REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME SEPTEMBER 1989

Test of the acute lethal toxicity of pollutants to marine fish and invertebrates

Reference Methods For Marine Pollution Studies No.43

Prepared in co-operation with

FAO IAEA

UNEP 1989

NOTE: This document has been prepared in co-operation between the Food and Agriculture Organization of the United Nations (FAO), the International Atomic Energy Agency (IAEA) and the United Nations Environment Programme (UNEP) under project FP/5102-88-03.

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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 draft issue of the Reference Method for Marine Pollution Studies No. 43 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, 13-15 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 the measurement of the acute lethal toxicity of pollutants to marine animals (fish and invertebrates) by a static (non-continuous flow) method. Procedures are given for the determination of the toxicity curve (survival time-concentration relationship) and for the estimation of median lethal concentrations (LC50). The method is suitable for use with fish and macro-invertebrate species. It is not suitable for planktonic organisms nor for determining the toxicity of oil, oil dispersants or other petroleum products. Those methods are described in Reference Methods Nos. 44 and 45, respectively.

2. REFERENCES

FAO (1977) Manual of Methods in Aquatic Environment Research. Part 4. Bases for selecting biological tests to evaluate marine pollution. FAO Fish. Tech. Pap. (No.164) 31pp.

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., Rome, FAO.

WARD, G.S. and 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

The test animals are exposed, in groups of approximately ten, to each of several concentrations of the pollutant. The animals are observed, at intervals, for several days, the test solutions being renewed regularly. A record is maintained of the survival times of individual animals exposed to each concentration of pollutant. The median survival time of each group of animals is determined from a graphical plot of the raw data after a log-probability transformation. Median survival times and their confidence limits are plotted against concentrations of test substance to give a toxicity curve. Additionally the same experimental data can be used to estimate the median lethal concentration (LC50) of the test substance to the animals after different periods of exposure.

NOTE: In some countries experiments on living animals are subject to certain legal restrictions. Investigators should ensure that they hold any necessary licences or permissions before commencing any experiments.

4. REAGENTS

4.1 Sea Water

This water may be natural sea water, artificial sea water made from commercial sea salt, or synthetic sea water made up by dissolving the appropriate chemicals in demineralised water. It is strongly recommended that natural sea water be used whenever possible. Commercial sea salts (e.g."Instant Ocean") are acceptable as second choice. Synthetic sea water (see section 4.1.3) should only be used if there is no alternative. Investigators should be aware that such synthetic sea water may be contaminated with trace heavy metals even when high-grade chemicals are used.

4.1.1 Natural sea water: Depending upon its origin, natural sea water may require settling and/or filtration before use. It should be drawn from an uncontaminated area and should not have been in contact with metallic pipes or pumps.

4.1.2 Artificial sea water made from commercial sea salts (as supplied for aquaria): Should be made up exactly according to the manufacturer's recommendations (some adjustment to salinity may be made, according to local conditions). Sufficient sea water should be made up to last for the entire duration of the experiment or experiments. Before use, the sea water should be aerated vigorously for at least 48 hours to ensure equilibration with air, and the pH should stabilise at 8.1 - 8.2. After equilibration, any precipitate which has formed should be removed by settling or filtration. Avoid contact with metallic implements.

NOTE: Many species do not thrive in freshly-prepared artificial sea water. Ideally, the water should be "conditioned" in a recirculating laboratory aquarium for as long as possible before it is used for experimental purposes.

4.1.3 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

Dissolve the substances in the order shown in distilled water, and make up to 1000g. Note that this formula gives sea water of S=35 salinity. If other salinities are required, adjust the amount of distilled water pro rata, e.g., for sea water of S=37 salinity, dissolve the above chemicals with distilled water to 946g.

Analytical grade reagents should be used. Sufficient sea water should be made up to last for the entire duration of the experiment or experiments. Equilibration should be carried out as in section 4.1.2. Any precipitate formed should be removed by settling or filtration. Avoid contact with metal implements.

NOTE: Sea water made by this method is very expensive.

