REGIONAL SEAS

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Estimation of the toxicity of pollutants to marine phytoplanktonic and zooplanktonic organisms

Reference Methods For Marine Pollution Studies No.44

Prepared in co-operation with

FAO IAEA

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources and has requested the development of regional action plans. The Regional Seas Programme at present includes ten regions and has over 120 coastal States participating in it. (1), (2)

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment and of its resources, and of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies are being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (3). The Methods recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared in co-operation with the relevant specialized bodies of the United Nations system as well as other organizations and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (ISO) is followed as closely as possible.

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, of analytical instrumentation and the actual need of the users. In order to facilitate these revisions the users are invited to convey their comments and suggestions to:

Marine Environmental Studies Laboratory
International Atomic Energy Agency
International Laboratory of Marine Radioactivity
2, Avenue Prince Hereditaire Albert
MC98000 MONACO

which is responsible for the technical co-ordination of the development, testing and intercalibration of Reference Methods.

PREFACE cont'd.

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(1) UNEP: Achievements and planned development of the UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No. 1 UNEP, 1982.

(2) P. HULM: A Strategy for the Seas. The Regional Seas Programme: Past and Future UNEP, 1983.

(3) UNEP/IAEA/IOC: Reference Methods and Materials: A programme of comprehensive support for regional and global marine pollution assessments. UNEP 1988.

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This issue of the Reference Method for Marine Pollution Studies No. 44 was prepared in co-operation with the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA). It includes comments received from a number of scientists who reviewed the method and particularly the conclusions of the FAO/UNEP Consultation meeting on the toxicity of selected substances to marine organisms held in Villefranche-sur-mer, France, 13-14 October, 1988. The assistance of all those who contributed to the preparation of this reference method is gratefully acknowledged.

CONTENTS

1. SCOPE AND FIELD OF APPLICATION

This reference method describes procedures for estimating the toxicity of pollutants to marine phytoplankton and zooplankton. Procedures are given for estimating the median effective concentrations (EC50) of toxicants to phytoplankton, and the minimum algistatic concentration (MAC-5). For zooplankton, procedures are given for determining median lethal concentrations.

2. REFERENCES

REISH, D.J. and OSHIDA, P.S. (1986) Manual of Methods in Aquatic Environment Research. Part 10. Short-term static bioassays. FAO Fish. Tech. Pap. (No.247) 62pp.

UNEP/FAO/IAEA (1989) Test of the acute lethal toxicity of pollutants to marine fish and invertebrates. Reference Methods for Marine Pollution Studies No.43, UNEP.

WARD, G.S. & PARRISH, P.R.(1982) Manual of Methods in Aquatic Environment Research. Part 6. Toxicity Tests. FAO Fish. Tech. Pap.(No.185) 23pp., Rome, FAO.

3. PRINCIPLES

Organisms are exposed to each of a range of concentrations of the test substance. For phytoplankton, the median effective concentration (EC50) is estimated in terms of the number of individuals surviving, the biomass of individuals surviving, or the chlorophyll content of the individuals surviving. For zooplankton, the median lethal concentration (LC50) is estimated by conventional log-probit analysis of the mortality data.

4. REAGENTS

4.1 For phytoplankton tests

Filtered sea water

Distilled water

KNO_3

KH_2PO_4

$ZnSO_4.7H_2O$

$CuSO_4.5H_2O$

$CoSO_4.7H_2O$

$Na_2MoO_4.2H_2O$

Sodium EDTA

Crystalline vitamin B12

Biotin

Thiamine hydrochloride

Toxicant stock solution

4.2 For zooplankton tests

Filtered sea water

Toxicant stock solution

NOTE: Maintenance of zooplankton in the laboratory frequently requires the maintenance of phytoplankton cultures as a food source.

5. APPARATUS

5.1 For phytoplankton tests

Autoclave

Erlenmeyer flasks (various sizes)

Microscope

Constant temperature room or enclosure

Fluorescent lighting system

Haematocytometer

Automatic particle counter (optional)

Orbital shaker (optional)

Fluorometer (optional)

Logarithmic-probability graph paper

Salinometer or salinity probe of adequate accuracy

Spectrophotometer (optional)

5.2. For zooplankton tests
Culture vessels of 10-50 litre capacity

Microscope

Constant temperature room or enclosure

Pasteur pipettes

Glass test vessels of appropriate size

Logarithmic-probability graph paper

6. MAINTENANCE OF STOCK PHYTOPLANKTON CULTURES

6.1 Laboratories undertaking toxicity tests with phytoplankton should establish and maintain stock cultures of the required species. It is generally easier and more reliable to obtain the initial cultures from a reputable biological supplier than to attempt to isolate pure cultures from field collections.

6.2 Preparation of stock solution and sea water

NOTE: Any established culture medium may be used, but the medium described in this section is recommended as an example.

6.2.1 Solution A - sea water: Use sea water from an uncontaminated source, filtered through a 0.45 um filter to remove algae, bacteria and particulate matter. Artificial sea water may be used if necessary. Synthetic sea water may be made up from the following formula:

Chemical	Quantity (g)
NaCl	23.926
Na_2SO_4	4.008
KCl	0.677
$NaHCO_3$	0.196
KBr	0.098
H_3BO_3	0.026
NaF	0.003
$MgCl_2.6H_2O$	10.83
$CaCl_2.2H_2O$	1.52
$SrCl_2.2H_2O$	0.02
Distilled water	to 1000g

Adjust the salinity to 30-32 by diluting with double-distilled water.

NOTE: Many experienced workers have reported that 0.22 μm filters are superior to 0.45 μm filters, and their use is recommended if they are available.

6.2.2. Solution B - nitrate stock solution: Dissolve 5g KNO_3 in 1 litre of distilled water. This solution may be stored in a refrigerator for up to 4 months.

6.2.3. Solution C - phosphate stock solution: Dissolve 0.68g KH_2PO_4 in 1 litre of distilled water. This solution may be stored in a refrigerator for up to 4 months.

