

OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Biodegradability in Seawater

GENERAL INTRODUCTION

1. When the original OECD Test Guidelines were developed, it was not known to what extent results from the screening tests for ready biodegradability using freshwater, 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. (1)).
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 seawater have been developed.
3. The methods described here use natural seawater both as the aqueous phase and as the source of micro-organisms. In an endeavour to conform with the methods for ready biodegradability in freshwater, the use of ultra-filtered and centrifuged seawater was investigated, as was the use of marine sediments as inocula. These investigations were unsuccessful. The test medium therefore is natural seawater pre-treated to remove coarse particles.
4. In order to assess ultimate biodegradability with the Shake Flask Method, relatively high concentrations of the test substance have to be used because of the poor sensitivity of the dissolved organic carbon (DOC) analytical method. This in turn necessitates the addition to the seawater 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 micro-organisms already present in the seawater. 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. For these reasons the methods are proposed under a new subsection "Biodegradability in Seawater".

APPLICATION

6. The results of the tests, which would be applied because the pattern of use and disposal of the chemical in question indicated a route to the sea, give a first impression of biodegradability in seawater. 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 a potential but indicates that further study is necessary, for example, using as low a concentration of the test compound as possible.

7. In either case, if a more definitive value for the rate or degree of biodegradation in seawater 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. Also, non-fortified, non-pre-treated seawater taken from the location of interest could be used and primary biodegradation could be followed by specific chemical analysis. For ultimate biodegradability, ^{14}C -labelled chemicals would be necessary in order that the rates of the disappearance of soluble organic ^{14}C and the production of $^{14}\text{CO}_2$ at environmentally realistic concentrations could be measured.

CHOICE OF METHODS

8. The selection of which method to use depends on a number of factors; the following Table is given to help the selection. While chemicals of water solubility below the equivalent of about 5 mg C/l cannot be tested in the Shake Flask Method, at least, in principle, poorly soluble chemicals may be tested in the Closed Bottle Method.

TABLE: ADVANTAGES AND DISADVANTAGES OF THE SHAKE FLASK AND CLOSED BOTTLE TEST

METHOD	ADVANTAGES	DISADVANTES
SHAKE FLASK	<ul style="list-style-type: none"> - simple apparatus except C analyser - 60 d duration is not a problem - no interference from nitrification - can be adapted for volatile chemicals 	<ul style="list-style-type: none"> - needs C analyser - uses 5-40 mg DOC/l, could be inhibitory - DOC determination is difficult at low concentrations in seawater (chloride effect) - DOC sometimes high in seawater
CLOSED BOTTLE	<ul style="list-style-type: none"> - simple apparatus - simple end determination - uses low concentration of test compound (2 mg/l) thus less chance of inhibition - easily adapted for volatile chemicals 	<ul style="list-style-type: none"> - could be difficult to maintain air-tightness of bottles - wall growth of bacteria can lead to false values - blank O_2 uptake values can be high especially after 28 days; could be overcome by ageing the seawater - possible interference from O_2 uptake by nitrification

SHAKE FLASK METHOD

INTRODUCTION

1. This method is a seawater variant of the Modified OECD Screening Test (2). It was finalised as a result of a ring test organized for the EEC by the Danish Water Quality Institute (3).
2. In common with the accompanying marine 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 biodegradability of chemicals in marine environments.

PRINCIPLE OF THE METHOD

3. A pre-determined amount of the test substance is dissolved in the test medium to yield a concentration of 5-40 mg dissolved organic carbon (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 compounds. The solution of the test substance in the test medium is incubated under agitation in the dark or in diffuse light under aerobic conditions at a fixed temperature (controlled to $\pm 2^{\circ}\text{C}$) 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 carried out beyond this normal temperature range. The recommended maximum test duration is about 60 days. Degradation is followed by DOC measurements (ultimate degradation) and, in some cases, by specific analysis (primary degradation).

INFORMATION ON THE TEST SUBSTANCE

4. In order to know whether the test may be applied to a particular substance, some of its properties 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. Also, the test substance should not significantly adsorb onto glass surfaces. Information on the purity or the relative proportions of major components of the test material is required in order that the results obtained can be interpreted, especially when the result lies close to the "pass" level.
5. Information on the toxicity of the test substance to bacteria, for example as measured in short-term respiration rate tests (4), 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 18 is more suitable.

REFERENCE COMPOUNDS

6. Suitable reference compounds must be used to check the microbial activity of the seawater sample. Sodium benzoate, sodium acetate and aniline are examples of chemicals which may be used for this purpose. The reference compounds must be degraded within a reasonably short time span, otherwise it is recommended that the test be repeated using another seawater sample.
7. In the EEC ring test where seawater samples were taken at different locations and at different times of the year (3), 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, whilst the t_{50} ranged from 1 to 10 days.

REPRODUCIBILITY AND SENSITIVITY OF THE METHOD

8. The reproducibility of the method was established in the ring test (3). The lowest concentration of test substance, for which this method can be used with DOC analysis, is largely determined by the detection limit of the organic carbon analysis (about 0.5 mg C/l, at present) and the concentration of dissolved organic carbon in the seawater used (usually of the order of 3-5 mg/l for water from the open sea). The background concentration of DOC should not exceed about 20% of the total DOC concentration after addition of test material. If this is not feasible, the background concentration of DOC may sometimes be reduced by ageing the seawater 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.