NOTE: Many species do not thrive in freshly-prepared synthetic sea-water. Ideally, the water should be "conditioned" in a recirculating laboratory aquarium for as long as possible before it is used for experimental purposes.

4.2 **The test substance**

The test substance should be obtained in as pure a form as possible, preferably analytical reagent grade.

4.2.1 If the test substance is readily soluble in water the stock solution should be made up in distilled deionised water. If the test substance is stable in solution a single stock solution may be made up at the start of the experiment. Where the test substance is not stable in solution (e.g. subject to oxidation or other chemical or biological transformation) fresh stock solution should be made up immediately before each use.

4.2.2 If the test substance is an organic substance which is not readily soluble in water (e.g. certain pesticides), aqueous stock solutions can be obtained by first dissolving the test substance in a small quantity of a suitable solvent. Acetone ethanol or propylene glycol are recommended as these have low toxicity to aquatic organisms.

Weigh out the required quantity of test substance. Dissolve it in a small but known quantity of the solvent. Add this solution, slowly, to distilled water, preferably using a magnetic stirrer to aid rapid mixing. Check the solution carefully for any signs of precipitation, and reject any solutions in which precipitation occurs. If no precipitation occurs, make up the solution to the required volume with more distilled water.

NOTE: Where an organic solvent is used to make an aqueous solution of a chemical, two controls are required in the experiment. One should contain no test substance or solvent. The other must contain a quantity of solvent which is equivalent to the concentration of solvent in the vessel containing the highest concentration of test substance tested.

4.2.3 The strength of the 'stock solution' required will vary according to the conditions of the experiment, but generally a concentration of 1000 mg l^{-1} (active molecule or ion) is appropriate for water-soluble test substances, and 1-10 mg l^{-1} for pesticides.

NOTE: Where the range of concentrations to be tested includes high values, addition of the stock solution may significantly increase the volume of fluid in the experimental vessels, and decrease the salinity of the test solution; therefore a more concentrated stock solution is required.

4.3 **Analytical reagents**

It is highly desirable (see sections 7.6, 7.7) that the concentration of test substance in the test vessels be measured periodically during the experiment. If this is to be done, the appropriate analytical reagents will be required.

5. APPARATUS

5.1 **The test vessels**

A sufficient number of test vessels of appropriate size (see section 7.1) is required. They should preferably be constructed of glass, and be thoroughly cleaned, acid-washed and rinsed with sea-water before use. Polythene, PVC or fibreglass vessels may be used if glass is unavailable. However, caution should be exercised when using new containers, as toxic substances are frequently released from them into the water. New plastic or fibreglass vessels should be soaked several times in new sea water for several weeks before their first use. Some test materials readily adsorb to plastic surfaces, resulting in lower test concentrations during the experiment. The shape of the vessels is not important, but they should allow for a sufficient depth of water in relation to the size of the animals, and should be large enough to accommodate the required water volume while leaving sufficient dead space at the top to prevent the animals from being able to jump out.

5.2 Aeration equipment

Each test vessel should be gently aerated during the test with a single air stone connected to a suitable oil-free air supply.

5.3 A constant-temperature room or enclosure of appropriate size.

5.4 Logarithmic-probability graph paper.

5.5 Log-log graph paper (desirable but not essential).

5.6 Random number tables or a random number generator.

5.7 A pH meter.

5.8 A salinometer.

5.9 If required, apparatus for the determination of the concentration of the test substance in the test solutions.

6. THE EXPERIMENTAL ANIMALS

6.1 Depending upon the purpose of the experiment, any available fish or macro-invertebrate species which can be maintained in the laboratory for the required period may be used. They should be obtained from an uncontaminated area or from aquaculture and as far as possible should all be of similar size. If age and sex can be determined they should also be of similar age and of the same sex.

NOTE: Detailed instructions on the care and maintenance of many commonly-used test animals are given in the references listed in section 2.

6.2 Acclimation to laboratory conditions

6.2.1. Animals should be transferred to stock tanks in the laboratory as soon as possible after capture. They should be kept in quarantine for at least 14 days.

6.2.2 During this period they may gradually be acclimated to the temperature of the experiment provided that the temperature change does not exceed $1^{o}C$ per day.

