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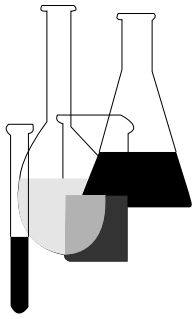
Fate, Transport and Transformation Test Guidelines

OPPTS 835.3160 Biodegradability in Sea Water



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 ''Environmental Test Methods and Guidelines.''

OPPTS 835.3160 Biodegradability in sea water.

(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 OECD guideline 306, Biodegradability in Sea Water.

(b) **General introduction.** (1) When the original Organization for Economic Cooperation and Development (OECD) Test Guidelines were developed, it was not known to what extent results from the screening tests for ready biodegradability using fresh water and sewage effluent or activated sludge as inoculum could be applied to the marine environment. Variable results on this point have been reported (e.g., see paragraph (e)(1) of this guideline).

(2) Many industrial waste waters, containing a variety of chemicals, reach the sea either by direct discharge or via estuaries and rivers in which the residence times are low compared with the period necessary for complete biodegradation of many of the chemicals present. Because of the growing awareness of the need to protect the marine environment against increasing loads of chemicals and the need to estimate the probable concentration of chemicals in the sea, test methods for biodegradability in sea water have been developed.

(3) The methods described here use natural sea water both as the aqueous phase and as the source of microorganisms. In an endeavor to conform with the methods for ready biodegradability in fresh water, the use of ultrafiltered and centrifuged sea water was investigated, as was the use of marine sediments as inocula. These investigations were unsuccessful. The test medium therefore is natural sea water pre-treated to remove coarse particles.

(4) This guideline consists of two test methods: the Shake Flask Method and the Closed Bottle Method. In order to assess ultimate biodegradability with the Shake Flask Method, relatively high concentrations of the test substance must be used because of the poor sensitivity of the dissolved organic carbon (DOC) analytical method. This in turn necessitates the addition to the sea water of mineral nutrients (N and P), the low concentrations of which would otherwise limit the removal of DOC. It is also necessary to add the nutrients in the Closed Bottle Method because of the concentration of the added test substance.

(5) Hence, the methods are not tests for ready biodegradability since no inoculum is added in addition to the microorganisms already present in the sea water. Neither do the tests simulate the marine environment

since nutrients are added and the concentration of test substance is very much higher than would be present in the sea.

(c) **Application.** (1) The methods in this guideline are intended for application when the pattern of use and disposal of the chemical substance in question indicates a route to the sea. If the result is positive (>70% DOC removal; >60% ThOD—theoretical oxygen demand), it may be concluded that there is a potential for biodegradation in the marine environment. However, a negative result does not preclude such potential but indicates that further study is necessary, for example using as low a concentration of the test substance as possible.

(2) In either case, if a more definitive value for the rate or degree of biodegradation in sea water at a particular site is required, other more complex and sophisticated, and hence more costly, methods would have to be applied. For example, a simulation test could be applied using a concentration of test substance nearer to the likely environmental concentration, or non-fortified, non-pretreated sea water taken from the location of interest could be used and primary biodegradation followed by specific chemical analysis. For ultimate biodegradability, ^{14}C-labeled test substance would be necessary to allow rates of disappearance of soluble organic ^{14}C and production of $^{14}CO_2$ to be measured at environmentally realistic concentrations of that substance.

(d) **Choice of methods.** The selection of test method depends on a number of factors. The following table 1 is given to help the investigator select a test method. Whereas chemicals having water solubility below the equivalent of about 5 mg C/L cannot be tested using the Shake Flask Method, in principle they may be tested using the Closed Bottle Method.

Table 1.—Advantages and Disadvantages of the Shake Flask and Closed Bottle Methods

Method	Advantages	Disadvantages
Shake flask	– simple apparatus except for C analyzer – 60 d duration is not a problem – no interference from nitrification – can be adapted for volatile chemicals	– needs C analyzer – uses 5–40 mg DOC/L; could be inhibitory – DOC determination is difficut at low concentrations in sea water (chloride effect) – DOC sometimes high in sea water
Closed bottle	– simple apparatus – simple end determination – uses low concentration of test compound (2 mg/L), thus less chance of inhibition – easily adapted for volatile chemicals	– can be difficut to maintain airtightness of bottles – wall growth of bacteria can lead to false values – blank 0_2 uptake values can be high, especially after 28 days; can be overcome by aging the sea water – possible interference from 0_2 uptake by nitrification

(1) **Shake flask method**—(i) **Introduction.** (A) This method is a sea water variant of the Modified OECD Screening Test (see paragraph (e)(2) of this guideline). It was finalized as a result of a ring test organized for the Europeon Economic Community (EEC) by the Danish Water Quality Institute (see paragraph (e)(3) of this guideline).

(B) In common with the accompanying sea water Closed Bottle Method, the results from this test are not to be taken as indicators of ready biodegradability, but are to be used specifically for obtaining information about the biodegadability of chemicals in marine environments.

(ii) **Principle of the method.** A predetermined amount of the test substance is dissolved in the test medium to yield a concentration of 5–40 mg DOC/L. If the limits of sensitivity of organic carbon analyses are improved, the use of lower concentrations of test substance may be advantageous, particularly for inhibitory substances. The solution of the test substance in the test medium is incubated with agitation in the dark or in diffuse light under aerobic conditions at a fixed temperature (controlled to ± 1°C) which will normally be within the range 15–20°C. In cases where the objective of the study is to simulate environmental situations, tests may be performed at temperatures outside this range. The recommended maximum test duration is 60 days. Degradation is followed by DOC measurements (ultimate degradation) and, in some cases, by specific analysis (primary degradation).

(iii) **Information on the test substance.** (A) To detemine whether the test may be applied to a particular substance, certain properties of the

substance must be known. The organic carbon content of the substance must be established, its volatility must be such that significant losses do not occur during the course of the test, and its solubility in water should be greater than the equivalent of 25–40 mg C/L. Further, the test substance should not significantly sorb to glass surfaces. Information on the purity or the relative proportions of major components of the test substance will be useful in interpreting the results obtained, especially when the result lies close to the ''pass'' level.

(B) Information on the toxicity of the test substance to bacteria, for example as measured in short-term respiration rate tests (see paragraph (e)(4) of this guideline), may be useful when selecting appropriate test concentrations, and may be essential for the correct interpretation of low biodegradation values. However, such information is not always sufficient for interpreting results obtained in the biodegradation test, and the procedure described in paragraph (d)(1)(vi)(F)(*3*) of this guideline may be more suitable.

(iv) **Reference substance.** (A) Suitable reference substances shall be used to check the microbial activity of the sea water sample. Sodium benzoate, sodium acetate and aniline are examples of chemicals that may be used for this purpose. If reference substances are not degraded within a reasonably short time, it is recommended that the test be repeated using another sea water sample.