6.2.4 Solution D - Trace metal mixture:

(a) Dissolve 30mg $ZnSO_4.7H_2O$; 25mg $CuSO_4.5H_2O$; and 20mg $CoSO_4.7H_2O$ in 1 litre of distilled water.

(b) Dissolve 5g $FeCl_3.6H_2O$ and 2g $MnSO_4$ in 1 litre of distilled water. Ignore any precipitate.

(c) Dissolve 25mg $Na_2MoO_4.2H_2O$ in 1 litre distilled water.

(d) Dissolve 50g of sodium ethylene diamine tetra acetate (EDTA) in 1 litre of distilled water.

NOTE: These solutions must be made up separately.

6.2.5 Solution E - vitamin B12: Dissolve 10mg crystalline vitamin B12 in 100ml distilled water. Store in freezer until required.

6.2.6 Solution F - Biotin: Dissolve 10mg biotin in 100ml distilled water. Store in freezer until needed.

6.2.7 Solution G - Thiamine hydrochloride: Dissolve 100mg thiamine hydrochloride in 100ml distilled water. Store in freezer until needed.

6.3 **Preparation of final solutions**

To prepare the stock solutions for use, carry out the following operations.

6.3.1 Obtain the appropriate quantity of sea water (solution A).

6.3.2 Take 1 ml of solution B and add it to 999ml distilled water.

6.3.3 Take 1 ml of solution C and add it to 999ml distilled water.

6.3.4. Take 100ml of EDTA solution (section 6.2.4.) and 10ml of each of the other solutions described in 6.2.4., and add them to 800 ml distilled water. Adjust to pH 7.5 with dilute NaOH and add distilled water to 1000 ml.

6.3.5 Thaw solution E, take 1 ml and re-freeze the remainder. Add this 1ml to 99ml distilled water.

6.3.6 Thaw solution F, take 1 ml and re-freeze the remainder. Add this 1ml to 99ml distilled water.

6.3.7 Thaw solution G, take 1 ml and re-freeze the remainder. Add this 1ml to 99ml distilled water.

NOTE: These diluted solutions should be made up immediately before use, as they do not keep. When the required amount of culture medium has been made up, the diluted solutions must be discarded. The diluted heavy metal mixture, however, can be stored in a refrigerator for up to 4 months.

6.4 **Preparation of culture medium**

NOTE: In this section, the solutions referred to are the diluted solutions prepared as described in 6.3 from the original stock solutions described in 6.2.

NOTE: Many experienced workers recommend the addition of silicate to the culture medium, particularly when diatoms are to be cultured. In this case, it is recommended that sodium silicate is added to the culture medium in a quantity equivalent to 3% of the total dissolved salts.

6.4.1 To each litre of sea water, add:

10 ml nitrate solution (B), (6.3.2)

1 ml trace metal mixture solution (D), (6.3.4).

6.4.2 Autoclave this mixture.

6.4.3. When cool, aseptically add to this mixture the following:
1 ml vitamin B12 solution (E), (6.3.5).
1 ml biotin solution (F), (6.3.6).
1 ml thiamine hydrochloride solution (G) (6.3.7).

NOTE: Vitamin solutions must NOT be autoclaved.

6.4.4 Autoclave the required quantity of phosphate solution (C) (6.3.3).

6.4.5 Add 10ml of phosphate solution to each litre of autoclaved culture medium.

6.4.6 If any precipitate occurs, it may be necessary to filter the medium through sterile 0.45 μm filters before use (but see note to paragraph 6.2.1)

6.4.7 The medium is now ready for use. To avoid contamination, all glassware should be autoclaved and aseptic technique employed when handling the medium.

6.5 Dispense the medium into 500ml Erlenmeyer flasks and inoculate each flask aseptically with 5-15ml of algal culture. Place a cotton wool plug in the mouth of each flask. Incubate at 20°C under cool white fluorescent lighting (e.g. "Grow-lux") at a light intensity of approximately 2000 lux, on an orbital shaker at 100 revolutions per minute.

NOTE: Different species of algae may require different lighting conditions, and the lighting conditions required will also depend upon the size and characteristics of the experimental vessels and of the room or enclosure in which the experiment is carried out. The conditions recommended should produce good results, but investigators are strongly advised to carry out preliminary experiments to determine precisely the optimum lighting conditions for the species under investigation and for the particular circumstances of their experiments.

NOTE: Agitation of algal cultures with the aid of an orbital shaker is strongly recommended and is the preferred method. If an orbital shaker is not available, alternative means of agitation of the cultures may be employed, for example manual shaking at least twice per day or agitation by aseptic aeration. In the latter case, the use of UV light for the sterilisation of the air in the enclosure is strongly recommended.

6.6 To maintain a continuous supply of healthy cells, aliquots of each stock culture should be transferred weekly, under aseptic conditions, to freshly-prepared culture medium. Each culture should be checked periodically by microscopic examination to ensure that they have not been contaminated with other species. Contaminated cultures should be rejected.

7. TEST PROCEDURE FOR PHYTOPLANKTON

7.1 Two tests are carried out in each case: a preliminary range-finding test and a definitive test.

7.2 Tests may be carried out using the same medium as is used for maintenance of stock cultures. Some workers, however, prefer to use sea water as the test medium; and some to use sea water supplemented with certain nutrients. Since different species have different characteristics and environmental requirements, no specific recommendation is given here. Whichever medium is chosen should be clearly stated. Ward & Parrish (see References, section 2) describe a suitable test medium.

7.3 The physical conditions (lighting and temperature) should be the same as the stock culture conditions, unless the purpose of the experiment requires that they be different.

7.4 Where the experimental conditions (test medium or physical conditions) differ from those of the stock culture (other than the presence of the toxicant), cultures of the test algae should be acclimated to the test conditions for an appropriate period (usually a few days before the experiment begins. This is done by inoculating culture flasks as described in section 6, but incubating them under the conditions that will prevail during the experiment. Inoculations of the experimental cultures should be made from the acclimated cultures.