DESCRIPTION OF THE METHOD

Apparatus

9. Normal laboratory apparatus and:
- (a) Shaking machine accommodating 0.5-2 litre Erlenmeyer flasks, either with automatic temperature control or used in a constant temperature room at 15-20°C controlled to $\pm 2^\circ\text{C}$;
 - (b) Narrow neck, 0.5-2 litre Erlenmeyer flasks;
 - (c) Membrane filtration apparatus, or centrifuge;
 - (d) Membrane filters, 0.2-0.45 μm ;
 - (e) Carbon analyser;
 - (f) Equipment for specific analysis (optional).

Seawater

10. Collect a sample of seawater in a thoroughly cleansed container and transport to the laboratory, preferably within one or two 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.

11. Provide the following information for the seawater sample itself:
- date of collection;
 - depth of collection;
 - appearance of sample - turbid, etc.;
 - temperature at the time of collection;

- salinity;
- DOC;
- delay between collection and use in the test.

12. If the DOC content of the seawater sample is found to be high (paragraph 8), it is recommended that the seawater be aged for about a week prior to use. Age by storing under aerobic conditions at the test temperature and in the dark or in diffuse light. If necessary, maintain aerobic conditions by gentle aeration. During ageing, the content of easily degradable organic material is reduced. In the ring test (3), no difference was revealed between the degradation potential of aged and freshly collected seawater samples. Prior to use, pre-treat the seawater to remove coarse particles, e.g. by filtration through a nylon filter or coarse paper filter (not membrane or GF-C filters), or by sedimentation and decanting. The procedure used must be reported. Carry out pre-treatment after ageing, if used.

Stock solutions for mineral nutrients

13. Prepare the following stock solutions, using analytical grade reagents:

- | | | |
|-----|-----------------------------------------------------------------------------------------------------------|---------|
| (a) | Potassium dihydrogen orthophosphate, KH_2PO_4 | 8.50 g |
| | Dipotassium hydrogen orthophosphate, K_2HPO_4 | 21.75 g |
| | Disodium hydrogen orthophosphate dihydrate, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ | 33.30 g |
| | Ammonium chloride, NH_4Cl | 0.50 g |

Dissolve and make up to 1 litre with distilled water.

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|-----|---------------------------------------------|---------|
| (b) | Calcium chloride, CaCl_2 | 27.50 g |
|-----|---------------------------------------------|---------|

Dissolve and make up to 1 litre with distilled water.

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|-----|--------------------------------------------------------------------------------------|---------|
| (c) | Magnesium sulphate heptahydrate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 22.50 g |
|-----|--------------------------------------------------------------------------------------|---------|

Dissolve and make up to 1 litre with distilled water.

- | | | |
|-----|--------------------------------------------------------------------------------------|--------|
| (d) | Iron (III) chloride hexahydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ | 0.25 g |
|-----|--------------------------------------------------------------------------------------|--------|

Dissolve and make up to 1 litre with distilled water.

Precipitation in solution (d) may be prevented by adding one drop of concentrated HCl or 0.4 g ethylenediaminetetra-acetic acid (EDTA, disodium salt) per litre. If a precipitate forms in a stock solution, replace it with freshly made solution.

Preparation of test medium

14. Add 1 ml of each of the above stock solutions per litre of pre-treated seawater.

Inoculum

15. Do not add a specific inoculum in addition to the micro-organisms already present in the seawater. Determine (optionally) the number of colony-forming heterotrophs in the seawater test medium (and preferably also in the original seawater samples) e.g. by plate count, using marine agar. This is particularly desirable for samples from coastal or polluted sites. Check the heterotrophic microbial activity in the seawater by performing a test with a reference compound.

Preparation of flasks

16. Ensure that all glassware is scrupulously clean, not necessarily sterile, (e.g. using alcoholic hydrochloric acid), rinsed and dried before use in order to avoid contamination with residues from previous tests. The flasks must also be cleaned before first use.

17. 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 substances in the test medium - they may be conveniently added via a concentrated stock solution - to give the desired starting concentrations of normally 5-40 mg DOC/l. Test the reference compound normally 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 seawater medium is not greatly altered.

18. 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.

19. 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 $\pm 2^\circ\text{C}$), and shield the vessels from light in order to avoid growth of algae. Ensure that the air is free of toxic materials.

Physical-chemical control test (optional)

20. If abiotic degradation or loss mechanisms are suspected, such as hydrolysis (a problem with specific analysis only), volatilization, or adsorption, it is advisable to perform a physical-chemical control experiment. This can be done by adding mercury (II) chloride (HgCl_2)⁽¹⁾ (50-100 mg/l) to vessels with test substance in order to stop 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).

Number of flasks

21. In a typical run, the following flasks are used:

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|--------------|---|--------------------------------------------------------------------------------------|
| Flasks 1 & 2 | - | containing test substance (test suspension); |
| Flasks 3 & 4 | - | containing seawater only (blank); |
| Flask 5 | - | containing reference compound (procedure control); |
| Flask 6 | - | containing test and reference substance (toxicity control) - optional; |
| Flask 7 | - | containing test substance and sterilising agent (abiotic sterile control)- optional. |

⁽¹⁾ Mercury (II) chloride (HgCl_2) is a very toxic substance which should be handled with suitable precautions. Aqueous wastes containing this chemical should be disposed of appropriately; they should not be discharged into the waste water system.