NOTE: Some species are particularly sensitive to temperature changes and may require longer acclimation periods.

6.2.3. Most animals can be maintained satisfactorily under conditions of continuous low-level illumination. Avoid unpredictable alterations in lighting conditions e.g. sudden switching on and off of the lights. If possible a consistent photoperiod regime should be employed e.g. 14 hours light 10 hours dark (the photoperiod regime should roughly correspond to that in the natural environment when the animals were collected). During the "dark" phase it is useful to facilitate observation to maintain a very low level of illumination rather than complete darkness.

NOTE: Some species are particularly sensitive to the level of illumination and variations in the illumination level. Failure to maintain organisms under laboratory conditions is sometimes due to an unsatisfactory light regime.

6.2.4. During the quarantine period the animals should be observed closely and an attempt made to establish normal feeding on a suitable diet.

6.2.5. At the end of the quarantine period if more than 20% of the animals have died show visible signs of disease or appear moribund the entire batch should be rejected and fresh animals sought for the experiments

6.3. **Selection of animals for experiments**

If the stock animals are judged generally acceptable discard any individuals which are moribund not feeding normally or showing signs of disease or any other abnormality.

6.4. **Discontinuation of feeding**

Animals selected for an experiment should not be fed for 48 hours prior to the start of an experiment.

6.5. **Weighing and measuring of animals**

A representative sample (minimum 30) of the test animals should be carefully weighed and measured and the results reported as mean values $\pm$ standard deviation, stating "n" (the number of animals measured).

7. THE EXPERIMENTAL CONDITIONS

7.1 **Volume of test solution**

Each experimental vessel should contain a minimum of 1 litre of test solution per gram of animal tissue. Preferably however, the volume of test solution should be between 2 and 3 litres per gram of animal tissue. Volumes lower than one litre per gram of tissue should be avoided.

7.2 Temperature

The temperature chosen for the experiment will depend upon the purpose of the experiment, and other factors. The temperature should not vary by more than $\pm 1^{o}C$ during the experiment, but variations of up to $\pm$ $2^{o}C$ are acceptable at nominal concentrations of $15^{o}C$ or above.

7.3 Salinity

The salinity of the test solutions should be adjusted to suit local conditions and/or the purpose of the experiment. Normally salinity fluctuation during the experiment should not exceed $\pm$ 1% of that chosen.

7.4 pH

Sea water is normally well-buffered and its pH should not vary during the experiment. The pH of the test solutions should be between 8.1 and 8.2 and should not vary by more than $\pm$ 0.05 during the experiment.

7.5 Duration of the experiment

Experiments should last a minimum of 96 hours. Experiments of shorter duration are of negligible value.

NOTE: Experiments should be continued for as long as possible, until mortalities have ceased or until a lethal threshold is established (i.e. until the toxicity curve becomes asymptotic to the time axis).

7.6 Renewal of test solutions

Renew the test solutions at least once every 24 hours. For test substances which are known to be chemically or biologically unstable, renewal is required at least twice every 24 hours. Failure to renew solutions invalidates the experiment.

Renewal is best carried out by having a duplicate set of test vessels to which the animals are transferred. The original containers can then be emptied, cleaned, and refilled. Test substance should be added immediately before the animals are transferred to the new containers.

If duplicate containers are not available, gently siphon out the old solution and replace immediately with fresh solution. Take great care to disturb the animals as little as possible, and avoid sudden temperature changes.

7.7 Measurement of the concentration of test substance

If possible, the concentration of test substance in each test vessel should be regularly measured by a suitable analytical procedure. Measure the actual concentration of test substance in each tank at the beginning of the experiment, and immediately before and immediately after each renewal of the test solution.

NOTE: Where concentrations of test substance decline by more than 20% between renewals of the test solutions, more frequent renewal is required. Results of the experiment should be expressed in terms of measured concentrations rather than nominal concentrations.

7.8 **Feeding of animals during experiments**

Normally animals should not be fed during experiments. However with small animals when the experiment continues for seven days or more, it is frequently desirable to offer food to avoid control mortalities due to starvation. In this case, a very small quantity of food should be offered once daily, about an hour before the test solutions are renewed.