7.5 All glassware should be thoroughly cleaned, acid-washed, rinsed in distilled water and autoclaved before use.

7.6 The preliminary test is carried out in exactly the same way as the definitive test, except that the range of toxicant concentrations is different.

7.7 Select the range of toxicant concentrations. At least 5 concentrations, plus a control, are required.

For the preliminary test, successive toxicant concentrations should be one order of magnitude apart, e.g. 0.01, 0.1, 1, 10, 100 mg l^{-1}. The purpose of the preliminary test is to establish the approximate toxicant concentration which is effective.

In the definitive test, more closely-spaced concentrations should be selected, and they should follow on approximately logarithmic series. For example, if the preliminary test indicates that the effective concentration of the toxicant is about 1 mg l^{-1}, the range 0.1, 0.2, 0., 1, 2, 5, 10 mg l^{-1} may be selected.

7.8 Prepare a minimum of three replicate series of test vessels. For the definitive test, five replicates are preferred.

7.8.1 The test vessels should normally be 250 ml Erlenmeyer flasks containing 100 ml of test medium. Smaller flasks may be used if necessary, but appropriate adjustments should be made pro rata to the quantities contained. Small flasks are unlikely to produce consistent results.

7.8.2. Determine the required volume of inoculum in each test vessel according to the procedure described in 7.9. This volume should not exceed 10 ml.

7.8.3. Determine the required volume of test medium. This is given by subtracting the volume of inoculum from the volume of test solution required.

7.8.4. Add the required volume of test medium to each test vessel, and afterwards the required volume of inoculum to each test vessel.

7.8.5 Add the required amount of toxicant and agitate the flask.

NOTE: The toxicant should be added from a concentrated stock solution, and should be a very small volume (< 1ml). If the volume of toxicant solution required exceeds 1 ml, a more concentrated toxicant stock solution should be used.

7.9 Determination of the inoculum volume

7.9.1 Determine the algal cell concentration in the stock solution. This may be done using a haemocytometer or, if available, an automatic particle counter. In either case, the instructions supplied with the apparatus must be followed exactly.

NOTE: Not all automatic particle counters are suitable for use with algal cells. They must be fitted with the appropriate accessories and specifically set up for algal cells. Otherwise they will produce erratic and erroneous results. In case of doubt, consult the manufacturer.

7.9.2 Calculate the required volume of inoculum. If it exceeds 10ml, the stock culture must be concentrated.

Example: Assume the stock culture contains 34000 cells/ml, the test flasks will contain 100ml medium, and the required initial cell density is 10,000 cells/ml. The total number of cells required is $100 \times 10{,}000 = 10^6$. The required inoculum volume is therefore

$$\frac{10^6}{34000} = 29.4(\text{ml}).$$

This volume is too large (since a large proportion of the test medium will be made up of exhausted medium from the stock culture). Therefore the stock culture must be concentrated.

NOTE: The figure of 10 000 cells per ml as the required initial cell density is used as an example and is not a recommendation.

7.9.3 The stock culture is concentrated by centrifuging, discarding the supernatant, and resuspending the algal cells into a smaller volume of medium.

7.9.4 Estimate the cell density of the concentrated stock culture as described in 7.9.1. Re-calculate the required volume of test medium. Repeat the process until the required volume of inoculum is less than 10ml.

7.10 Seal each flask with a cotton wool plug. Note the time. Place the flasks on an orbital shaker at 100 rpm, under appropriate conditions of lighting and temperature. NOTE: If an orbital shaker is not available, alternative methods of agitation are acceptable (see section 6.5).

7.11 Leave the flasks for 96 hours.

7.12 After 96 hours, determine the density of algal cells in each flask, using the same method as was used to determine the density of algal cells in the stock cultures.

NOTE: In many circumstances, it is advantageous to make more frequent observations of algal population density and to plot the growth curve. For example, it sometimes happens that the effect of the test substance on the algal population is to alter the shape of the growth curve, while total algal growth at 96 hours is unaffected. It is therefore recommended that where possible, algal population densities should be estimated several times during the 96 hour experimental period, and the resultant growth curves plotted and compared.

For similar reasons, it is frequently advantageous to use experimental periods other than 96 hours (longer or shorter as the case may be depending upon the purposes of the experiment, the characteristics of the algal species used and the toxic properties of the test substance).

7.13 If the biomass of the algal cells is to be determined, use one of the following procedures.

7.13.1 Filter the contents of each flask through a pre-weighed 0.45µm filter. Dry at 60°C for several hours. Weigh each filter paper. Subtract the weight of the filter paper from the weight of the filter paper and algal cells to determine the biomass of algal cells.

7.13.2 Alternatively, centrifuge the contents of each flask, discard the supernatant, and wash the cells three times with distilled water. Repeat the centrifuging between each washing. Transfer the biomass to a pre-weighted crucible and dry at 60°C for several hours. Subtract the weight of the crucible from the weight of the crucible and biomass.

7.14. **Estimation of chlorophyll-a content**

Two methods of estimating chlorophyll-a are widely used, the spectrophotometric method and the fluorimetric method. The spectrophotometric method is more accurate, but if a spectrophotometer is not available the fluorimetric method is usually adequate.

The spectrophotometric method is described in section 7.14.1, and the fluorimetric method in section 7.14.2.

NOTE: In sections 7.14.1. and 7.14.2, only a general outline of these methods can be given. This is because of differences in the precise characteristics of the analytical instruments found in different laboratories. Also, the characteristics of chlorophyll vary between different species of algae and according to the expoerimental conditions. Investigators will therefore need to optimise the method according to their own conditions. It is recommended that investigators consult an appropriate manual (e.g. "A Practical Handbook of Sea Water Analysis" by J.D.H. Strickland and T.R. Parsons, Fisheries Research Board of Canada, 1972, in particular section IV.3).

7.14.1. Take 1. ml of algal suspension and add 9 ml of acetone. Leave for 10 minutes (shaking the mixture at regular intervals to encourage extraction), centrifuge the solution and read the optical density of the supernatants in the spectrophotometer at 660 nm using a blank of 90% acetone in sea water.