DOC analysis

22. In the course of the test, withdraw samples at suitable intervals for DOC analysis (Annex 1). 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 as the rate of biodegradation varies. Carry out the DOC determination in duplicate on each sample.

Sampling

23. The required volume of the samples depends upon the analytical method (specific analysis), on the carbon analyser used, and on the procedure (membrane filtration or centrifugation) selected for sample treatment before carbon determination (paragraphs 25 and 26). 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.

24. 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, acidify to pH 2 before storing).

25. Membrane filters (0.2-0.45 µm) are suitable if it is ensured that they neither release carbon nor adsorb the substance in the filtration step e.g. polycarbonate membrane filters. Some membrane filters are impregnated with surfactants for hydrophilization and may release considerable quantities of dissolved carbon. Prepare such filters by boiling in deionised water for three consecutive periods, each of one hour. After boiling, store the filters in deionised water. Discard the first 20 ml of the filtrate.

26. Centrifugation of the samples may be chosen as an alternative to membrane filtration. Centrifuge at 40 000 m.s² (~ 4000 g) for 15 minutes, preferably in a refrigerated centrifuge.

Note: The differentiation of Total Organic Carbon (TOC) over DOC (TOC/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 per litre), the centrifugation error seems to be comparatively small.

Frequency of sampling

27. If analyses are performed immediately after sampling, assess the next sampling time by considering the result of the analytical determination.

28. If samples are preserved (paragraph 24) for analysis at a later time, take more samples than the required minimum number of five. Analyse the last samples first, and by a step-wise "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 analysed, and in this situation, the "backwards" strategy may save considerable analytical costs.

29. 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.

DATA AND REPORTING**Treatment of results**

30. Record the analytical results on the attached data sheet (Annex 2), and calculate the biodegradation values for both test and reference substances from the equation:

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

where:

D_t	=	degradation in percentage DOC or specific compound removal at time t,
C_0	=	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.

31. 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 per litre, and round up the means of the D_t values to the nearest whole per cent.

32. Illustrate the course of the degradation graphically in a diagram as shown in the figure in "Validity and interpretation of results". 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}).

Test report

33. The test report must contain the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Test conditions:

- location and description of the sampling site; pollutional and nutrient status (colony count, nitrate, ammonium, phosphate if appropriate);
- characteristics of the sample (date of sampling, depth, appearance, temperature, salinity, DOC (optional), delay between collection and use in the test);
- method used (if any) for ageing of the seawater;
- method used for pre-treatment (filtration/sedimentation) of the seawater;
- method used for DOC determination;
- method used for specific analysis (optional);
- method used for determining the number of heterotrophs in the seawater (plate count method or alternative procedure) (optional);
- other methods (optional) used to characterise the seawater (ATP measurements, etc.).

Results:

- analytical data reported on a data sheet (Annex 2);
- the course of the degradation test is 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 per cent removal (t_{50}). The lag phase may be estimated graphically as shown in the figure in the "Validity and interpretation of results" section or conveniently taken as the time needed for 10 per cent degradation;
- percentage degradation measured after 60 days, or at end of test.

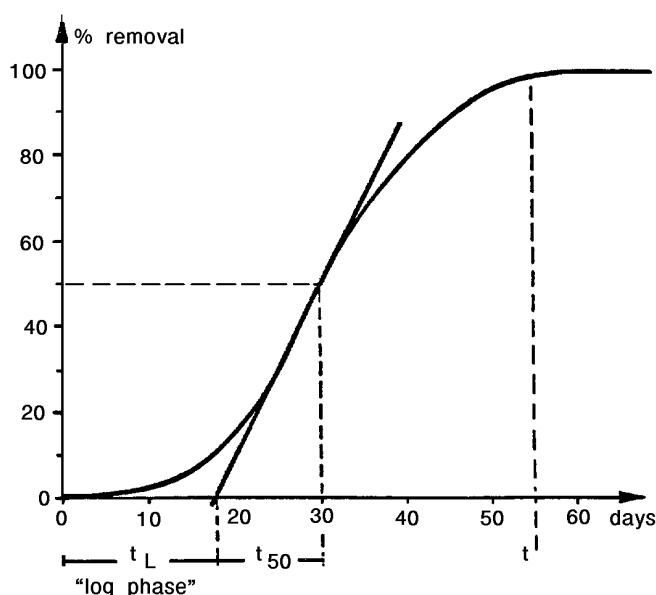
Discussion of results.

Validity and interpretation of results

34. 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 (3) (refer to section on "Reference Compounds", paragraph 7). If results obtained with reference compounds are atypical, the test should be repeated using another seawater 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.

35. Owing to the relatively high test concentrations used as compared with most natural systems (and consequently an unfavourable ratio between the concentrations of test substances and other carbon sources), the method is to be regarded as a preliminary test which can be used to indicate whether or not a substance is easily biodegradable. 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 in order for this to be established.

An example of a theoretical degradation experiment illustrating a feasible way of estimating the values of t_L (length of "lag phase") and t_{50} (time interval, starting at t_L , needed to reach 50 per cent removal, is given in the figure below.



CLOSED BOTTLE METHOD

INTRODUCTION

1. This method is a seawater variant of the Closed Bottle Test (5) and was finalised as a result of a ring test organised for the EEC by the Danish Water Quality Institute (3).
2. In common with the accompanying marine 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.