NOTE: Excess food should never be allowed to remain in the test vessels after the solutions are renewed.

7.9 **Illumination**

The conditions of illumination should be the same as those used during the acclimation period.

7.10 **Selection of concentrations to be tested**

A minimum of six test concentrations, plus control, is normally required.

The concentration values selected obviously depend on the purpose of the experiment, and upon the characteristics of the test substance and the animals. The range of concentrations required may be estimated from previous experience, from information in the literature and from preliminary experimentation. The range of concentrations should be sufficient to cause most of the animals to die within periods ranging from a few hours to several days. Within the chosen range, it is frequently most useful to select experimental concentration values which follow a roughly logarithmic series. For example, if the range 1 to 100 mg l^{-1} is selected, the experimental concentrations chosen should be 1, 2, 5, 10, 20, 50 and 100 mg l^{-1}. If the range 0.5 to 5 mg l^{-1} is selected, the concentrations 0.5, 0.7, 1.0, 1.3, 2, 3 and 5 mg l^{-1} will be most useful.

NOTE: Where an organic solvent has been used to make an aqueous solution or an organic test substance, two controls are required (see section 4.2.2.).

7.11 **Number of animals required**

Each experimental vessel (and the control) should initially contain the same number of animals. A sample size of 20 is optimum, but samples of 10 may be routinely used.

NOTE: Sample sizes above 20 are uneconomical and add little to the accuracy of the final result. Sample sizes below 10 produce results of limited accuracy; in special cases a minimum of 8 animals per vessel may be used. Experiments involving fewer individuals per vessel are invalid.

7.12 **Termination of the experiment**

Continue the experiment for at least 96 hours. Preferably however, the experiment should continue for 7-14 days, or until a lethal threshold is apparent. If at any time control mortalities exceed 10%, the experiment must be terminated. If control mortalities exceeding 10% occur within 96 hours, the experiment is invalid. The use of correction factors for control mortality is not acceptable.

8. THE EXPERIMENTAL PROCEDURE

8.1 **Commencing the experiment**

8.1.1 Place the experimental vessels in the controlled-temperature enclosure. Fill each container with the required quantity of sea water (see section 7.1). Number each tank.

8.1.2 Install the aeration devices so that each vessel receives constant gentle aeration.

8.1.3 Check that the salinity, pH and temperature of the sea water are within acceptable limits. (see sections 7.2, 7.3, 7.4).

8.1.4 Randomly assign animals to each of the experimental vessels until each vessel contains the required number (usually 10) of animals. This is best done as follows.

Assume there are 8 vessels in the experiment. Using random number tables, or a random number generator, select a number between 1 and 8 inclusive. Place one animal from the stock tank into the vessel corresponding to the number selected. Repeat this process until each tank contains 10 animals. When a tank contains 10 animals, obviously no further animals are added even if that number is selected.

8.1.5. Assign a concentration of test substance to each vessel and label the vessel accordingly.

8.1.6. Add the required amount of stock solution of the test substance to each vessel to give the desired final concentration.

NOTE: The stock solution must be added slowly, with thorough mixing, to avoid exposing the animals to concentrated stock solution. Begin with the lowest concentration.

8.1.7 Note the time and date. The experiment has now begun.

8.1.8 Re-check the salinity and pH of the seawater in each vessel to ensure that the addition of the test substance has not altered their values.

8.1.9 If required, take a sample of water from each vessel (including control) for analysis of the concentration of test substance.

8.2 **Maintenance of the experiment**

IMPORTANT NOTE: All of the operations in section 8.2 must be carried out each day of the experiment, including weekends and holidays. Failure to do so will invalidate the experiment.

8.2.1 At least once daily, check that the temperature, salinity and pH of the water in each vessel are within the prescribed limits.

8.2.2 If required, take samples from each tank for analysis of the concentration of test substance, before and after each renewal of solution.

8.2.3 If required, feed the experimental animals with a minimal ration of suitable food one hour before the renewal of the test solutions.

