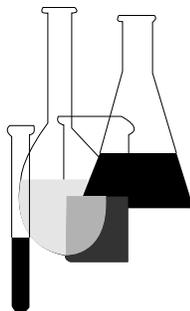




Fate, Transport and Transformation Test Guidelines

OPPTS 835.3200
Zahn-Wellens/EMPA
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 835.3200 Zahn-Wellens/EMPA 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 40 CFR 796.3360 Inherent Biodegradability: Modified Zahn-Wellens Test and OECD guideline 302 B Inherent Biodegradability: Zahn-Wellens/EMPA Test.

(b) **General concepts.** The original Zahn-Wellens test under paragraph (1)(7) of this guideline was adopted in 1981 as OECD Guideline 302 B for determining inherent biodegradability. Later proposals were made by Switzerland and Germany to modify this guideline by merging it with elements contained in a test developed by EMPA (Swiss Federal Laboratories for Materials Testing and Research) under paragraph (1)(6) of this guideline, hence the change in name of the test. The merged version of the test was further changed with respect to the mineral medium used. The medium retained is identical with that which is used in the DOC Die-Away, CO₂ Evolution, Manometric Respirometry, and Modified OECD screening methods of for determining ready biodegradability (835.3110).

(c) **Principle of the test.** (1) A mixture containing the test substance, mineral nutrients, and a relatively large amount of activated sludge in aqueous medium is agitated and aerated at 20–25 °C in the dark or in diffuse light for up to 28 days. Blank controls, containing activated sludge and mineral nutrients but no test substance, are run in parallel. The biodegradation process is monitored by determination of DOC (dissolved organic carbon) (or COD (chemical oxygen demand)) in filtered samples taken at daily or other time intervals. The ratio of eliminated DOC (or COD), corrected for the blank, after each time interval, to the initial DOC value is expressed as the percent biodegradation at the sampling time. The percent biodegradation is plotted against time to give the biodegradation curve.

(2) Specific analysis of the test substance may be useful in cases where molecular changes, caused by biochemical reactions (primary biodegradation) are to be detected.

(d) **Information on the test substance.** It is necessary to know the water solubility and vapor pressure of the test substance and it is also advisable to know its foaming properties. The chemical structure should be known if the measured values of DOC or COD are to be checked. Information on the toxicity of the test substance to bacteria is useful for selecting appropriate test concentrations and in interpreting results showing poor biodegradability under paragraph (1)(5) of this guideline. The test is usually performed only after failure to pass a test for ready

biodegradability. Thus, the physical and inhibitory properties may have already been ascertained.

(e) **Applicability of the method.** Chemicals which are nonvolatile and are soluble in water to at least 50 mg DOC/L may be assessed by this method, provided also that they do not significantly adsorb, are not lost by foaming and do not inhibit bacteria at the concentration tested.

(f) **Sensitivity.** The limits of sensitivity are given by the sensitivity of the DOC determination (normally 0.5–1 mg C/L) or the COD determination (15 mg O₂/L) and also by the variability of the blank. The relatively high concentration of test substance (50–400 mg DOC/L) gives the advantage of greater analytical reliability.

(g) **Reference compounds.** In order to check the functional capability of the activated sludge, a test using a reference compound of known biodegradability should be run in parallel with each series. For this purpose, ethylene glycol, diethylene glycol, lauryl sulfonate, and aniline are recommended. Biodegradation of these compounds must reach at least 70 percent (DOC or COD) within 14 days.

(h) **Reproducibility.** The test has been shown to have good reproducibility in ring tests.

(i) **Description of the method**—(1) **Apparatus.** (i) Cylindrical glass vessels with a volume of 1–5 L, each equipped with a stirrer made of inert material rotating about 5 to 10 cm above the bottom of the vessel (a magnetic stirrer with a 7–10 cm long rod can also be used) and a glass tube of 2–4 mm i.d. to introduce air at about 1 cm above the bottom of the vessel, or vessels of the same size equipped with a glass frit at the bottom, permitting aeration and agitation.

(ii) A supply of compressed air passed through a cotton wool strainer and a wash-bottle containing water, or from an aeration pump delivering air free from dust, oil, and organic impurities.

(iii) Normal laboratory equipment, especially a centrifuge (capable of at least 1,000 g), pH meter, dissolved oxygen measuring apparatus, and membrane filters (pore size 0.2–0.45 μm).