PRINCIPLE OF THE METHOD

3. A pre-determined amount of the test substance is dissolved in the test medium in a concentration of usually 2-10 mg of test substance per litre (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 $\pm 1^\circ\text{C}$ within a range of 15-20°C. In those cases where the objective of the study is to simulate environmental situations, tests may be carried out beyond this normal temperature range providing suitable adjustments are made for temperature control. The degradation is followed by oxygen analyses over a 28-day period.
4. 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 (paragraphs 15 and 40) the test could be prolonged.

INFORMATION ON THE TEST SUBSTANCE

5. In order to know whether the test may be applied to a particular substance, some of its properties must be known. The empirical formula is required so that the theoretical oxygen demand (ThOD) may be calculated (see Annex 3); otherwise the chemical oxygen demand (COD) of the compound must be determined to serve as the reference value. The use of COD is less satisfactory since some chemicals are not fully oxidised in the COD test.
6. The solubility of the substance should be at least 2 mg/l, though in principle less soluble compounds could be tested (e.g. using ultra sonication) as could volatile compounds. Information on the purity or the relative proportions of major components of the test material is required in order that the results obtained can be interpreted, especially when the result lies close to the "pass" level.
7. Information on the toxicity of the substance to bacteria e.g. as measured in short-term respiration tests (4) may be very 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 27 is more suitable.

REFERENCE COMPOUNDS

8. Suitable reference compounds must be used to check the microbial activity of the seawater

sample. Aniline, sodium acetate or sodium benzoate (for example) may be used for this purpose. A degradation of these compounds of at least 60 per cent (of their ThOD) must occur within a reasonably short time span, otherwise it is recommended that the test be repeated using another seawater sample.

9. In the EEC ring-test where seawater samples were taken at different locations and at different times of the year, the lag phase (t_L) and the time to achieve 50 per cent degradation (t_{50}), not including 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.

REPRODUCIBILITY

10. The reproducibility of the methods was established in the EEC ring test (3).

DESCRIPTION OF THE METHOD

Apparatus

11. Normal laboratory equipment and:
- (a) 250-300 ml BOD bottles with glass stoppers or narrow neck 250 ml bottle with glass stoppers may be used;
 - (b) Several 2-, 3- and 4- litre bottles with litre marks for the preparation of the experiment and for the filling of the BOD bottles;
 - (c) Waterbath or constant temperature room for keeping the bottles at constant temperature ($\pm 1^\circ\text{C}$) with the exclusion of light.
 - (d) Equipment for analysis of dissolved oxygen;
 - (e) Membrane filters, 0.2-0.45 μm (optional);
 - (f) Equipment for specific analysis (optional).

Seawater

12. Collect a seawater sample in a thoroughly cleansed container and transport to the laboratory, preferably within one or two days of collection. During transport do not allow the temperature of the sample to exceed significantly the temperature to be used in the test.

13. Identify the sampling location precisely and describe it in terms of its pollutional and nutritional status. Especially for coastal or polluted waters, include in this characterisation a heterotrophic microbial colony count and the determination of concentrations of dissolved nitrate, ammonium and phosphate.

14. Provide the following information for the seawater sample itself:

- date of collection;
- depth of collection;
- appearance of sample - turbid etc.;
- temperature at the time of collection;
- salinity;

- dissolved organic carbon (DOC);
- delay between collection and use in the test.

15. If the DOC content of the sample is found to be high or if it is thought that the blank BOD after 28 days would be more than 30 per cent of that of the reference substances, it is recommended that the seawater be aged for about a week prior to use.

16. Age the sample by storing it under aerobic conditions at the test temperature and in the dark or in diffuse light. If necessary, maintain aerobic conditions by gentle aeration. During ageing, the content of easily degradable organic material is reduced. In the ring-test (3), no difference was revealed between the degradation potential of aged and freshly collected seawater samples.

17. Prior to use, pretreat the seawater to remove coarse particles e.g. by filtration through a nylon filter or a coarse paper filter (not membrane or GF-C filters), or by sedimentation and decanting. Report the procedure used. Pretreat after ageing, if used.

Stock solutions for mineral nutrients

18. Prepare the following stock solutions using analytical grade reagents:

- | | | |
|-----|--------------------------------------------------------------------------------------------------------------|---------|
| (a) | Potassium dihydrogen orthophosphate, KH_2PO_4 | 8.50 g |
| | Dipotassium hydrogen orthophosphate, K_2HPO_4 | 21.75 g |
| | Disodium hydrogen orthophosphate dihydrate,
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ | 33.30 g |
| | Ammonium chloride, NH_4Cl | 0.50 g |

Dissolve and make up to 1 litre with distilled water.

- | | | |
|-----|---------------------------------------------|---------|
| (b) | Calcium chloride, CaCl_2 | 27.50 g |
|-----|---------------------------------------------|---------|

Dissolve and make up to 1 litre with distilled water.

- | | | |
|-----|--------------------------------------------------------------------------------------|---------|
| (c) | Magnesium sulphate heptahydrate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 22.50 g |
|-----|--------------------------------------------------------------------------------------|---------|

Dissolve and make up to 1 litre with distilled water.

- | | | |
|-----|--------------------------------------------------------------------------------------|--------|
| (d) | Iron (III) chloride hexahydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ | 0.25 g |
|-----|--------------------------------------------------------------------------------------|--------|

Dissolve and make up to 1 litre with distilled water.