(B) In the EEC ring test (see paragraph (e)(3) of this guideline), the lag phase (t_L) and time to achieve 50 per cent degradation (t_{50}) excluding the lag phase were 1 to 4 days and 1 to 7 days, respectively, for sodium benzoate. For aniline the t_L ranged from 0 to 10 days and the t_{50} from 1 to 10 days.

(v) **Reproducibility and sensitivity of the method.** The reproducibility of the method was established in the ring test (see paragraph (e)(3) of this guideline). The lowest concentration of test substance for which this method can be used with DOC analysis is largely determined by the detection limit in the organic carbon analysis (about 0.5 mg C/L, at present) and the concentration of dissolved organic carbon in the sea water used (usually on the order of 3–5 mg/L for water from the open ocean). The background concentration of DOC should not exceed about 20% of the total DOC concentration after addition of test substance. If this is not feasible, the background concentration of DOC may sometimes be reduced by aging the sea water prior to testing. If the method is used with specific chemical analysis only (by which primary degradation is measured), the investigator must document, by supplying additional information, whether ultimate degradability can be expected. This additional information may consist of the results from other tests for ready or inherent biodegradability.

(vi) **Description of the method**—(A) **Apparatus.** Normal laboratory apparatus and:

(*1*) Shaking machine accommodating 0.5- to 2-L Erlenmeyer flasks, either with automatic temperature control or used in a constant temperature room at 15–20°C controlled to ±1°C.

(*2*) Narrow neck, 0.5- to 2-L Erlenmeyer flasks.

(*3*) Membrane filtration apparatus, or centrifuge.

(*4*) Membrane filters, 0.2–0.45μm.

(*5*) Carbon analyzer.

(*6*) Equipment for specific analysis (optional).

(B) **Sea water.** (*1*) Collect a sample of sea water in a thoroughly cleaned container and transport it to the laboratory, preferably within 1–2 days of collection. During transport do not allow the temperature of the sample to exceed significantly the temperature to be used in the test. Identify the sampling location precisely and describe it in terms of its pollutional and nutrient status. Especially for coastal waters, include in this characterization a heterotrophic microbial colony count and the determination of the concentrations of dissolved nitrate, ammonium and phosphate.

(*2*) Provide the following information for the sea water sample itself:

(*i*) Date of collection.

(*ii*) Depth of collection.

(*iii*) Appearance of sample—turbid, etc.

(*iv*) Temperature at the time of collection.

(*v*) Salinity.

(*vi*) DOC.

(*vii*) Delay between collection and use in the test.

(*3*) If the DOC content of the sea water sample is found to be high (see paragraph (d)(1)(v) of this guideline), it is recommended that the seawater be aged for approximately one week prior to use. This is accomplished by storing the sample under aerobic conditions at the test temperature and in the dark or in diffuse light. If necessary, maintain aerobic conditions by aerating gently. During aging, the content of easily degradable organic material is reduced. In the ring test (see paragraph (e)(3) of this guideline), no difference was revealed between the degradation potential of aged and freshly collected sea water samples. Prior to use, pretreat the

sea water to remove coarse particles, e.g., by filtering through a nylon or coarse paper filter (not membrane or GF-C filters), or by ceatrifuging gently. The procedure used must be reported. Pretreat after aging, if used.

(C) **Stock solutions for mineral nutrients.** (*1*) Prepare the following stock solutions using analytical grade reagents:

(*i*) Potassium dihydrogen orthophosphate, KH_2PO_2. . . 8.50 g

Dipotassium hydrogen orthophosphate, K_2HPO_4. . . 21.75 g

Disodium hydrogen orthophosphate dihydrate,

$Na_2HPO_4.2H_2O$. . . 33.30 g

Ammonium chloride, NH_4Cl. . . 0.50 g

Dissolve and make up to 1 L with distilled water.

(*ii*) Calcium chloride, $CaCl_2$. . . 27.50 g

26Dissolve and make up to 1 L with distilled water.

(*iii*) Magnesium sulfate heptahydrate, $MgSO_4.7H_2O$ . . . 22.50 g

Dissolve and make up to 1 L with distilled water.

(*iv*) Iron (III) chloride hexahydrate, $FeCl_3.6H_2O$. . . 0.25 g

Dissolve and make up to 1 L with distilled water.

(*2*) Precipitation in solution (see paragraph (d)(1)(vi)(C)(*1*) (*iv*) of this guideline) may be prevented by adding one drop of concentrated HCl or 0.4 g ethylenediaminetetraacetic acid (EDTA, disodium salt) per L. If a precipitate forms in a stock solution, replace it with freshly made solution.

(D) **Preparation of test medium.** Add 1 mL of each of the stock solutions described in paragraphs (d)(1)(vi)(C)(*1*)(*i*) through (d)(1)(vi)(C)(*1*)(*iv*) of this guideline per L of pretreated sea water.

(E) **Inoculum.** Do not add any inoculum in addition to the microorganisms already present in the sea water. Optionally, determine the number of colony-forming heterotrophs in the sea water test medium (and preferably also in the original sea water samples) using a suitable method, such as plate counts with marine agar. This is particularly desirable for samples from coastal or polluted sites. Check the heterotrophic microbial activity in the sea water by using a reference compound.

(F) **Preparation of flasks.** (*1*) Ensure that all glassware is scrupulously clean (e.g., using alcoholic hydrochloric acid), rinsed and dried

before use in order to avoid contamination with residues from previous tests. Flasks must also be cleaned before they are used for the first time.

(*2*) Evaluate test substances in duplicate flasks simultaneously, together with a single flask for the reference compound. Carry out a blank test, in duplicate, with neither test nor reference substance, for the determination of analytical blanks. Dissolve the test substance in the test medium—it may be conveniently added via a concentrated stock solution—to give the desired starting concentration of 5–40 mg DOC/L. Test the reference substance at a starting concentration corresponding to 20 mg DOC/L. If stock solutions of test and/or reference substances are used, ensure that the salinity of the sea water medium is not greatly altered.

(*3*) If toxic effects can be expected or cannot be ruled out, it may be advisable to include an inhibition experiment, in duplicate, in the test design. Add the test and reference substances to the same vessel, the concentration of the reference compound being normally the same as in the control test (i.e., 20 mg DOC/L) in order to allow comparison.

(*4*) Dispense adequate amounts of test solutions into the Erlenmeyer flasks (up to about half the flask volume is a convenient amount) and subsequently provide each flask with a loose cover (e.g., aluminium foil) that makes gas exchange between the flask and the surrounding air possible. (Cotton wool plugs are unsuitable if DOC analysis is used). Place the vessels on the shaker and shake continuously at a gentle rate (e.g., 100 rpm) throughout the test. Control the temperature (15–20°C and within ±2°C), and shield the vessels from light in order to avoid growth of algae. Ensure that the air is free of toxic materials.

(G) **Physical-chemical control test (optional).** If abiotic degradation or loss mechanisms are suspected, such as hydrolysis (a problem with specific analysis only), volatilization, or sorption, it is advisable to perform a physical-chemical control. This can be done by adding mercury (II) chloride ($HgCl_2$)[1] (50–100 mg/L) to vessels with test substance in order to inhibit microbial activity. A significant decrease in DOC or specific compound concentration in the physical-chemical control test indicates abiotic removal mechanisms. (If mercury chloride is used, attention should be paid to interferences or catalyst poisoning in DOC analysis).