(iv) Analytical equipment for determining DOC (refer to paragraph (1)(1) of this guideline) or COD (refer to paragraph (1)(2) of this guideline).

(2) **Reagents.** Use analytical grade reagents throughout.

(3) **Water.** Deionized or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu²⁺ ions) is used. It should contain only minimal amounts of organic carbon so that high blank values are eliminated. Contamination may result from inherent impurities and also from the ion-exchange resins and lysed materials from bacteria and algae.

For each test series use only one batch of water, previously checked by DOC analysis.

(4) **Stock solutions for mineral medium.** (i) Prepare the following stock solutions:

(A) Dissolve 8.5 g potassium dihydrogen orthophosphate, KH_2PO_4 , 21.75 g dipotassium hydrogen orthophosphate, K_2HPO_4 , 33.4 g disodium hydrogen orthophosphate dihydrate, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 0.5 g ammonium chloride, NH_4Cl , in water and make up to 1 L. The pH of the solution should be 7.4.

(B) Dissolve 27.5 g calcium chloride, anhydrous, CaCl_2 or 36.4 g calcium chloride dihydrate, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, in water and make up to 1 L.

(C) Dissolve 22.5 g magnesium sulphate heptahydrate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, in water and make up to 1 L.

(D) Dissolve 0.25 g iron(III) chloride hexahydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, in water and make up to 1 L.

(ii) Note: In order to avoid having to prepare this solution immediately before use, add one drop of concentrated HCl or add 0.4 g ethylenediaminetetraacetic acid (EDTA, disodium salt) per liter. If a precipitate forms in a stock solution, replace with a freshly made solution.

(5) **Preparation of mineral medium.** Mix 10 mL of solution (A) with 800 mL water, add 1 mL each of solutions (B), (C) and (D) and make up to 1 L.

(6) **Inoculum.** Collect a fresh sample of activated sludge from a sewage treatment works (BOD_5 of effluent should be < 25 mg/L) and wash twice with mineral medium or tap water. Separate the sludge by centrifuging for 3–5 min at about 1,000 g or by allowing the sludge to settle. In special cases, to get as many different species and strains as possible, mix samples from different sources (e.g. other treatment plants, soil extracts, river water, etc.) and treat the mixture as above. Use the sludge within 6 h of sampling, otherwise disperse it in mineral medium and aerate until required. Check the activity of the sludge with the procedural control using a reference compound, as described under paragraph (i)(7)(iv) of this guideline.

(7) **Preparation of vessels.** (i) Before starting the test, make certain with appropriate methods that no inhibition of sludge occurs at the chosen concentration of test substance if this is not already known (see paragraphs (1)(3) and (4) of this guideline). If an inhibitory effect is found, reduce the concentration of test substance to a level which is unlikely to be inhibitory.

(ii) To an appropriate number of test vessels introduce 500 mL mineral medium and the appropriate amounts of test substance and inoculum to reach respectively between 50 and 400 mg DOC/L (between 100 and 1,000 mg COD/L) and 0.2–1.0 g dry matter/L in the final volume. Ensure that the ratio between inoculum and test compound (as DOC) lies between 2.5:1 and 4:1. Make up to the required volume with mineral medium. The final volume, between 1 and 5 L, depends on the number of samples to be taken for DOC or COD determinations and the volumes necessary for the analytical procedures. Normally a volume of 2 L is satisfactory.

(iii) Set up one or two blank vessels in parallel to contain only activated sludge and mineral medium with volumes identical to those of the test suspensions.

(iv) Also, set up one vessel in parallel with each test series as a procedural control, using one of the reference compounds in place of the test substance. If information on abiotic degradation is required, a sterile uninoculated solution of the test chemical can be prepared.

(8) **Number of vessels.** (i) The following vessels are used in a typical run:

(A) One or two containing test substance and inoculum (test suspension).

(B) One or two containing inoculum alone (inoculum blank).

(C) One containing reference compound and inoculum (procedure control).

(ii) It is mandatory to follow DOC in the test suspension and inoculum blanks in parallel. It is advisable to follow DOC in the other vessel in parallel as well but this may not always be possible.