Precipitation in solution (d) may be prevented by adding one drop of concentrated HCl or 0.4 g ethylenediaminetetra-acetic acid (EDTA, disodium salt) per litre. If a precipitate forms in a stock solution, replace it with freshly made solution.

Preparation of test medium

19. Add per litre of pre-treated seawater 1 ml of each of the above stock solutions. Saturate the test medium with air at the test temperature by aerating with clean compressed air for about 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 enclosed with this test guideline (Annex 4).

Inoculum

20. Do not add a specific inoculum in addition to the micro-organisms already present in the seawater. Determine (optionally) the number of colony-forming heterotrophs in the seawater test medium (and preferably also in the original seawater sample), e.g. by plate count using a marine agar. This is particularly desirable for samples from coastal or polluted sites. Check the heterotrophic microbial activity in the seawater by performing a test with a reference compound.

Preparation of test bottles

21. Perform all necessary manipulations including ageing and pre-treatment of the seawater at the chosen test temperature between 15 to 20°C, ensuring cleanliness, but not sterility of all glassware.

22. 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 (blanks, reference and test substances), i.e. prepare two bottles for each determination. Perform analyses at least on days 0, 5, 15 and 28 (four determinations).

For oxygen analyses, four determinations require a total of $3 \times 2 \times 4 = 24$ bottles (blank, reference and test substance), and thus about 8 litres of test medium (for one concentration of test material).

23. Prepare separate solutions of test and reference substances in large bottles of sufficient volume (paragraph 11) by first adding test and reference substances either directly or by using 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 seawater medium is not significantly altered.

24. Select concentrations of test and reference substances by taking into account:

- (a) the solubility of dissolved oxygen in seawater at the prevailing test temperature and salinity (see the enclosed nomogram - Annex 4);
- (b) the blank BOD of the seawater; and
- (c) the expected biodegradability of the test substance.

25. At 15°C and 20°C and 32 parts per thousand salinity (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 O₂/l or more, if the seawater is not aged. Therefore in order to ensure a significant oxygen concentration remaining after oxidation of the test substance, use a starting concentration of test compound of about 2-3 mg/l (depending on the ThOD) for the compounds that are expected to become completely degraded under the conditions of the test (such as reference compounds). Test less degradable substances at higher concentrations, up to about 10 mg/l, provided that toxic effects do not occur. It can be advantageous to run parallel tests with a low (about 2 mg/l) and a high (about 10 mg/l) concentration of test substance.

26. An oxygen blank must be determined in parallel in bottles containing neither test or reference substance.

27. If inhibitory effects are to be determined, prepare the following series of solutions in separate large bottles (paragraph 13):

- (a) 2 mg per litre of an easily-degradable compound, e.g. any of the reference compounds mentioned;

- (b) x mg per litre of test substance (x is usually 2);
- (c) 2 mg per litre of the easily-degradable compound plus x mg per litre of test substance.

Physical-chemical control test (optional)

28. If the option of using specific analyses is used, a physical-chemical experiment may be performed in order to check whether the test material is removed by abiotic mechanisms, such as hydrolysis or adsorption. A physical-chemical control test may be performed by adding mercury (II) chloride (HgCl_2)⁽¹⁾ (50-100 mg/l) to duplicate flasks with test material in order to stop microbial activity. A significant decrease in specific compound concentration in the course of the test indicates abiotic removal mechanisms.

Number of BOD bottles in a typical run

29. In a typical run the following bottles are used:
- at least 8 containing test substance;
 - at least 8 containing nutrient-fortified seawater only;
 - at least 8 containing reference compound, and when necessary
 - 6 bottles containing test and reference substances (toxicity control).

PROCEDURE

30. 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 analyse the zero controls (time zero) for dissolved oxygen (paragraph 33) or preserve them for later chemical analysis by precipitation with MnCl_2 (manganese (II) chloride) and NaOH (sodium hydroxide).

31. Incubate the remaining parallel BOD bottles at the test temperature (15-20°C), keep in the dark, and remove from the incubation area at appropriate time intervals, (e.g. after 5, 15 and 28 days as a minimum) and analyse for dissolved oxygen (paragraph 33).

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

Dissolved oxygen determination

33. Determine the concentration of dissolved oxygen using a chemical or electrochemical method which is recognised nationally or internationally.

⁽¹⁾Mercury (II) chloride (HgCl_2) is a very toxic substance which should be handled with suitable precautions. Aqueous wastes containing this chemical should be disposed of appropriately; they should not be discharged directly into the waste water system.

DATA AND REPORTING**Treatment of Results**

34. Record analytical results on the attached data sheets (Annex 5).
35. Calculate the BOD as the difference of the oxygen depletion between a blank and a solution of test compound 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. The degradation is defined as the ratio of the biochemical oxygen demand to either, preferably, the theoretical oxygen demand (ThOD) or the chemical oxygen demand (COD) and expressed as a percentage (see paragraph 36).
36. Calculate the biodegradation values for each sampling time for both test and reference compounds using one or other of the equations:

$$\% \text{ biodegradation} = \frac{\text{mg O}_2/\text{mg tested substance}}{\text{mg ThOD}/\text{mg tested substance}} \times 100$$

$$\% \text{ biodegradation} = \frac{\text{mg O}_2/\text{mg tested substance}}{\text{mg COD}/\text{mg tested substance}} \times 100$$

where:

ThOD = theoretical oxygen demand (calculation, Annex 3)

COD = chemical oxygen demand, determined experimentally.