(H) **Number of flasks.** In a typical experiment, the following flasks are used:

(*1*) Flasks 1 & 2—containing test substance.

(*2*) Flasks 3 & 4—containing sea water only (blank).

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[1]Mercury (II) chloride ($HgCl_2$) is a very toxic substance that should be handled with suitable precautions. Aqueous wastes containing this chemical should be disposed of appropriately; they should not be discharged down the drain.

(*3*) Flask 5—containing reference substance.

(*4*) Flask 6—containing test and reference substance (toxicity control)—optional.

(*5*) Flask 7—containing test substance and sterilizing agent (abiotic sterile control)—optional.

(I) **DOC analysis.** In the course of the test, withdraw samples at suitable intervals for DOC analysis as described in paragraphs (d)(1)(vi)(I)(*1*)(*i*) through (d)(1)(vi)(I)(*1*)(*x*) of this guideline. Always take samples at the start of the test (day 0) and at day 60. A minimum of five samples in total are required to describe the time-course of degradation. No fixed time schedule for sampling can be stated since the rate of biodegradation varies. Carry out the DOC determination in duplicate on each sample.

(*1*) **Determination of organic carbon in sea water.** (*i*) For the determination of organic carbon of a water sample, the organic compounds in the sample are oxidized to carbon dioxide using one of the following three techniques:

(*a*) Wet oxidation by persulfate/UV-irradiation.

(*b*) Wet oxidation by persulfate/elevated temperature (116–130°C).

(*c*) Combustion.

(*ii*) Evolved CO_2 is then quantified employing infrared spectrometry or titrimetry. Alternatively, CO_2 is reduced to methane, which is quantified using a flame ionization detector (FID).

(*iii*) The persulfate/UV-method is commonly used for the analysis of ''clean'' water with a low content of particulate matter. The persulfate/elevated temperature and combustion methods can be applied to most kinds of water samples, the former being most suitable for samples with low levels of nonvolatile organic carbon (NVOC), and the combustion method being applicable to samples with NVOC content well above 1 mg C/L.

(*2*) **Interferences.** (*i*) All three methods depend on eliminating or compensating for inorganic carbon (IC) present in the sample. Purging of CO_2 from the acidified sample is the most frequently used method to eliminate IC, although this also results in a loss of volatile organic compounds (see paragraph (e)(5) of this guideline). Complete elimination or compensation of IC must be ensured for each sample matrix, and it may be necessary to determine volatile organic carbon (VOC) separately from NVOC.

(*ii*) High chloride concentrations result in decreased oxidation efficiency using the persulfate/UV-method (see paragraph (e)(6) of this guideline). Application of an oxidation reagent modified by the addition of mercury (II) nitrate may remove this interference. It is recommended that the maximum tolerable sample volume be used to evaluate each type of chloride-containing sample. High salt concentrations in samples analyzed using the combustion method can cause salt coating of the catalyst and excessive corrosion of the combustion tube. Precautions should be taken according to the manufacturer's manual.

(*iii*) Highly turbid samples as well as samples containing particulate matter may be incompletely oxidized by the persulfate/UV-method.

(*3*) **An example of a suitable method.** (*i*) Nonvolatile organic carbon is determined by oxidation with persulfate/UV-irradiation and subsequent quantification of evolved CO_2 employing non-dispersive infrared spectrometry.

(*ii*) The oxidation reagent is modified in accordance with the suggestions given in paragraph (e)(6) of this guideline, as described in the manufacturer's manual:

(*a*) 8.2 g of $HgCl_2$ and 9.6 g of $Hg(NO_3)_2.H_2O$ are dissolved in several hundred mL of low-carbon concentration reagent water.

(*b*) 20 g of $K_2S_2O_8$ are dissolved in the mercuric salt solution.

(*c*) 5 mL of concentrated HNO_3 are added to the mixture.

(*d*) The reagent is diluted to 1000 mL.

(*iii*) The interference from chloride is removed using a 40-µL sample volume for 10% chloride and 200-µL sample volume for 1.9% chloride. Samples with high chloride concentrations and/or larger sample volumes can be analyzed using this method provided that build-up of chloride in the oxidation vessel is prevented. Determination of volatile organic carbon can subsequently be performed, if relevant, for the sample type in question.

(*iv*) Automated systems have also been described in the literature (see paragraph (e)(7) of this guideline).

(J) **Sampling.** (*1*) The required volume of the samples depends upon the analytical method (specific analysis), the carbon analyzer used, and the procedure (membrane filtration or centrifugation) selected for sample treatment before carbon determination (see paragraphs (d)(1)(vi)(J)(*3*) and (d)(1)(vi)(J)(*4*) of this guideline). Before sampling ensure that the test medium is mixed well and that any material adhering to the wall of the flask is dissolved or suspended.

(*2*) Membrane filter or centrifuge immediately after sampling. If necessary, store the filtered or centrifuged samples at 2–4°C for up to 48 hours or below -18°C for longer periods. If it is known that the substance will remain unaffected by acidification, acidify to pH 2 before storing.

(*3*) Membrane filters (0.2–0.45 μm) are suitable if it is ensured that they neither release carbon nor sorb the substance in the filtration step. Polycarbonate filters are generally suitable. Some membrane filters are impregnated with surfactants for hydrophilization and may release considerable quantities of dissolved carbon. Prepare such filters by boiling in deionized water for three consecutive periods, each of one hour. After boiling, store the filters in deionized water. Discard the first 20 mL of the filtrate.

(*4*) Centrifugation of the samples may be an acceptable alternative to membrane filtration. Centrifuge at 40,000 $m.s^{-2}$ (~4000 g) for 15 minutes, preferably in a refrigerated centrifuge.

Note: The differentiation of Total Organic Carbon (TOC) from DOC by centrifugation at very low concentrations does not seem to work, since either not all bacteria are removed, or carbon as part of the bacterial plasma is redissolved. At higher test concentrations (> 10 mg C/L) the centrifugation error seems to be comparatively small.

(K) **Frequency of sampling.** (*1*) If analyses are performed immediately after sampling, determine the next sampling time by considering the result of the analytical determination.

(*2*) If samples are preserved (see paragraph (d)(1)(vi)(J)(*2*) of this guideline) for analysis at a later time, take more samples than the required minimum number of five. Analyze the last samples first, and by a stepwise ‘‘backwards’’selection of appropriate samples for analysis, it is possible to obtain a good description of the biodegradation curve with a relatively small number of analytical determinations. If no degradation has taken place by the end of the test, no further samples need to be analyzed, and in this situation, the ‘‘backwards’’ strategy may save considerable analytical costs.

(*3*) If a plateau on the degradation curve is observed before the 60th day, end the test. If degradation has obviously started by day 60, but has not reached a plateau, extend the experiment for a further period.