(j) **Procedure.** (1) For practical reasons, do not start the test immediately before a week-end. Run the test, normally for up to 28 days, in the dark or in diffuse light at 20–25 °C. Aerate the suspensions with purified, humidified air and, if necessary, stir to ensure that sludge does not settle and that the concentration of dissolved oxygen does not fall below mg/L. Check the pH value at regular intervals (e.g. on each day of sampling) and adjust to pH 6.5–8.0 with NaOH (40 g/L) or H₂SO₄ (50 g/L) if necessary.

(2) **Sampling.** Follow the biodegradation of the test substance by determining the DOC or COD in samples of suspension taken:

(i) At 3 h ± 30 min after addition of the test substance in order to estimate any adsorption by the activated sludge (see example in Figure 1. under paragraph (m)(1) of this guideline).

(ii) On at least four occasions in the interval between the 1st and 27th day.

(iii) On the 27th and 28th days, or, if the plateau is attained in less than 28 days, on the last 2 days of the test run.

(iv) The volume of sample taken depends on the type of carbon analyzer to be used. Additional sampling may be necessary in order to describe the reaching of the plateau or if adaptation is to be followed.

(v) Replace losses due to evaporation immediately prior to each sampling.

(4) **Adaptation.** (i) If adaptation (see curve 1, Figure 2. under paragraph (m)(2) of this guideline) is to be followed, carry out analyses for DOC or COD at relatively short intervals (e.g. daily). Prolong the test beyond 28 days if adaptation occurs in the final days of the test period.

(ii) If more detailed knowledge of the behavior of the adapted sludge is needed, reexpose the same activated sludge to the test substance. To do this, stop aeration and agitation and allow the sludge to settle. Draw off the supernatant liquid, refill the vessel to the original volume with mineral medium, stir for 15 min and repeat this operation once more. Alternatively, isolate the sludge by centrifuging (refer to paragraph (i)(6) of this guideline). Repeat the test using the recovered sludge, which may be augmented with fresh sludge if insufficient recovered sludge is available to yield 0.2–1 g dry matter/L.

(5) **Analytical methods.** (i) Filter the samples of sludge suspensions (test, blank, and procedure control) as soon as they are taken, discarding the first 5 mL of filtrate. Use either carefully washed paper filters or membrane filters, which are suitable if they neither release nor adsorb organic compounds. Otherwise wash the membranes 3 times in deionized or distilled water at about 60 °C, and store in water. Sludges which are difficult to separate by filtration should be separated by centrifugation or other suitable separation techniques.

(ii) Determine the DOC or COD in duplicate in the filtered or centrifuged samples by any suitable methods e.g. refer to paragraphs (1)(1) and (2) of this guideline. If primary biodegradation is to be followed, use specific analyses, e.g. UV spectroscopy, in addition to DOC or COD. If the filtrates cannot be analyzed on the day of sampling, store at 2–4 °C for a maximum of 48 h, or at –18 °C for longer periods. Storage for long periods is not recommended.

(k) **Data and reporting—(1) Treatment of results.** (i) Calculate the percent degradation at time t from

$$D_t = [1 - (C_t - C_B)/(C_A - C_{BA})] \times 100$$

where:

D_t = percent degradation at time t ; C_A = concentration of DOC or COD in the test suspension measured after $3 \text{ h} \pm 30 \text{ min}$ of incubation, expressed as milligrams per liter; C_t = mean concentration of DOC or COD in the test suspension at time t , expressed as milligrams per liter; C_{BA} = mean concentration of DOC or COD in the blanks measured after $3 \text{ h} \pm 30 \text{ min}$ of incubation, expressed as milligrams per liter; C_B = mean concentration of DOC or COD in the blanks at time t , expressed as milligrams per liter.

(ii) Carry out the same calculation for the reference compound. Display the course of biodegradation graphically (as in Figures 1. and 2. under paragraph (m) of this guideline) and record all results on data sheets.

(2) **Validity and interpretation.** (i) The test is considered valid if the procedural control shows the removal of the reference compound by at least 70 percent within 14 days and if the removal of DOC (or COD) in the test suspension took place relatively gradually over days or weeks, since this indicates biodegradation.

(ii) Physicochemical adsorption can, in some cases, play a role and this is indicated when there is complete or substantial removal in the first 3 h and the difference between blanks and test solutions remains at an unexpected low value. In such cases additional information is obtained from a comparison between the 3-h value, the expected initial value calculated from the amount of test substance added and the value measured before the inoculum is added. If a more precise distinction between biodegradation (or partial degradation) and adsorption is to be drawn, carry out further testing, preferably running a respirometric test for ready biodegradation, using the supernatant of the acclimatized sludge as inoculum.