Note: Sometimes the two ways of calculation (percentage of the ThOD or percentage of the COD) do not give the same results; it is preferable to use ThOD, since some chemicals are not fully oxidised in the COD test.

37. Illustrate the course of the degradation test graphically in a diagram (see example in section on "Validity and interpretation of results". If there are sufficient data, calculate the lag phase (t_l) and the time (t_{50}) to reach 50 per cent removal from the end of the lag phase from the biodegradation curve.
38. If specific analysis is used (optional), state the percentage of primary degradation as the percentage of specific compound removal within the test period (corrected for analytical blanks).

Test Report

39. The test report must contain the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Test conditions:

- location and description of the sampling site: pollutional and nutrient status (colony count, nitrate, ammonium, phosphate if appropriate);

- characteristics of the sample (date of sampling, depth, appearance, temperature, salinity, DOC (optional), delay between collection and use in the test);
- method used (if any) for ageing of the seawater;
- method used for pre-treatment (filtration/sedimentation) of the seawater;
- method used for the COD determination (if performed);
- method used for the oxygen measurements;
- dispersion procedure for substances which are poorly soluble under the test conditions;
- method used for determining the number of heterotrophs in the seawater (plate count method or alternative procedure);
- method used for determining DOC in seawater (optional);
- method used for specific analysis (optional);
- other optional methods used to characterise the seawater (ATP measurements, etc.).

Results:

- analytical data reported on a data sheet (as attached, Annex 5);
- the course of the degradation test 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 per cent of the final oxygen uptake caused by oxidation of the test compound (t_{50}). The lag phase may be estimated graphically as shown in the attached figure, or conveniently taken as the time needed for 10 per cent degradation;
- per cent degradation measured after 28 days.

Discussion of results.

Validity and interpretation of results

40. The blank respiration should not exceed 30 per cent of the oxygen in the test bottle. If it is not possible to meet this criterion using freshly collected seawater, the seawater must be aged (stabilized) before use.

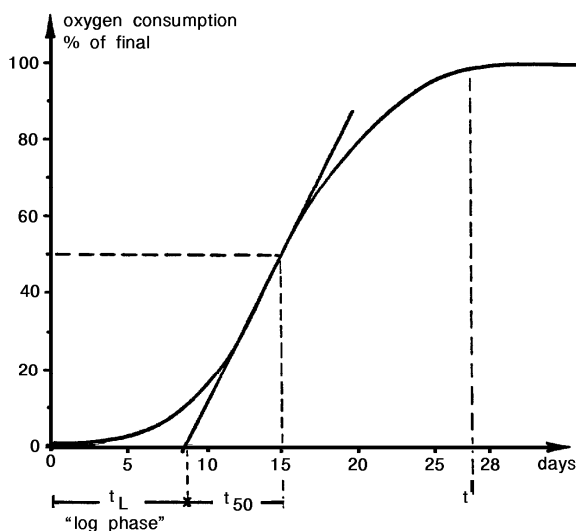
41. The possibility that nitrogen-containing compounds may affect the results should be considered.

42. Results obtained with the reference compounds sodium benzoate and aniline should be comparable to the results obtained in the ring-test (3) (paragraph 9). If results obtained with reference compounds are atypical, the test should be repeated using another seawater sample.

43. 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.

44. Owing to the relatively high test concentrations as compared with most natural systems, and consequently an unfavourable ratio between the concentrations of test substance and other carbon sources, the method is to be regarded as a preliminary test which can be used to indicate whether or not a substance is easily biodegradable. 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 in order for this to be established.

An example of a theoretical degradation experiment illustrating a feasible way of estimating the values of t_L (length of "lag phase") and t_{50} , time interval (starting at t_L), needed to reach 50% of the final oxygen uptake caused by oxidation of the test substance, is given below:



LITERATURE

- (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) OECD, Paris (1992). Test Guideline 301 D.

ANNEX 1DETERMINATION OF ORGANIC CARBON IN SEAWATERSHAKE FLASK METHOD

For the determination of organic carbon of a water sample, the organic compounds in the sample are oxidized to carbon dioxide using generally one of the following three techniques:

- wet-oxidation by persulphate/UV-irradiation;
- wet-oxidation by persulfate/elevated temperature (116-130°C);
- combustion.

Evolved CO₂ is quantified employing infra-red spectrometry or titrimetry. Alternatively, CO₂ is reduced to methane, which is quantified on a flame ionization detector (FID).

The persulfate/UV-method is commonly used for the analysis of "clean" water with low content of particulate matter. The latter two methods can be applied to most kinds of water samples, the persulfate/elevated temperature-oxidation being most suitable for low-level samples, and the combustion technique being applicable for samples with non-volatile organic carbon (NVOC) content well above 1 mg C/l.

Interferences

All three methods are dependent on eliminating or compensating for inorganic carbon (IC) present in the sample. Purging of CO₂ from the acidified sample is the most frequently used method to eliminate the IC, although this also results in a loss of volatile organic compounds (1). The complete elimination or compensation of IC must be ensured for each sample matrix, and volatile organic carbon (VOC) must be determined in addition to NVOC dependent on the sample type.