An alternative procedure which avoids centrifuging is to read the optical density at 660 nm and at 750 nm non-selective background absorption). The chlorophyll content can then be estimated from $OD_{660} - OD_{750}$.

The spectrophotometer should be calibrated by constructing a calibration curve using known quantities of chlorophyll. In practice, pure chlorophyll is difficult to obtain and a useful alternative is to construct the calibration curve using the procedure described with suspensions containing known quantities of algal cells.

7.14.2. The recommended fluorimetric method is that of Strickland and Parsons (1972), based on the use of the Turner (or similar) fluorometer (see note to section 7.14). The fluorometer should be fitted with the high-sensitivity door, the F.4T4.-BL lamp, and glass filters (Wratten 47B or Corning CS.5-60 for the excitation light, and Corning CS.2-64. for the emitted light.

Extracts of chlorophyll, obtained as described in 7.14.1, are measured in the fluorometer with the scale zeroed for each door opening against a tube of 90. acetone. The quantity of chlorophyll-a present, in micrograms per litre, is

F X R

where F is the door factor and R is the reading on the fluorometer.

To determine the "door factor", the fluorometer must be calibrated against a spectrophotometer. Extract sufficient chlorophyll to give a reading of about 50 on door 3 of the fluorometer. Determine the concentration of chlorophyll in this extract by a spectrophotometric method (see 7.14.1). The door factor is given by

$$F = C / 3$$

where C is the concentration of chlorophyll, and R is the fluorimeter reading.

NOTE: This procedure calculates the factor for door 3. In practice, it is often useful to know the door factors for doors 10 and 30, so that a wider range of chlorophyll concentrations can be measured. To determine the door factors for doors 10 and 30, dilute the chlorophyll extract with 90% acetone to give known lower concentrations of chlorophyll. This concentration should be sufficient to give readings greater than 50 on the fluorimeter with the door to be calibrated (10 or 30). The door factors can then be calculated in the same fashion, using the expression above.

7.15 If the minimum algistatic concentration (MAC-5) is to be determined, proceed as follows.

7.15.1. Reseal the flasks and incubate under the same conditions for one more day.

7.15.2. Centrifuge the contents of each flask and discard the supernatant. Resuspend the algal cells in fresh test medium without the toxicant. Repeat this process twice more to remove toxicant.

7.15.3. Inoculate fresh flasks containing 100ml of test medium with a suspension of cells from each of the initial test flasks, to give a final cell concentration of approximately 10000 cells/ml, using the method described in 7.9.

7.15.4 Seal each flask with a cotton wool plug and re-incubate under the same experimental conditions for a further 9 days.

7.15.5. After 9 days, estimate the algal cell density in each flask using the procedure used previously.

NOTE: MAC-5 cannot be estimated if the test cultures have been used for biomass and/or chlorophyll determinations.

8. DATA ANALYSIS IN PHYTOPLANKTON TESTS

Three alternative methods of data analysis are presented.

8.1. Tabulate the data as shown in Table 1.

Table 1. Tabulation of data from a toxicity test with phytoplankton, showing the number of cells per ml of test medium after 96 hours.

Test concentration, mg l^{-1}	Replicates					Mean	Standard Deviation	Mean % reduction compared w/control
	1	2	3	4	5			
Control	115000	122000	114000	126000	119000	119200	4970	-
0.1	114000	116000	108000	119000	116000	114600	4090	3.85
0.32	98000	101000	99000	110000	96000	100800	5450	19.3
0.56	28000	47000	33000	41000	39000	37600	7335	68.5
1.0	16000	21000	18000	19000	17000	18200	1924	84.7
1.3	4000	7000	6000	8000	6000	6200	1483	94.8

NOTE: In the example given, data for the number of phytoplankton cells per ml of test medium are used. Exactly the same procedures are used for data on algal biomass or chlorophyll-a concentration.

8.2. To determine the concentrations of toxicant which exert a significant effect on the phytoplankton, compare each mean value with the control mean value using the Student Newman-Keuls test. This test is described in detail by Reish and Oshida (see references in section 2).

8.3. To determine the 96h EC50, calculate the percentage reduction in each concentration, compared with the control value. Using logarithmic-probability paper, plot a graph of percentage reduction (probability scale) against concentration of test substance (logarithmic scale). Proceed to paragraph 11.4. and continue the analysis.

NOTE: The data analysis method described in section 11 for zooplankton is exactly the same as the method used here, except that data for percentage reduction in cell numbers, biomass or chlorophyll-a are used in place of mortality data. The final result is expressed as the 96h EC50 (median effective concentration) instead of 96h LC50 (median lethal concentration).

NOTE: In some cases, exposure to pollutants may cause an increase in cell density, biomass or chlorophyll-a. This should not occur in a definitive test if the preliminary test has been properly carried out and interpretedcorrectly. However if it does occur, investigators should consider carrying out properly-designed biostimulation tests, since the present test cannot be used to investigate biostimulation.

8.4 Determination of minimum algistatic concentration (MAC-5).

NOTE: This is only possible if the additional procedure described in 7.5 has been carried out.

8.4.1 For each replicate at each test concentration, calculate

$$K = \frac{\log_2 \frac{N_1}{N_2}}{t},$$

where K = the growth constant,
N_1=number of cells at the end of the recovery period,
N_2=number of cells at the beginning of the recovery period,
t = the length of the recovery period in days (generally 9 days).

NOTE: Remember that the cells were exposed to the toxicant for 5 days, therefore the final result will be the 5-day minimum algistatic concentration (MAC-5).

8.4.2. Calculate the mean values of K and their standard deviations for each toxicant concentration.

8.4.3. Compare each mean value of K with the mean control value of K, using the Student Newman-Keuls test (see Reish & Oshida, references section 2), or Student's t-test.

8.4.4. The MAC-5 lies between the lowest concentration of test substance which gives a K-value which is significantly different from the control value, and the highest concentration whose K-value is not significantly different from the control.