(vii) **Data and reporting**—(A) **Treatment of results. (***1*) Record the results on the data sheet below, and calculate the biodegradation values for both test and reference substances from the equation:

$$D_t = \left[1 - \frac{C_t - C_{\mathrm{bl}(t)}}{C_0 - C_{bl(0)}}\right] \times 100$$

where:

D_t = degradation in percentage DOC or specific compound removal at time t;

C_o = starting concentration of DOC or specific compound in the test medium;

C_t = concentration of DOC or specific compound in the test medium at time t;

$C_{bl(0)}$ = starting concentration of DOC or specific compound in the blank;

$C_{bl(t)}$ = concentration of DOC or specific compound in the blank at time t.

BIODEGRADATION IN SEA WATER—SHAKE FLASK METHOD

DATA SHEET

LABORATORY:_____

DATE AT START OF TEST:_____

TEST SUBSTANCE:_____

Name:_____

Stock solution concentration (mg/L as chemical):_____

Initial concentration in medium, t_o (mg/L as chemical):_____

thus (mg DOC/L):_____

1. SEA WATER:

Source:_____

Date of collection:_____

Depth of collection:_____

Appearance at time of collection (e.g., turbid, etc.):_____

Salinity at collection (%):_____

Temperature at collection (°C):_____

DOC ‘‘x’’hours after collection (mg/L):_____

Pretreatment prior to testing (e.g., filtration, sedimentation, aging, etc.):_____

Microbial colony count of original sample (colonies/mL):_____

At start of test (colonies/mL):_____

Other characteristics:_____

2. CARBON DETERMINATIONS:

Carbon analyzer:

	Flask no.		DOC after n days (mg/L)				
			0	n_1	n_2	n_3	n_x
Test: nutrient-fortified sea water with test substance	1	a_1					
		a_2					
		mean, $C_{a(t)}$					
	2	b_1					
		b_2					
		mean, $C_{b(t)}$					
Blank: nutrient-fortified as waterout test substance	1	c_1					
		c_2					
		mean, $C_{c(t)}$					
	2	d_1					
		d_2					
		mean, $C_{d(t)}$					
		mean $(C_{c(t)} + C_{d(t)})/2$					

3. EVAULATION OF RAW DATA

Flask No.	Calculation of results	% Degradation after n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = 1 - \frac{C_{a(t)} - C_{bl(t)}}{C_o - C_{bl(o)}} \times 100$	0				
2	$D_2 = 1 - \frac{C_{b(t)} - C_{bl(t)}}{C_o - C_{bl(o)}} \times 100$	0				
Mean (*)	$D_t = \frac{D_1 + D_2}{2}$	0				

* D_1 and D_2 should not be averaged if there is a considerable difference.

Note: Similar formats may be used when degradation is followed by specific analysis and for the reference compound and toxicity controls.

4. ABIOTIC DEGRADATION (optional)

	Time (days)	
	0	t
DOC conc. (mg/L) in sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\% \text{ abiotic degradation} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

(*2*) State degradation as the percentage DOC removal (ultimate degradation) or specific compound removal (primary degradation) at time t. Calculate the DOC concentrations to the nearest 0.1 mg/L, and round up the means of the Dt values to the nearest whole per cent.

(*3*) Illustrate the time course of degradation graphically in a diagram as shown in the figure in paragraph (d)(1)(vii)(C)(*3*) of this guideline. If there are sufficient data, calculate from the curve the lag phase (t_L) and the time to reach 50 per cent removal from the end of the lag phase (t_{50}).

(B) **Test report.** (*1*) The test report shall contain the following information:

(*i*) Test substance. (*a*) Physical nature and, where relevant, physical/chemical properties.

(*b*) Identification of the substance.

(*ii*) Test conditions. (*a*) Location and description of the sampling site; pollutional and nutrient status (colony counts, and nitrate, ammonium and phosphate levels if appropriate).

(*b*) Characteristics of the sample (date of sampling, depth, appearance, temperature, salinity, DOC (optional), delay between collection and use in the test.

(*c*) Method used (if any) for aging the sea water.

(*d*) Method used for pretreatment (filtration/sedimentation) of the sea water.

(*e*) Method used for DOC determination.

(*f*) Method used for specific analysis (optional).

(*g*) Method used for determining the number of heterotrophs in the sea water (plate count method or alternative procedure) (optional).

(*h*) Other methods (optional) used to characterise the sea water (ATP measurements, etc.).

(*iii*) Results. (*a*) Analytical data reported on a data sheet as shown in paragraph (d)(1)(vii)(A)(*1*) of this guideline.

(*b*) The course of degradation represented graphically in a diagram showing the lag phase (t_L), slope, and time (starting from the end of the lag phase) to reach 50% removal (t_{50}). The lag phase may be estimated graphically as shown in the figure in paragraph (d)(1)(vii)(C)(*3*) of this guideline or conveniently taken as the time needed for 10% degradation.

(*c*) Percentage degradation measured after 60 days, or at end of test.

(*iv*) Discussion of results.

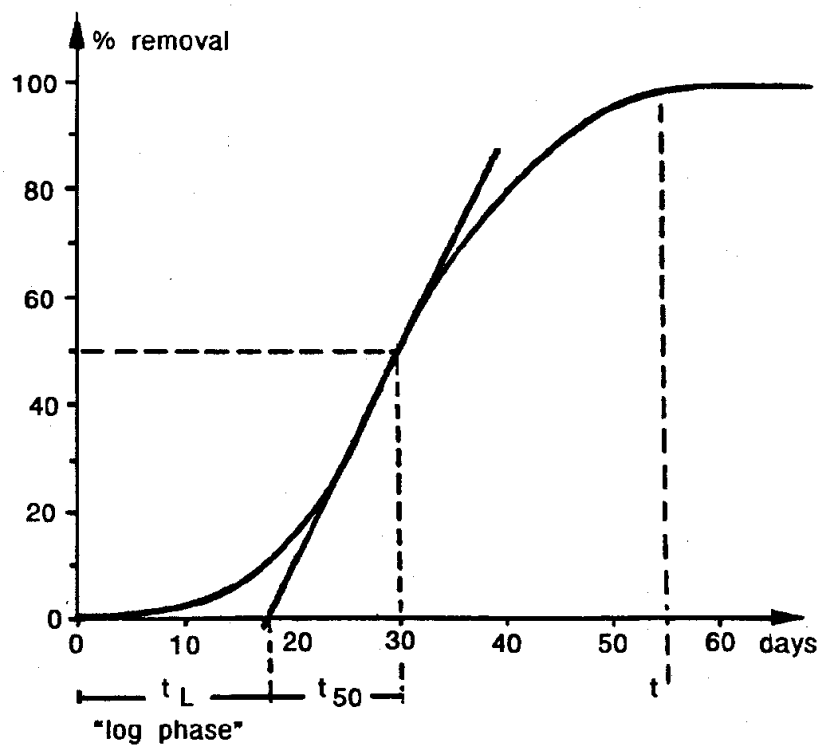
(C) **Validity and interpretation of results.** (*1*) The results obtained with the reference compounds (e.g., sodium benzoate, sodium acetate or aniline) should be comparable to results obtained in the ring test (see paragraphs (d)(1)(iv) and (e)(3) of this guideline. If results obtained with reference compounds are atypical, the test should be repeated using another sea water sample. Although results of inhibition tests may not always be straightforward to interpret because of the contribution of DOC by the test material, a significant reduction of the total DOC removal rate, compared with that of the control, is a positive sign of toxic effects.