High chloride concentrations result in decreased oxidation efficiency using the persulfate/UV-method (2). Application of an oxidation reagent modified by the addition of mercury (II) nitrate may, however, 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 sample analysed 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.

Highly turbid samples as well as samples containing particulate matter may be incompletely oxidized when employing the persulfate/UV-method.

An example of a suitable method

Non-volatile organic carbon is determined by oxidation with persulfate/UV-irradiation and subsequent quantification of evolved CO₂ employing non-dispersive infra-red spectrometry.

The oxidation reagent is modified in accordance with the suggestions given in (2) as described in the manufacturer's manual:

- a) 8.2 g HgCl₂ and 9.6 g Hg(NO₃)₂.H₂O are dissolved in several hundred millilitres of low carbon concentration reagent water.
- b) 20 g K₂S₂O₈ are dissolved in the mercuric salt solution.

- c) 5 ml HNO₃ (conc.) are added to the mixture.
- d) the reagent is diluted to 1000 ml.

The interference from chloride is removed using a 40 µl sample volume for 10 per cent chloride and 200 µl sample volume for 1.9 per cent chloride. Samples of high chloride concentrations and/or larger sample volumes can be analysed according to 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.

LITERATURE

- (1) ISO, Water quality - determination of total organic carbon. Draft International Standard ISO/DIS 8245, January 16, 1986.
- (2) American Public Health Association, Standard Methods for the Estimation of Water and Wastewater. American Water Works Association & Water Pollution Control Federation, 16th edition, 1985.

Also of interest (gives a description of an autoanalysis system):

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

ANNEX 2**BIODEGRADATION IN SEAWATER
SHAKE FLASK METHOD
DATA SHEET****1. LABORATORY:****2. DATE AT START OF TEST:****3. TEST SUBSTANCE:**

Name:

Stock solution concentration: mg/l as chemical

Initial concentration in medium, t₀: mg/l as chemical

: mg DOC/l

4. SEAWATER:

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,
ageing, etc.):

Microbial colony count - original sample: colonies/ml

- at start of test: colonies/ml

Other characteristics:

5. CARBON DETERMINATIONS:

Carbon analyser:

	Flask no.		DOC after n days (mg/l)				
			0	n ₁	n ₂	n ₃	n _x
Test: nutrient-fortified seawater with test substance	1	a ₁					
		a ₂					
		mean, C _{a(t)}					
	2	b ₁					
		b ₂					
		mean, C _{b(t)}					
Blank: nutrient-fortified seawater without test substance	1	c ₁					
		c ₂					
		mean, C _{c(t)}					
	2	d ₁					
		d ₂					
		mean, C _{d(t)}					
	mean, C _{bl(t)} = $\frac{C_{c(t)} + C_{d(t)}}{2}$						

6. EVALUATION OF RAW DATA:

Flask No.	Calculation of results	% Degradation after n days				
		0	n ₁	n ₂	n ₃	n _x
1	$D_1 = 1 - \frac{C_{a(t)} - C_{b(t)}}{C_a - C_{b(0)}} \times 100$	0				
2	$D_2 = 1 - \frac{C_{b(t)} - C_{b(t)}}{C_a - C_{b(0)}} \times 100$	0				
Mean (*)	$D_t = \frac{D_1 + D_2}{2}$	0				

* D₁ and D₂ 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.

7. ABIOTIC DEGRADATION (optional)

	Time (days)	
	0	t
DOC conc. (mg/l) in sterile control	C _{s(0)}	C _{s(t)}

$$\% \text{ abiotic degradation} = \frac{C_{s(0)} - C_{s(t)}}{C_{s(0)}} \times 100$$

ANNEX 3

CALCULATION OF THE THEORETICAL BIOCHEMICAL OXYGEN DEMANDCLOSED BOTTLE METHOD

The ThOD of the substance $C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ of the molecular weight MW is calculated according to:

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

This calculation implies that C is mineralised 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

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

Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolysed.

Sulphur is assumed to be oxidised to the state of +6.

Example:

Sodium n-dodecylbenzenesulphonate $C_{18}H_{29}SO_3Na$, MW = 348

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

In the case of nitrogen-containing substances the nitrogen may be eliminated as ammonia, nitrite, or nitrate corresponding to different theoretical biochemical oxygen demands.

$$ThOD_{NO2} = \frac{16[2c + \frac{1}{2}(h - cl) + 3s + \frac{3}{2}n + \frac{5}{2}p + \frac{1}{2}na - o]}{MW}$$