NOTE: It is obvious that the MAC-5 cannot be determined accurately, since it must always lie between two of the concentrations actually tested. Therefore tests to determine the MAC-5 should include closely-spaced concentrations, and should be expressed thus: "The MAC-5 is less than x mg l^{-1}, or "The MAC-5 lies between x and y mg l^{-1}".

9. MAINTENANCE OF ZOOPLANKTON CULTURES

9.1. Although a limited range of species may be available from commercial suppliers, zooplanktonic species are best obtained by collection from the field. It is frequently found that in a collection of zooplankton, a single species is overwhelmingly predominant.

9.2. Transport the collected material to the laboratory in aerated containers, taking care to protect the animals from large changes in temperature and strong sunlight.

9.3. Examine the individuals under a microscope, and select individuals of the species required. Transfer them, using a pipette, to a separate container.

9.4 **Culture conditions**

Many zooplankton species have been successfully cultured in the laboratory, but the precise requirements of many species are not known. Investigators should undertake a literature search to discover if the specific requirements of the species they wish to culture are known. Some experimentation will be required to establish optimal culture conditions. Laboratories which wish to undertake toxicity testing with zooplankton must first successfully establish stock cultures of the species concerned. The following general recommendations may be useful, and further information can be found in the references listed in Section 2.

9.4.1. The culture vessels should be between 10 l and 50 l capacity.

9.4.2. Filtered natural sea water from an uncontaminated source is the preferred medium. Synthetic sea water may be used, but many species do not thrive in freshly-prepared synthetic sea water. If synthetic seawater is used, it must be conditioned for at least a week before use.

9.4.3. Continuous aeration must be provided.

9.4.4. Feeding requirements vary between species and some experimentation will be required to determine the optimum quality and quantity of food. The feeding requirements of some commonly-used species are:

Acartia tonsa: feeds on a mixture of four species of phytoplankton - Skeletonema costatum, Thalassiosire pseudonara, Isochrysis galbana and Rhodomonas Baltica (see section 6 for details of phytoplankton culture methods).

Tigriopus sp.: feed on a suspension of finely-powdered fish food such as
Tetra Min.

Tisbe sp.: feed on Duniallela.

Mysidaceae sp.: feed on newly-hatched Artemia nauplii.

9.4.5. The temperature should be maintained constant, and be appropriate for the species concerned, taking into account its natural habitat.

9.4.6. Cultures of zooplankton need regular care and should be examined daily. Excess food and other detritus should not be allowed to accumulate, and the entire culture should be transferred periodically to a new vessel with fresh sea water.

9.4.7. Samples of the zooplankton culture should be examined periodically under the microscope to ensure that they are not contaminated with other zooplankton species. Contaminated cultures should be rejected.

9.4.8. In many species, laboratory cultures lose vitality after a few generations, so it will be necessary to establish new cultures periodically.

10. TEST PROCEDURE FOR ZOOPLANKTON

10.1. The test vessels should be made of glass, and sufficiently large to contain 100ml of test solution. It is advantageous if they are of such a size and shape to be placed on the stage of a binocular microscope, for examination of the animals during the test.

10.2. Select the range of test concentrations. At least 5 concentrations,plus a control, are required. The test concentrations should follow an approximately logarithmic series, e.g. 0.1, 0.2, 0.5, 1, 2, 5 mg l^{-1}

10.3. Prepare the test vessels by adding the appropriate quantity of toxicant stock solution to filtered sea water, so that each vessel contains 100ml of test solution of the required concentration.

10.4. Select the animals for the test.

10.4.1. A minimum of 10, and preferably 20, animals should be used in each test vessel. An equal number of animals should be used in each test vessel.

10.4.2. Examine each animal individually under a microscope. Reject any animal which appears diseased, injured or damaged, moribund, or in any way abnormal. Also reject females carrying eggs.

NOTE: Because of the short generation time of zooplankton species, it is possible to obtain more animals at the end of the experiment than were present at the beginning. Therefore it is particularly important to exclude gravid females from toxicity tests.

NOTE: If more than 20% of the animals are rejected owing to disease, injury or other abnormality, it is likely that the entire culture is unhealthy and animals from that culture should not be used for experiments.

10.4.3. Place the required number of animals into each test vessel,starting with the lowest concentration of toxicant. Note the time. The experiment has now begun.

10.4.4. Place the test vessels in a suitable room or enclosure where the temperature and other physical conditions are controlled at the required levels.

10.4.5. Decide upon the duration of the experiment. It will be assumed that this is 96 hours.

NOTE: Toxicity tests of duration less than 96 hours are of very limited value, as they tend to produce misleading conclusions. Ideally, toxicity tests should continue as long as possible. However, since zooplanktonic organisms have short generation times, there is a possibility that animals will reproduce during the test, thus invalidating the results. Therefore 96h is recommended as a standard duration.

10.4.6. After 96 hours, count and record the number of animals surviving in each test vessel.

NOTE: Particular care must be taken in deciding whether or not an animal is dead. Living animals will normally be moving, but animals which appear immobile should be examined carefully under a microscope. If any movement of the appendages, digestive tract or internal organs are observed, the animal should be counted as a survivor.

10.4. Estimation of 24h LC50, 48h LC50 or 72h LC50 may be made if the number of animals surviving in each test vessel at these times is recorded. The data analysis procedure is exactly the same as described in section 11 for the 96h LC50, except that mortality data corresponding to the earlier observation times are used.

NOTE: Some investigators may wish to determine median survival times (LT50) at each concentration of test substance, and construct a toxicity curve. The same experimental procedure is employed, but observations on mortality must be made more frequently.

11. DATA ANALYSIS FOR ZOOPLANKTON TESTS

The 96 hour LC50 and its confidence limits are estimated as follows.

11.1. Tabulate the data as shown in Table 2. The percentage mortality in each test concentration is required, and is equal to

$$\frac{N-x}{N} \times 100$$

where N = the initial number of animals in the test vessel and x = the number of animals still alive after 96 hours.