(*2*) Owing to the relatively high test concentrations used as compared with most natural systems and consequently an unfavorable ratio between the concentrations of test substances and other carbon sources, the method is to be regarded as a preliminary test that can be used to indicate whether or not a substance is easily biodegradable in sea water. Accordingly a

low result does not necessarily mean that the test substance is not biodegradable in marine environments, but indicates that more work will be necessary to establish this.

(*3*) An example of graphical presentation of data and estimation of the values of t_L (length of lag phase) and t_{50} (time, starting at t_L, needed to reach 50% removal) is given in the following Figure 1.

FIGURE 1.—TYPICAL BIODEGRADATION CURVE FOR THE SHAKE FLASK METHOD



(2) **Closed bottle method**—(i) **Introduction.** (A) This method is a sea water variant of the Closed Bottle Test (see paragraph (e)(8) of this guideline) and was finalized as a result of a ring test organised for the EEC by the Danish Water Quality Institute (see paragraph (e)(3) of this guideline).

(B) In common with the accompanying sea water Shake Flask Method, results of this test are not to be taken as indications of ready biodegradability, but are to be used specifically for obtaining information about the biodegradability of chemicals in marine environments.

(ii) **Principle of the method.** (A) A predetermined amount of the test substance is dissolved in the test medium at a typical concentration of 2 to 10 mg of test substance/L. One or more concentrations may be

used. The solution is kept in a filled closed bottle in the dark in a constant temperature bath or enclosure controlled to ±1°C within the range 15–20°C. In those cases where the objective of the study is to simulate environmental situations, tests may be performed at temperatures outside this range provided that the temperature is properly controlled. Degradation is followed by oxygen analyses over a 28-day period.

(B) The ring test showed that if the test was extended beyond 28 days no useful information could be gathered, in most cases, due to severe interferences. The blank biological oxygen demand (BOD) values were excessively high probably due to wall growth, caused by lack of agitation, and to nitrification. Thus, the recommended duration is 28 days, but if the blank BOD value remains within the 30 per cent limit (see paragraph (d)(2)(vi)(B)(*4*) of this guideline) the test could be prolonged.

(iii) **Information on the test substance.** (A) To determine whether the test may be applied to a particular substance, certain properties of the substance must be known. The empirical formula is required so that the theoretical oxygen demand (ThOD) may be calculated as described in paragraphs (d)(2)(iii)(A)(*1*)(*i*) through (*vi*) of this guideline; otherwise the chemical oxygen demand (COD) of the substance must be determined to serve as a surrogate for the ThOD. The use of COD is less satisfactory since some chemicals are not fully oxidized in the COD test.

(*1*) **Calulation of the theoretical oxygen demand.** (*i*) The ThOD of the substance $C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ of molecular weight MW is calculated as follows:

$$ThOD_{NH3} = \frac{16\left[2c + \frac{1}{2}(h - cl - 3n) + 3s + \frac{5}{2}p + \frac{1}{2}na - 0\right]}{MW}$$

(*ii*) This calculation implies that C is mineralized to CO_2, H to H_2O, P to P_2O_5 and Na to Na_2O. Halogen is eliminated as hydrogen halide and nitrogen as ammonia.

Example:

Glucose $C_6H_{12}O_{6'}$ MW = 180

$$\text{ThOD} = \frac{16(2 \times 6 + \frac{1}{2} \times 12 - 6)}{180} = 1.07 \text{ mg } O_2 \text{ / mg glucose}$$

(*iii*) Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolyzed.

(*iv*) Sulfur is assumed to be oxidized to the state of +6.

Example:

Sodium dodecylbenzenesulfonate $C_{18}H_{29}SO_3Na$, MW = 348

$$\text{ThOD} = \frac{16(36 + \frac{29}{2} + 3 + \frac{1}{2} - 3)}{348} = 2.34 \text{ mg } O_2 \text{ / mg substance}$$

(*v*) In the case of nitrogen-containing substances the nitrogen may be eliminated as ammonia, nitrite, or nitrate, and each corresponds to a different theoretical oxygen demand.

For nitrite,

$$\text{ThOD}_{NO_2} = \frac{16\left[2c + \frac{1}{2}(h - cl) + 3s + \frac{3}{2}n + \frac{5}{2}p + \frac{1}{2}na - o\right]}{MW}$$

For nitrate,

$$\text{ThOD}_{NO_3} = \frac{16\left[2c + \frac{1}{2}(h - cl) + 3s + \frac{5}{2}n + \frac{5}{2}p + \frac{1}{2}na - o\right]}{MW}$$

(*vi*) Suppose full nitrate formation had been observed by analysis in the case of a secondary amine: $(C_{12}H_{25})_2$ NH, MW = 353. Then

$$ThOD_{NO_3} = \frac{16(48 + 51/2 + 5/2)}{353} = 3.44 \text{ mg } O_2 / \text{mg substance}$$

(B) The solubility of the substance should be at least 2 mg/L, though in principle less soluble compounds could be tested (e.g., after sonication) as could volatile compounds. Information on the purity or the relative proportions of major components of the test substance will be useful in interpreting the results obtained, especially when the result lies close to the ''pass'' level.

(C) Information on the toxicity of the substance to bacteria, for example as measured in short-term respiration tests (see paragraph (e)(4) of this guideline), may be useful when selecting appropriate test concentrations, and may be essential for the correct interpretation of low biodegradation values. However, such information is not always sufficient for interpreting results obtained in the biodegradation test, and the procedure described in paragraph (d)(2)(vi)(F)(*7*) of this guideline may be more suitable.

(iv) **Reference substance.** (A) Suitable reference substances shall be used to check the microbial activity of the sea water sample. Aniline, sodium acetate and sodium benzoate are examples of substances that may be used for this purpose. Degradation of these compounds must reach at least 60 per cent with respect to their ThOD within a reasonably short time span; otherwise it is recommended that the test be repeated using another sea water sample.

(B) In the EEC ring test (see paragraph (e)(3) of this guideline), the lag phase (t_L) and the time to achieve 50 per cent degradation (t_{50}) excluding the lag phase were 0 to 2 days and 1 to 4 days, respectively, for sodium benzoate. For aniline the t_L and t_{50} values were 0 to 7 and 2 to 12 days, respectively.

(v) **Reproducibility.** The reproducibility of the method was established in the EEC ring test (see paragraph (e)(3) of this guideline).

