so that the concentration of the buffer in the final solution and the pH of the solution remain essentially unchanged. Do not employ heat in dissolving the chemical. The final concentration shall not be greater than one-half the chemical's solubility in water and not greater than 10^{-3} M.

(*2*) If the test chemical is too insoluble in pure water to permit reasonable handling and analytical procedures, it is recommended that the chemical be dissolved in reagent-grade acetonitrile and buffer solution and then added to an aliquot of the acetonitrile solution. Do not employ heat to dissolve the chemical in acetonitrile. The final concentration of the test chemical shall not be greater than one-half the chemical's solubility in water and not greater than 10^{-3} M. In addition, the final concentration of the acetonitrile shall be one volume percent or less.

(3) *Performance of the test.* Carry out all hydrolysis experiments by employing one of the procedures described in this paragraph. Prepare the test solutions as described in paragraph (b)(2)(i) of this section at pH 3.00, 7.00, and 11.00 ±0.05, and determine the initial test chemical concentration (C_o) in triplicate. Analyze each reaction mixture in triplicate at regular intervals, employing one of the following procedures:

(i) *Procedure 1.* Analyze each test solution at regular intervals to provide a minimum of six measurements with the extent of hydrolysis between 20 to 70 percent. Rates should be rapid enough so that 60 to 70 percent of the chemical is hydrolyzed in 672 hours.

(ii) *Procedure 2.* If the reaction is too slow to conveniently follow hydrolysis to high conversion in 672 hours but still rapid enough to attain at least 20 percent conversion, take 15 to 20 time points at regular intervals after 10 percent conversion is attained.

(iii) *Procedure 3.* (A) If chemical hydrolysis is less than 20 percent after 672 hours, determine the concentration (C) after this time period.

(B) If the pH at the end of concentration measurements employing any of the above three procedures has changed by more than 0.05 units from the initial pH, repeat the experiment using a solution having a test chemical concentration lowered sufficiently to keep the pH variation within 0.05 pH units.

(iv) *Analytical methodology.* Select an analytical method that is most applicable to the analysis of the specific chemical being tested under paragraph (b)(1)(xi) of this section.

(c) *Data and reporting*—(1) *Treatment of results.* (i) If Procedure 1 or 2 were employed in making concentration measurements, use a linear regression analysis with Equation 4 under paragraph (a)(2)(v)(B) of this section to calculate k_h at 25 °C for each pH employed in the hydrolysis experiments. Calculate the coefficient of determination (R^2) for each rate constant. Use Equation 3 under paragraph (a)(2)(v)(B) of this section to calculate the hydrolysis half-life using k_h.

(ii) If Procedure 3 was employed in making rate measurements, use the mean initial concentration (C_o) and the mean concentration of chemical (C) in Equation 4 under paragraph (a)(2)(v)(B) of this section to calculate k_h for each pH used in the experiments. Calculate the hydrolysis half-life using k_h in Equation 3 under paragraph (a)(2)(v)(B) of this section.

(iii) For each set of three concentration replicates, calculate the mean value of C and the standard deviation.

(iv) For test chemicals that are not ionized or protonated between pH 3 and 11, calculate k_A, k_B, and k_N using Equation 5.

(2) *Specific analytical and recovery procedures*. (i) Provide a detailed description or reference for the analytical procedure used, including the calibration data and precision.

(ii) If extraction methods were used to separate the solute from the aqueous solution, provide a description of the extraction method as well as the recovery data.

(3) *Test data report*. (i) For Procedures 1 and 2, report k_h, the hydrolysis half-life ($t_{1/2}$), and the coefficient of determination (R^2) for each pH employed in the rate measurements. In addition, report the individual values, the mean value, and the standard deviation for each set of replicate concentration measurements. Finally, report k_A, k_B, and k_N.

(ii) For Procedure 3, report k_h and the half-life for each pH employed in the rate measurements. In addition, report the individual values, the mean value, and the standard deviation for each set of replicate concentration measurements. Finally, report k_A, k_B, and k_N.

(iii) If, after 672 hours, the concentration (C) is the same as the initial concentration (C_o) within experimental error, then k_h cannot be calculated and the chemical can be reported as being persistent with respect to hydrolysis.

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PART 797—ENVIRONMENTAL EFFECTS TESTING GUIDELINES

Subpart A [Reserved]

Subpart B—Aquatic Guidelines

Sec.

AUTHORITY: 15 U.S.C. 2603.

SOURCE: 50 FR 39321, Sept. 27, 1985, unless otherwise noted.

Subpart A [Reserved]

Subpart B—Aquatic Guidelines

§ 797.1050 Algal acute toxicity test.

(a) *Purpose*. The guideline in this section is intended for use in developing data on the acute toxicity of chemical substances and mixtures (''chemicals'') subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes test procedures and conditions using freshwater and marine algae to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (U.S. EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions*. The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in part 792—Good Laboratory Practice Standards of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) *Algicidal* means having the property of killing algae.

(2) *Algistatic* means having the property of inhibiting algal growth.

(3) *ECx* means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(4) *Growth* means a relative measure of the viability of an algal population based on the number and/or weight of