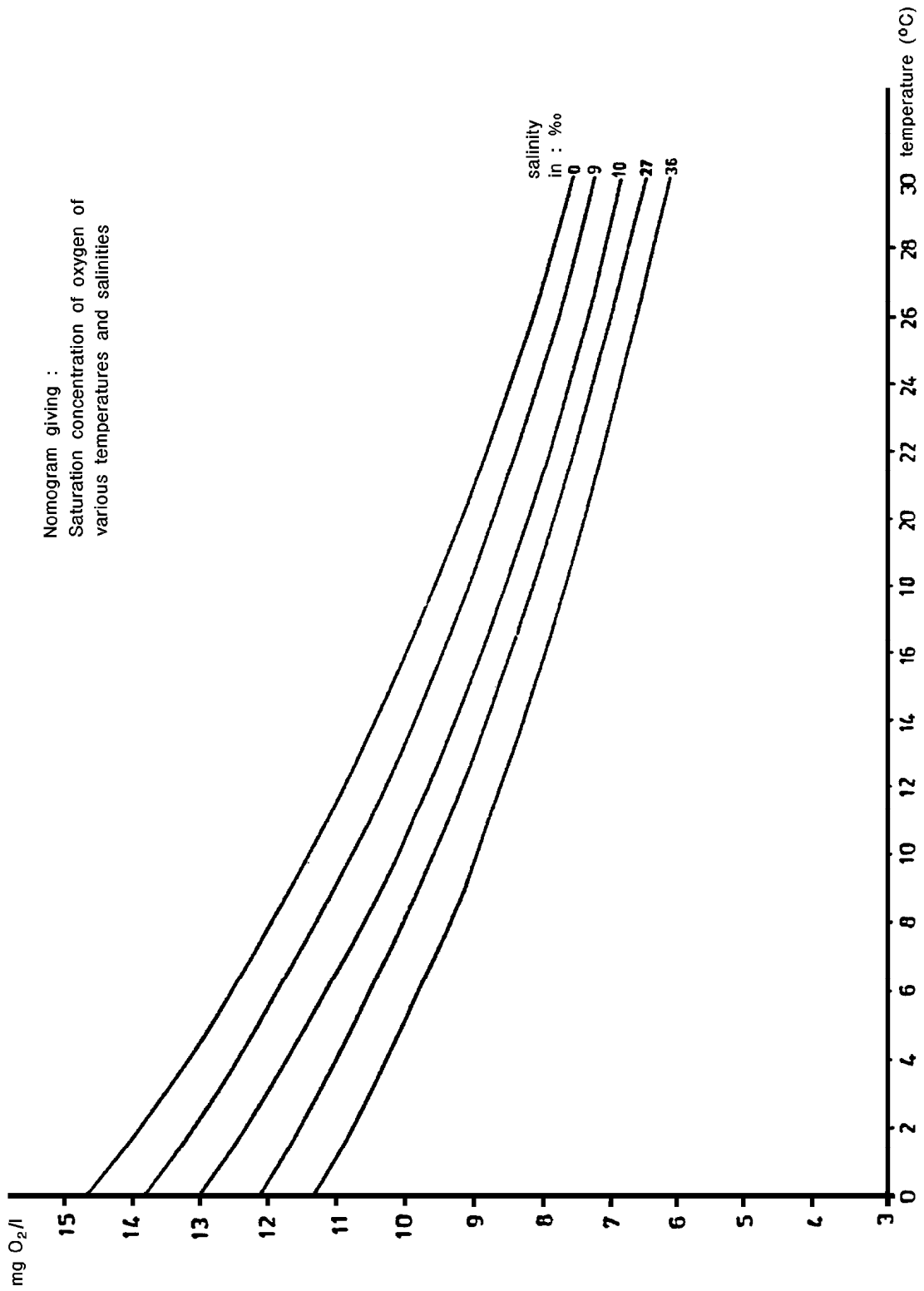
$$ThOD_{NO_3} = \frac{16[2c + \frac{1}{2}(h - cl) + 3s + \frac{5}{2}n + \frac{5}{2}p + \frac{1}{2}na - o]}{MW}$$

Suppose full nitrate formation had been observed by analysis in the case of a secondary amine:

$(C_{12}H_{25})_2NH$, MW = 353

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

ANNEX 4



ANNEX 5**BIODEGRADATION IN SEAWATER
CLOSED BOTTLE METHOD
DATA SHEET****1. LABORATORY:****2. DATE AT START OF TEST:****3. TEST SUBSTANCE:**

Name:
Stock solution concentration: mg/l
Initial conc. in seawater medium: mg/l
ThOD or COD: mg O₂/mg test substance

4. SEAWATER:

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

Pre-treatment prior to testing (e.g. filtration, sedimentation, ageing, etc.):

Microbial colony count - original sample: colonies/ml
- at start of test: colonies/ml

Other characteristics:

5. TEST MEDIUM:

Temperature after aeration: °C

O₂ concentration after aeration and
standing before start of test: mg O₂/l

6. **DO DETERMINATION:**

Method: Winkler/electrode

	Flask no.		mg O ₂ /l after n days			
			0	5	15	28
Test: nutrient - fortified seawater with test substance	1	a ₁				
	2	a ₂				
	Mean blank	$m_t = \frac{a_1 + a_2}{2}$				
Blank: nutrient - fortified seawater, but without test substance	1	c ₁				
	2	c ₂				
	Mean test	$m_b = \frac{c_1 + c_2}{2}$				

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

7. **DO DEPLETION: % DEGRADATION (%D):**

	DO depletion after n days		
	5	15	28
$(m_b - m_t)^{(1)}$			
$\%D = \frac{(m_b - m_t)^{(1)}}{\text{test substance (mg/l)} \times \text{ThOD}} \times 100$			

⁽¹⁾ This assumes that m_{b(0)} = m_{t(0)}, where

m_{b(0)} = blank value at day 0,
 m_{t(0)} = test substance value at day 0.

If m_{b(0)} does not equal m_{t(0)}, use (m_{t(0)} - m_{t(x)}) - (m_{b(0)} - m_{b(x)}), where

m_{b(x)} = blank value at day x,
 m_{t(x)} = test substance value at day x.