(vi) **Description of the method**—(A) **Apparatus.** Normal laboratory equipment and:

(*1*) 250- to 300-mL BOD bottles with glass stoppers or narrow neck 250-mL bottles with glass stoppers.

(*2*) Several 2-, 3- and 4-L bottles for the preparation of the experiment and for the filling of BOD bottles.

(*3*) Waterbath or constant temperature room for keeping the bottles at constant temperature (± 1°C)) with the exclusion of light.

(*4*) Equipment for analysis of dissolved oxygen.

(*5*) Membrane filters, 0.2 to 0.45 μm (optional).

(*6*) Equipment for specific analysis (optional).

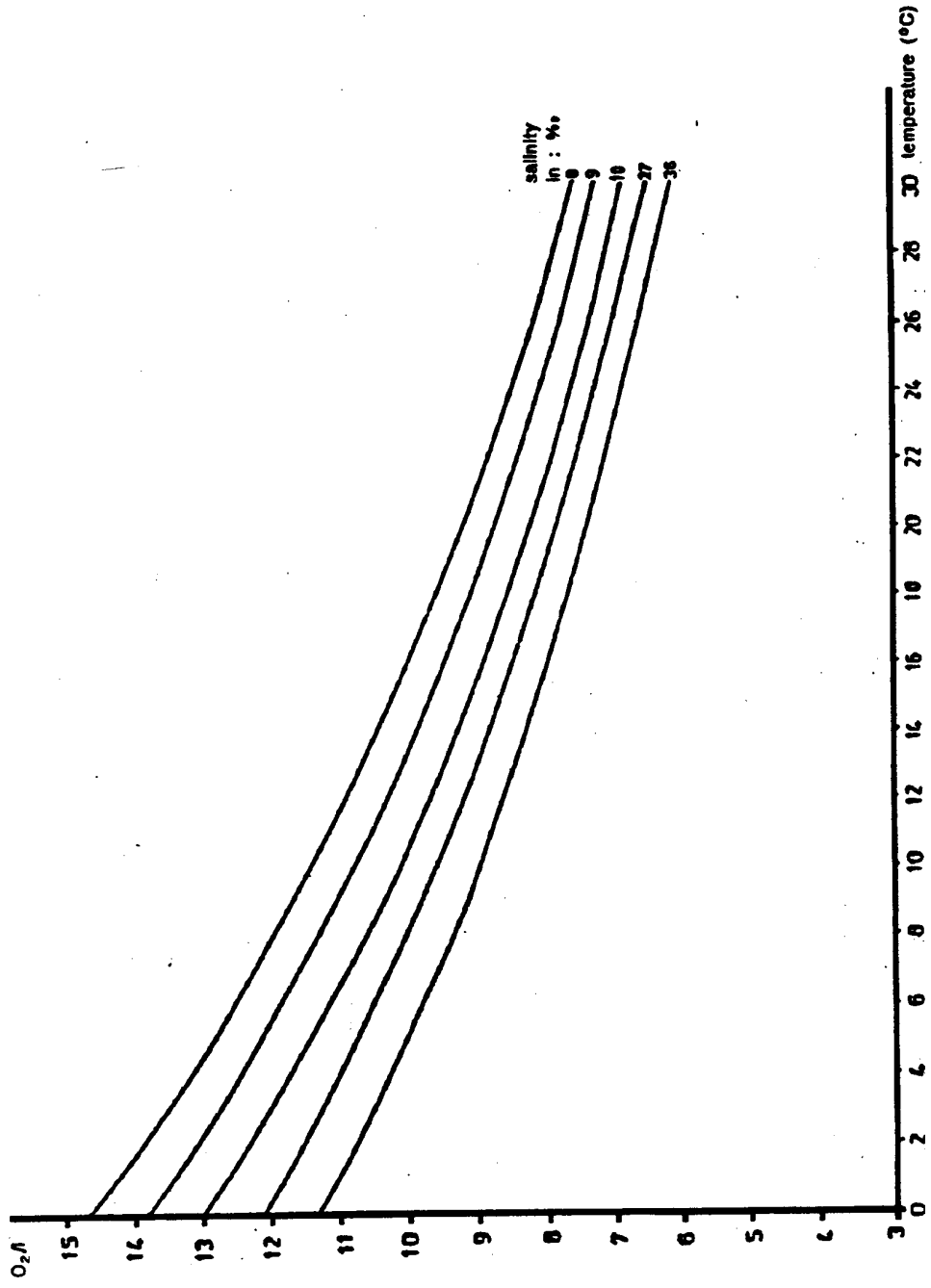
(B) **Sea Water.** (*1*) Collect a sea water sample and provide information on the sampling location and sample as described in paragraphs (d)(1)(vi)(B)(*1*) and (d)(1)(vi)(B)(*2*) of this guideline.

(*2*) If the DOC content of the sample is found to be high or if it is thought that the blank BOD after 28 days may be more than 30% of that of the reference substance, it is recommended that the sea water be aged for about a week prior to use. Follow the procedures described in paragraph (d)(1)(vi)(B)(*3*) of this guideline.

(C) **Stock solutions for mineral nutrients.** Prepare stock solutions as described in paragraph (d)(1)(vi)(C) of this guideline.

(D) **Preparation of test medium.** Add 1 mL of each of the stock solutions described in paragraph (d)(1)(iv)(C) of this guideline per L of sea water. Saturate the test medium with air at the test temperature by aerating with clean compressed air for approximately 20 minutes. Determine the concentration of dissolved oxygen for control purposes. The saturated concentration of dissolved oxygen as a function of salinity and temperature may be read from the nomogram in the following Figure 2.

FIGURE 2.—NOMOGRAM OF SATURATION CONCENTRATION OF OXYGEN VS. TEMPERATURE AT VARIOUS SALINITIES



(E) **Inoculum.** See paragraph (d)(1)(vi)(E) of this guideline.

(F) **Preparation of test bottles.** (*1*) Perform all necessary manipulations including aging and pretreatment of the sea water at the chosen test temperature in the range of 15 to 20°C, ensuring cleanliness but not sterility of all glassware.

(*2*) Prepare groups of BOD bottles for the determination of the BOD of the test and reference substances in simultaneous experimental series. Perform all analyses on duplicate bottles (i.e., duplicate blanks, duplicate reference substance bottles and duplicate test substance bottles). A minimum of five sampling times in total are required to describe the time course of degradation. For oxygen analyses, five sampling times require a total of 3 x 2 x 5 = 30 bottles (blank, reference substance and test substance), and thus about 10 L of test medium.

(*3*) Prepare separate solutions of test and reference substances in large bottles of sufficient volume (see paragraph (d)(2)(vi)(A)(*2*) of this guideline) by first adding test and reference substances either directly or by adding a concentrated stock solution to the partly filled large bottles. Add further test medium to give the final desired concentrations. If stock solutions of test and/or reference substances are used, ensure that the salinity of the sea water medium is not significantly altered.