11.2. Using logarithmic-probability graph paper, plot a graph of percentage mortality (probability scale against concentration of test substance (logarithmic scale as shown in Fig. 1. Ignore zero and 100% mortality values.

Table 2: A hypothetical example of the data required to estimate the 96h LC50 of a test substance, and to test the goodness of fit of the probit line shown in Fig. 1.

Concentration of test substance mg l^{-1}	No. of Animals Tested	Observed % Mortality 96h	Expected % Mortality	Contribution to chi^2
1	10	10	12	0.004
2	10	30	24	0.02
5	10	50	45	0.01
10	10	60	64	0.007
20	10	80	79	0.001
50	10	100	-	-
			Total chi^2 =	0.042

Since the number of animals tested per concentration is 10, the chi^2 value is 0.042 x 10 = 0.42. The number of concentrations tested is 6, excluding the control (note that 0% and 100% values are not used in plotting the line). The number of degrees of freedom is therefore (6-2) = 4. Using Table 3, it can be seen that the initial value of chi^2 with 4 degrees of freedom is 9.49. Since 0.42 < 9.49, the line shown in Fig. 1 is a good fit and the 96h LC50 and its confidence limits can be estimated from this line.

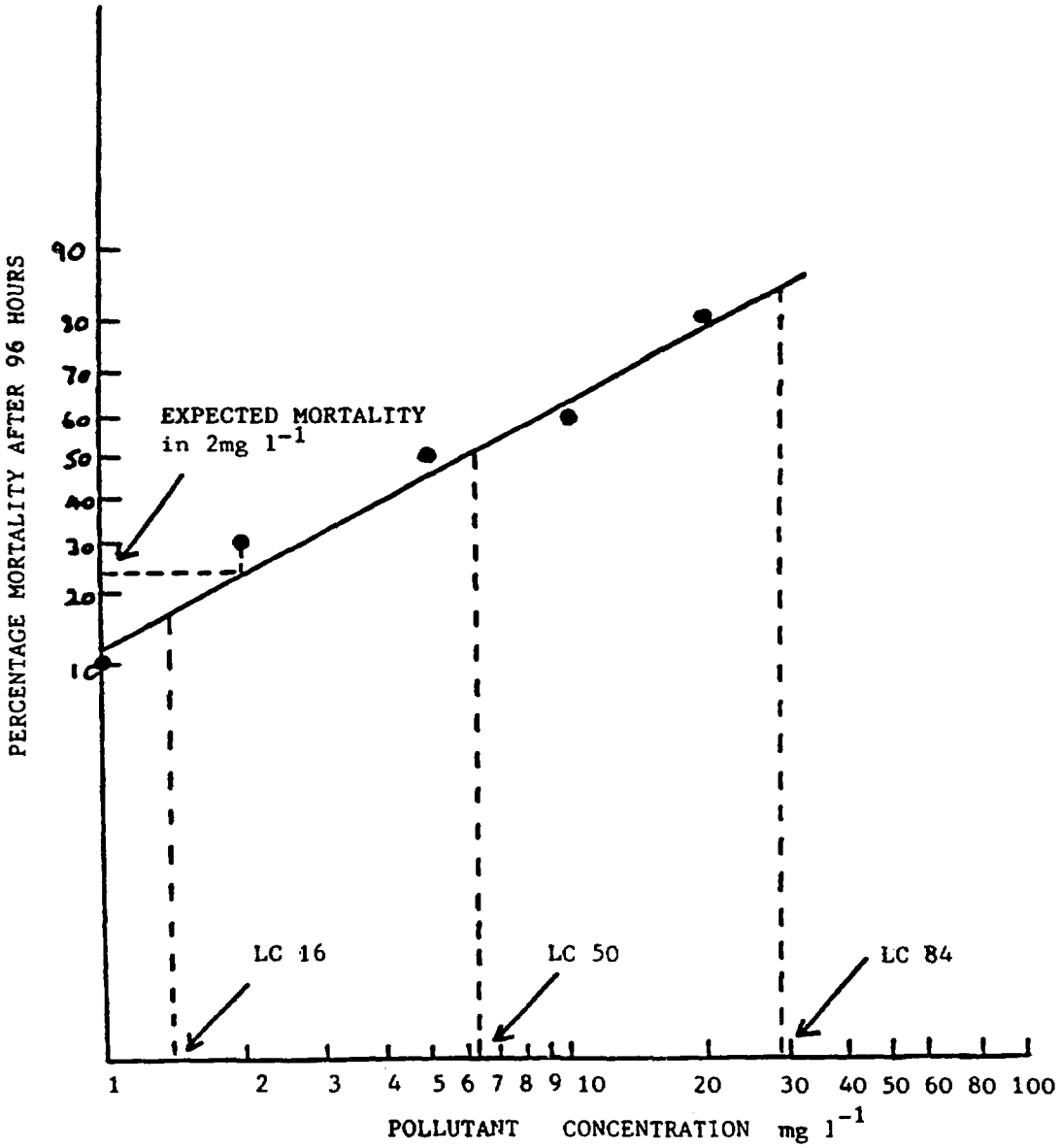


FIGURE 1: Estimation of the 96h LC50 value using the data given in Table 2. The diagram shows how values of LC16, LC84 are read off from the line and how the expected mortalities at each concentration are determined.

NOTE: LC50 values cannot be calculated by this procedure unless the graph includes at least one point exceeding 50% mortality, and at least one point below 50% mortality.

11.3. Fit a line, by eye, to the points on the graph and test its goodness of fit by the procedure described in 11.8.

NOTE: In most cases the number of points on the graph will be small. If there are fewer than four points on the graph, the LC50 value cannot be calculated accurately, and the investigator should consider repeating the experiment with more closely-spaced pollutant concentrations. Because the number of points on the graph is small, it is essential that the goodness of fit of the line is tested by the procedure described in 11.8. If the line is not a good fit, further lines must be drawn and tested until a line of good fit is obtained.