(iii) Low and 0-values of removal of the test substance may be due to its inhibition of bacteria; eliminate this possibility by testing for inhibition at the concentration used if this has not already been done (refer to paragraph (i)(7)(i) of this guideline).

(3) **Test report.** The test report must include the following information:

(i) Test substance: (A) Physical nature and, where relevant, physicochemical properties.

(B) Identification data.

(ii) Inoculum: Source, concentration, status of adaptation.

(iii) Test conditions: (A) Analytical methods used.

(B) Procedure control and compound used in the control.

(iv) Results: (A) Biodegradation curve.

(B) Toxicity evaluations.

(C) The degree of biodegradation attained at the end of the test after 28d, or earlier if complete degradation is attained in less than 28 days, as “inherent biodegradability in the static test after x days.”

(D) Any significant difference between the DOC (or COD) in the first sample at 3 h after starting the test and the value calculated from the amount of test compound added as “adsorbed by the activated sludge.”

(E) The adaptation phase (days), the biodegradation phase (days) and the endpoint of biodegradation reached after x days as identified from the biodegradation curve.

(v) Discussion of the results.

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

(1) DIN 38409, Teil 3. Bestimmung des gelösten organischen Kohlenstoffgehaltes (DOC) (1983).

(2) ISO Standard 6060 (1986). Water Quality-Determination of Chemical Oxygen Demand.

(3) ISO Standard 8192. Water Quality-Test for inhibition of oxygen consumption by activated sludge (1986).

(4) OECD. Activated Sludge, Respiration Inhibition Test. Test Guideline 209, Paris (1984).

(5) Reynolds, L. et al. Evaluation of the toxicity of substances to be assessed for biodegradability. *Chemosphere* 16:2259 (1987). *Chemiker Zeitung* 98:228–232 (1974).

(6) Schefer W. and Wälchli O. Prüfung der biologischen Eliminierbarkeit organisch-chemischer Abwasser-Inhaltstoffen. *Zur Wasser- und Abwasserforschung* 13, 205–209 (1980).

(7) Zahn R. und Wellens H. Ein einfaches Verfahren zur Prüfung der biologischen Abbaubarkeit von Produkten und Abwasserinhaltsstoffen.

(m) **Figures.**

FIGURE 1.—EXAMPLES OF BIODEGRADATION CURVES

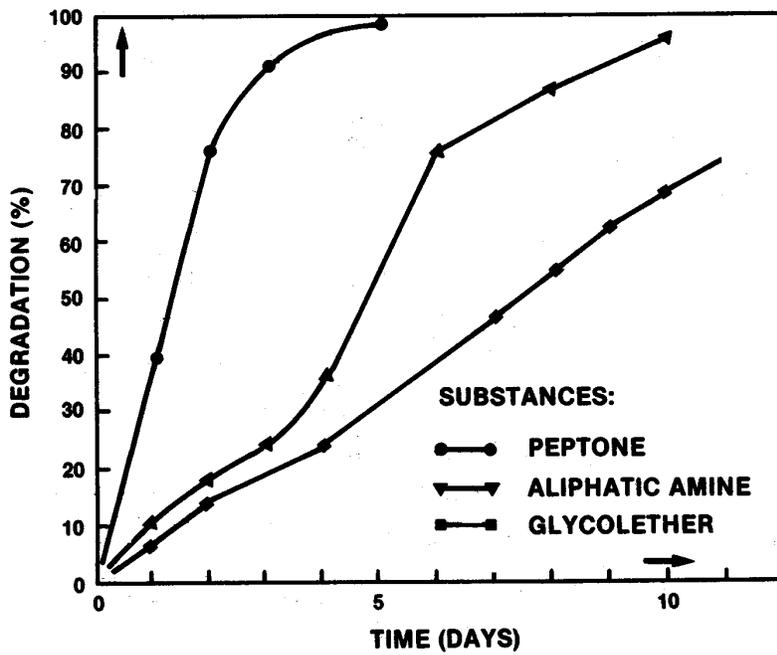


FIGURE 2.—EXAMPLE OF SLUDGE-ADAPTATION