(*4*) Select concentrations of test and reference substances by taking into account:

(*i*) The solubility of dissolved oxygen in sea water at the prevailing test temperature and salinity (see the nomogram in paragraph (d)(2)(vi)(D) of this guideline).

(*ii*) The blank BOD of the sea water.

(*iii*) The expected biodegradability of the test substance.

(*5*) At 15°C and 20°C and 32 parts per thousand salinity (i.e., that of ocean water), the solubility of dissolved oxygen is about 8.1 and 7.4 mg/l, respectively. The oxygen consumption of the seawater itself (blank respiration) may be 2 mg O_2/L or more if the sea water is not aged. Therefore, in order to ensure that a significant amount of oxygen remains after oxidation of the test substance, use a starting concentration of test compound of approximately 2 to 3 mg/L (depending on the ThOD) for the compounds that are expected to be completely degraded under the conditions of the test, such as reference substances. Use higher initial concentrations for less degradable substances, up to about 10 mg/L, provided that toxic effects do not occur. It can be advantageous to run parallel tests with low (about 2 mg/L) and high (about 10 mg/L) concentrations of test substance.

(*6*) An oxygen blank must be determined at each time point using parallel bottles containing neither test nor reference substance.

(*7*) If inhibitory effects are to be determined, prepare the following series of solutions in separate large bottles (see paragraph (d)(2)(vi)(A)(*2*) of this guideline):

(*i*) 2 mg/L of an easily degradable compound, e.g., any of the reference substances listed in paragraph (d)(2)(iv)(A) of this guideline.

(*ii*) x mg/L of test substance (x is usually 2).

(*iii*) 2 mg/L of the easily degradable compound plus x mg/L of test substance.

(G) **Physical-chemical control test (optional).** If the option of using specific analyses is used, a physical-chemical control test may be performed to determine whether the test substance is removed by abiotic mechanisms, such as hydrolysis or sorption. A physical-chemical control test may be performed by adding mercury (II) chloride ($HgCl_2$)[2] (50 to 100 mg/L) to duplicate flasks with test substance in order to inhibit microbial activity. A significant decrease in specific compound concentration in the course of the test indicates abiotic removal.

(H) **Number of BOD bottles.** In a typical experiment the following bottles are used:

(*1*) At least 10 containing test substance.

(*2*) At least 10 containing nutrient-fortified sea water only.

(*3*) At least 10 containing reference substance.

(*4*) 6 bottles containing test and reference substances (toxicity control)—optional.

(vii) **Procedure.** (A) After preparation, immediately siphon each solution from the lower quarter (not from the bottom) of the appropriate large bottle, to fill the respective group of BOD bottles. Immediately analyze the time zero samples for dissolved oxygen (see paragraph (d)(2)(vii)(D) of this guideline), or preserve them for later chemical analysis by precipitation with $MnCl_2$ (manganese (II) chloride) and NaOH (sodium hydroxide).

(B) Incubate the remaining BOD bottles at the test temperature (15–20°C) in the dark, and remove bottles for analysis at appropriate time intervals. No fixed time schedule can be stated since the rate of biodegradation

[2]Mercury (II) chloride ($HgCl_2$) is a very toxic substance that should be handled with suitable precautions. Aqueous wastes containing this chemical should be disposed of appropriately; they should not be discharged down the drain.

varies. Analyze for dissolved oxygen (see paragraph (d)(2)(vii)(D) of this guideline).

(C) Membrane filter (0.2–0.45 μm) or centrifuge for 15 minutes samples for specific analyses (optional). Store these samples for up to 48 hours at 2–4°C, or for longer periods at -18°C, if they are not analyzed immediately. If it is known that the test substance will remain unaffected by acidification, acidify to pH 2 before storing.

(D) Determine the concentration of dissolved oxygen using a chemical or electrochemical method that is recognized nationally or internationally.

(viii) **Data and reporting**—(A) **Treatment of results.** (*1*) Record analytical results on the data sheet below.

BIODEGRADATION IN SEA WATER—CLOSED BOTTLE METHOD

DATA SHEET

LABORATORY:_____

DATE AT START OF TEST:_____

TEST SUBSTANCE:_____

Name:_____

Stock solution concentration (mg/L):_____

Initial concentration in medium, t_o (mg/L):_____

ThOD or COD (mg O_2/mg test substance):_____

1. SEA WATER:

Source:_____

Date of collection:_____

Depth of collection:_____

Appearance at time of collection (e.g., turbid, etc.):_____

Salinity at collection (%):_____

Temperature at collection (°C):_____

DOC ‘‘x’’hours after collection (mg/L):_____

Pretreatment prior to testing (e.g., filtration, sedimentation, aging, etc.):_____

Microbial colony count of original sample (colonies/mL):_____

At start of test (colonies/mL):_____

Other characteristics:_____

Test medium:

Temperature after aeration (°C):_____

O_2 concentration after aeration and standing before start of test

(mg O_2/L):_____

2. DO DETERMINATION:

Method: Winkler/electrode

	Flask no.		mg O_2 after n days				
			0	n_1	n_2	n_3	n_4
Test: nutrient-fortified sea water with test substance	1	a_1					
	2	a_2					
	Mean blank	$m_t = (a_1 + a_2)/2$					
Blank: nutrient-fortified sea water but without test substance	1	c_1					
	2	c_2					
	Mean test	$m_b = (c_1 + c_2)/2$					

Note: Similar format may be used for reference compound and toxicity controls.

3. DO DEPLETION: % DEGRADATION (%D):

	DO depletion after n days			
	n_1	n_2	n_3	n_4
$(m_b - m_t)^{(1)}$				
%D = $(m_b - m_t)^{(1)}$ x 100/test substance (mg/l) x ThOD				

(1) This assumes that $m_{b(o)} = m_{t(o)}$, where

$m_{b(o)}$ = blank value at day 0,

$m_{t(o)}$ = test substance value at day 0.

If $m_{b(o)}$ does not equal $m_{t(o)}$, use $(m_{t(o)} - m_{t(x)}) - (m_{b(o)} - m_{b(x)})$, where

$m_{b(x)}$ = blank value at day x,

$m_{t(x)}$ = test substance value at day x.

(*2*) Calculate the BOD as the difference in the oxygen depletion between the blank and a solution of test substance under the conditions of the test. Divide the net oxygen depletion by the concentration (w/v) of the substance in order to express the BOD as mg BOD/mg test substance. Extent of degradation is defined as the ratio of the BOD to either the ThOD or the COD, but preferably the former, and is expressed as a percentage (see paragraph (d)(2)(viii)(A)(*3*) of this guideline).

(*3*) Calculate the extent of biodegradation for each sampling time, for both test and reference substances, using one of the following equations:

$$\% \text{ biodegradation } = \frac{\text{mg } O_2 \text{ / mg tested substance}}{\text{mg ThOD / mg tested substance}} \times 100$$

$$\% \text{ biodegradation } = \frac{\text{mg } O_2 \text{ / mg tested substance}}{\text{mg COD / mg tested substance}} \times 100$$

where:

ThOD = theoretical oxygen demand (for method of calculation, see paragraph (d)(2)(iii)(A) of this guideline).