11.4. If the line is a good fit, read off values for LC16, LC50 and LC84 as shown in Fig.1. Calculate S, the slope function of the line, according to the formula

$$S = \frac{\frac{LC84}{LC50} + \frac{LC50}{LC16}}{2} .$$

11.5. Determine the value of N. For zooplankton tests, this value is defined as the total number of animals tested at concentrations whose expected mortalities were between 16% and 84%. For phytoplanktopn tests, this value is defined as the total number of replicates tested at concentrations whose expected effects were between 16% and 84% reduction in growth, population density or chlorophyll-a concentration.

NOTE: In Fig. 1. there are five points on the graph. The expected mortality at concentration 1mg l^{-1} is less than 16%. The expected mortalities at the other four concentrations all lie between 16% and 84%. Since 10 animals were tested at each concentration, N = 10 x 4 = 40.

11.6 Calculate f, where

$$f = \text{antilog } \frac{2.77 \log S}{N} = S\ 2.77/\ N.$$

11.7 Calculate the upper and lower 95% confidence limits of the LC50. The LC50 value is read from the line (see Fig. 1), provided that the line is a good fit (see 11.8).

Upper confidence limit = LC50 x f
Lower confidence limit = LC50 / f.

11.8 Testing the goodness of fit of a probit line

NOTE: In some cases, a line of good fit cannot be constructed. If, after following this procedure, a line of good fit cannot be obtained, the LC50 value cannot be determined by this method. In such cases other methods of data analysis are required, or modification of the experimental procedure should be considered.

11.8.1. Tabulate the observed mortality values and the expected mortality values for each point on the graph (see Table 2).

NOTE: The observed values are the actual mortality values recorded during the experiment. The expected values are those which are read, at the appropriate concentration, from the fitted line on the graph as shown in Fig. 1.

11.8.2. Using the nomograph in Fig. 2, determine the contribution to chi^2 of each point on the graph (see Table 2).

11.8.3. Determine the sum of the individual contributions to chi^2. In zooplankton tests, multiply this number by the number of animals tested per concentration, i.e. if 10 animals were tested at each concentration, multiply the total chi^2 value by 10. For phytoplankton tests, multiply the sum of the individual contributions to chi^2 by the number of replicates tested per concentration (NOT by the number of algal cells tested per concentration). (See Table 2).

NOTE: Although the number of animals tested at each concentration should be the same, it occasionally happens that this is not the case. In this circumstance, calculate the ratio:

$$\frac{\text{Total Number of animals tested}}{\text{Number of concentrations tested}}$$

and multiply the total chi^2 by this value. When making this calculation, do not include the controls.

11.8.4. Determine the number of degrees of freedom: This is given by N-2, where N is the number of concentrations tested. Do not include controls in this calculation, or concentrations in which no animals have died, or (for phytoplankton tests, concentrations where no significant reduction in comparison with the control value occurred.

11.8.5. Using Table 3, read the value of chi2 for the appropriate number of degrees of freedom.

11.8.6. If the chi^2 value of the line is equal to or greater than the appropriate value given in Table 3, the line is not a good fit. In this case, construct a new line and repeat the procedure for testing its goodness of fit. Repeat this procedure until you obtain the line which has the minimum value of chi^2. Continue the analysis as described in 11.4.

NOTE: In a few cases, a line of good fit cannot be constructed and the analysis cannot proceed. Repeat the entire experiment, if necessary using a different range of concentrations of test substance.