COD = chemical oxygen demand, determined experimentally.

NOTE: Sometimes the two methods of calculation (percentage of the ThOD and percentage of the COD) do not give the same results. It is preferable to use ThOD because some chemicals are not fully oxidized in the COD test.

(*4*) Illustrate the time course of degradation graphically by means of a diagram; see the example in paragraph (d)(2)(viii)(C)(*6*) of this guideline. If there are sufficient data, calculate the lag phase (t_L) and the time from the end of the lag phase that is required to reach 50% degradation (t_{50}).

(*5*) If specific analysis is used (optional), express the percentage of primary degradation as the percentage of specific substance removed within the test period, corrected for analytical blanks.

(B) **Test report. (***1*) The test report must contain the following information:

(*i*) Test substance:

(*a*) Physical nature and, where relevant, physical/chemical properties.

(*b*) Identification of the substance.

(*ii*) Test conditions:

(*a*) Location and description of the sampling site: pollutional and nutrient status (colony counts, and nitrate, ammonium and phosphate levels if appropriate).

(*b*) Characteristics of the sample: date of sampling, depth, appearance, temperature, salinity, DOC (optional), delay between collection and use in the test.

(*c*) Method used (if any) for aging the sea water.

(*d*) Method used for pretreatment (filtration/sedimentation) of the sea water.

(*e*) Method used for COD determination (if performed).

(*f*) Method used for oxygen measurements.

(*g*) Method of dispersion for substances that are poorly soluble under the test conditions.

(*h*) Method used for determining the number of heterotrophs in the sea water (plate count method or alternative procedure).

(*i*) Method used for determining DOC in sea water (optional).

(*j*) Method used for specific analysis (optional).

(*k*) Other optional methods used to characterize the sea water (ATP measurements, etc.).

(*iii*) Results:

(*a*) Analytical data reported on a data sheet (see paragraph (d)(2)(viii)(A)(*1*) of this guideline).

(*b*) The time course of degradation represented graphically in a diagram showing the lag phase, (t_L), slope, and time starting from the end of the lag phase to reach 50% of the final oxygen uptake caused by oxidation of the test compound (t_{50}). The lag phase may be estimated graphically as shown in Figure 3 in paragraph (d)(2)(viii)(C)(*6*) of this guideline, or conveniently in taken as the time needed for 10% degradation.

(*c*) % degradation measured after 28 days.

(*iv*) Discussion of results.

(C) **Validity and interpretation of results.** (*1*) The blank respiration should not exceed 30% of the oxygen in the test bottle. If it is not possible to meet this criterion using freshly collected sea water, the seawater must be aged (stabilized) before use.

(*2*) The possibility that nitrogen-containing compounds may affect the results should be considered.

(*3*) Results obtained with the reference substances sodium benzoate and aniline should be comparable to the results obtained in the ring test

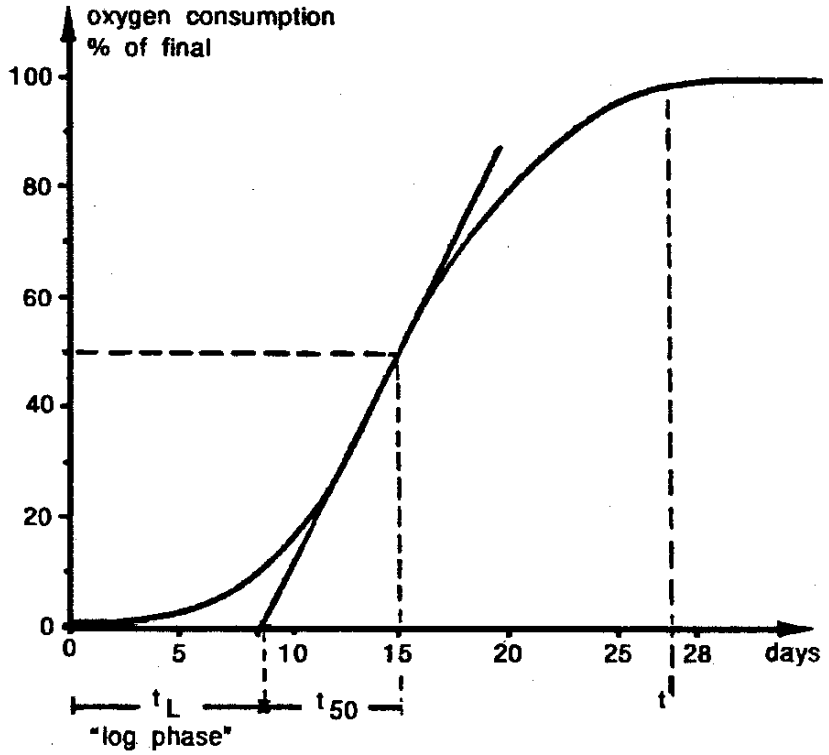
(see paragraphs (d)(2)(iv)(B) and (e)(3) of this guideline). If results obtained with reference compounds are atypical, the test should be repeated using another sea water sample.

(*4*) The test substance can be considered to be inhibitory to bacteria at the concentration used if the BOD of the mixture of reference and test substances is less than the sum of the BOD of the separate solutions of the two substances.

(*5*) Owing to the relatively high test concentrations as compared with most natural systems, and consequently an unfavorable ratio between the concentrations of test substance and other carbon sources, the method is regarded as a preliminary test that can be used to indicate whether or not a substance is easily biodegradable in sea water. Accordingly, a low result does not necessarily mean that the test substance is not biodegradable in marine environments, but indicates that more work will be necessary to establish this.

(*6*) An example of graphical presentation of data and estimation of the values of t_L (length of lag phase) and t_{50} (time, starting at t_L, needed to reach 50% of the final oxygen uptake caused by oxidation of the test substance), is given in the following Figure 3:

FIGURE 3.—TYPICAL BIODEGRADATON CURVE FOR THE CLOSED BOTTLE METHOD



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

(1) de Kreuk J.F. and Hanstveit A.O. 1981. Determination of the biodegradability of the organic fraction of chemical wastes. Chemosphere 10 (6): 561–573.

(2) OECD, Paris. 1992. Test Guideline 301 E.

(3) Nyholm N. and Kristensen P. 1987. Screening Test Methods for Assessment of Biodegradability of Chemical Substances in Seawater. Final Report of the ring test programme 1984–1985, March 1987, Commission of the European Communities.

(4) OECD, Paris. 1984. Test Guideline 209.

(5) International Standards Organization (ISO). 1986. Water quality—determination of total organic carbon. Draft International Standard ISO/DIS 8245, January 16.

(6) American Public Health Association. 1985. Standard Methods for the Examination of Water and Wastewater, 16th ed.

(7) Schreurs W. 1978. An automated colorimetric method for the determination of dissolved organic carbon in seawater by UV destruction. Hydrobiological Bulletin 12: 137–142.

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