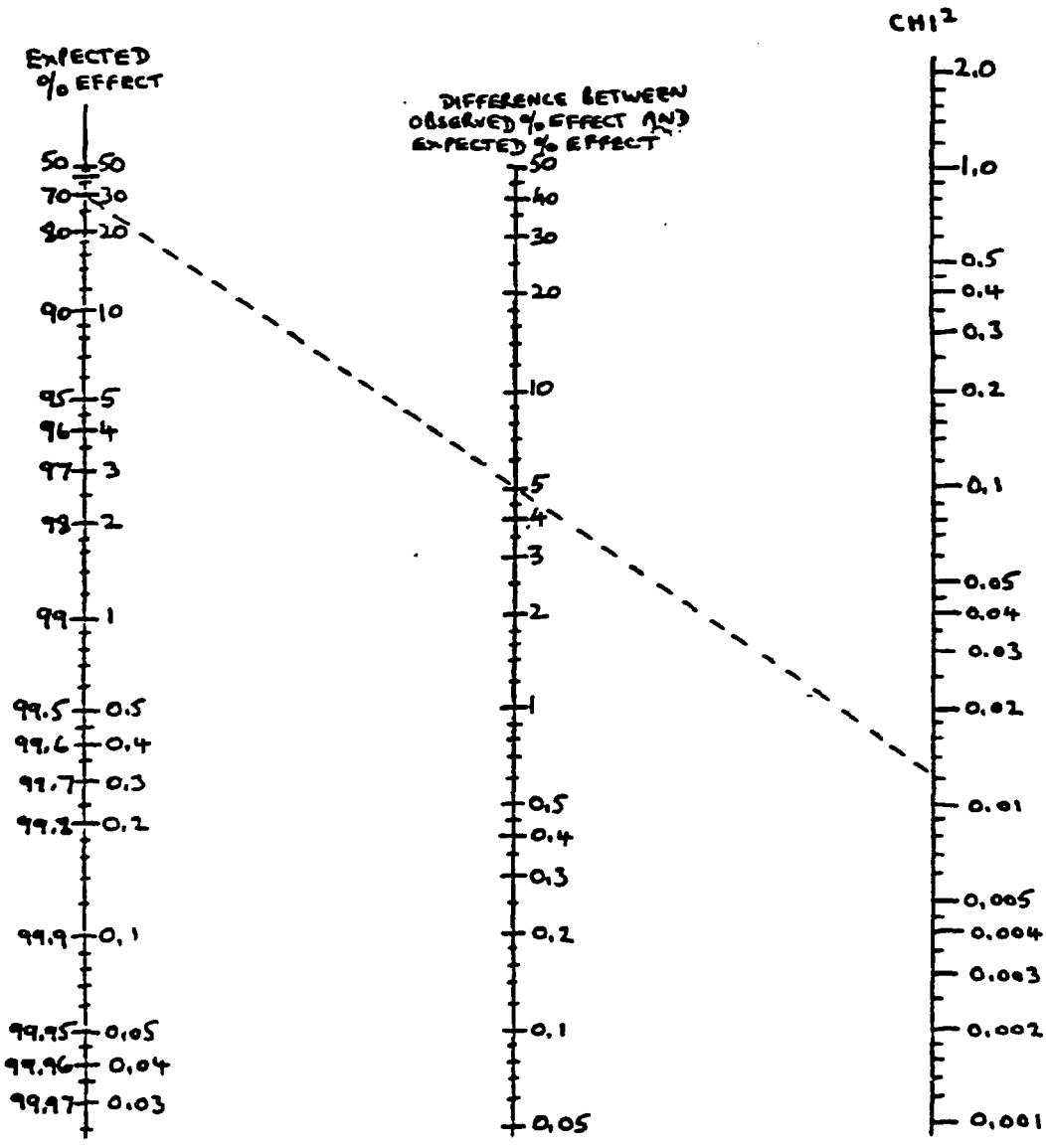
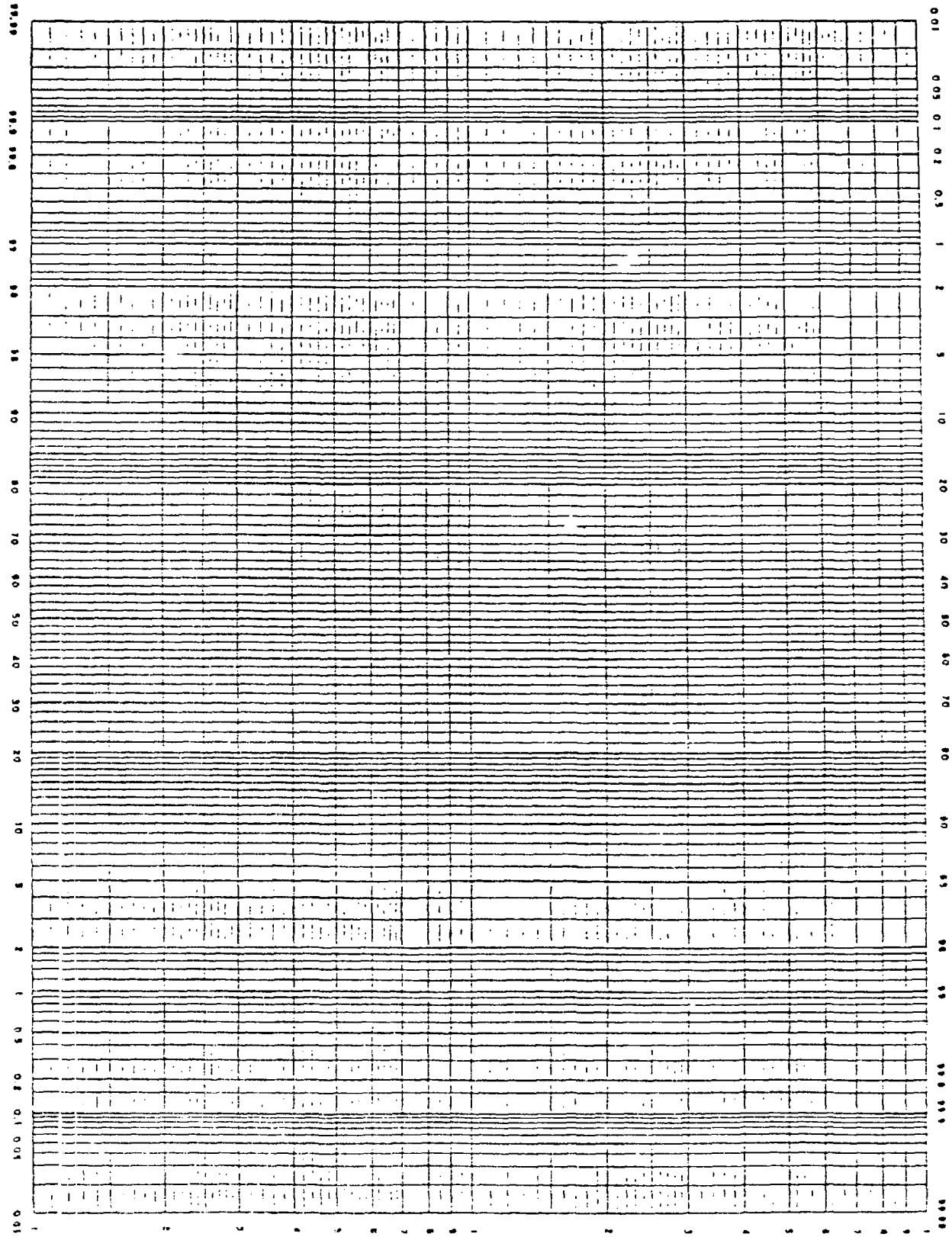


FIGURE 2. Nomograph for the determination of chi^2 (see 11.8). To use the nomograph, construct a straight line (or use a ruler) which connects the expected % effect value (on the left-hand axis) to the value of the difference between the observed and expected values (on the central scale). Project this line forward until it intersects the chi^2 axis on the right of the diagram. The chi^2 value is read from the intersection.

In the diagram, one example is shown. If the expected mortality is 30%, and the observed mortality is 25%, the chi2 value is 0.012. Remember that this chi^2 value must be multiplied by the number of animals tested per concentration, and added to the chi^2 values calculated in similar fashion for the other points on the graph.

Table 3. Critical values of chi2 for testing the goodness of fit of a probit line by the procedure described in 11.8 (if the chi2 value of the line is equal to or greater than the value shown in the table for the appropriate number of degrees of freedom, te line is not a good fit and must be rejected)

Degrees of freedom	chi2
1	3.84
2	5.99
3	7.82
4	9.49
5	11.1
6	12.6
7	14.1
8	15.5
9	16.9
10	18.3



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