8.2.4 Renew the test solutions at least once per day as described in section 7.6.

NOTE: In some circumstances (see sections 7.6 and 7.7) the test solutions must be renewed more frequently.

8.2.5 Ensure that any dead animals and all excess food, faecal matter and other detritus are removed from the tanks.

8.2.6 Check that the air supply to each tank is functioning correctly.

8.3 **Observations and data recording**

8.3.1 Record the measured values of temperature, salinity and pH for each vessel on each occasion they are measured.

8.3.2 Record occasions on which animals are offered food.

8.3.3. Record the values of concentration of test substance in each tank on each occasion they are measured.

8.3.4. Observations on mortality should be made at least at the following intervals (in hours): 0.25,0.5,0.75,1,2,4,8,14±2,24,33±3,48. Thereafter observations must be made at least once every 24. hours and where possible more frequently.

NOTE: For purposes of this experiment, death of an animal is defined as the permanent cessation of spontaneous movement and the failure to respond to a mild mechanical stimulus, e.g. touching with a glass rod. Particular care is required with certain species, e.g. some molluscs, in which death is difficult to recognise. Special criteria of death may be required for these species. In all cases the criterion of death used should be clearly stated.

NOTE: In many species a dead individual will be eaten by the surviving animals. Therefore the number of live animals should be counted and subtracted from the initial number of animals.

8.3.5 Note and report the occurrence of any precipitation which occurs in the test vessels.

NOTE: Many water-soluble substances tend to precipitate in sea water at concentrations which are used in toxicity experiments. The occurrence of precipitation does not necessarily invalidate the experiment, but it should be reported as it may be significant in the interpretation of the results.

8.3.6 If at any time control mortalities exceed 10%, the experiment must be terminated.

9. DATA ANALYSIS

9.1 Tabulation of mortality data

Construct a table of cumulative percentage mortality in each test vessel at each observation time, as in the example given in Table 1.

Table 1: An example of data from a toxicity test, tabulated to show the cumulative percentage mortality at each concentration as the experiment progresses

Time from start of experiment (h)	Concentration of test substance, mg l^{-1}						
	1	2	5	10	20	50	Control
1	0	0	0	0	0	0	0
2	0	0	0	0	0	10	0
4	0	0	0	0	10	20	0
8	0	0	0	0	30	40	0
16	0	0	0	10	40	60	0
24	0	0	10	20	50	70	0
36	0	0	20	30	60	80	0
48	0	10	30	40	60	90	0
72	0	20	40	50	70	100	0
96	10	30	50	60	80	100	0
120	20	40	60	80	90	100	0
144	40	60	80	100	100	100	0
168	50	70	100	100	100	100	0

9.2 Calculation of median survival times (LT50)

NOTE: LT50 can only be calculated if more than half of the animals in a particular concentration of test substance have died. If half or less than half of the animals in a vessel have died the data from that vessel must be rejected.

9.2.1 For each vessel in which more than half of the animals have died, plot a graph of cumulative percentage mortality (probability scale) against elapsed time (logarithmic scale) as shown in Fig. 1, using logarithmic-probability graph paper.

The example shown in Fig. 1. includes some features which commonly cause confusion to inexperienced investigators. Note that in this experiment, of the animals exposed to 20 mg l^{-1}, 10% (i.e. one of a sample of 10) had died after 4 hours, and 30% (i.e. three of a sample of 10) had died after 8 hours (see Table 1). These points are plotted on the graph vertically one above the other. If, however, the number of animals which die between observations exceeds 20% of the sample, the experiment is invalidated and must be repeated with more frequent observations.

Secondly, note from Table 1 that after 36 hours, 60% of the animals exposed to 20 mg l^{-1} had died. After 48 hours, no further mortalities had occurred. Thus 60% mortality (corrected to 55% as described in paragraph 9.2.1.(b)) at 36. hours is plotted on this graph. No point is plotted at 48 hours. Thus there should be one point on the graph for each animal in the sample.

Thirdly, note that in constructing the line fitted to the graph, particular attention should be paid to the points representing mortality values between 20% and 80%. Points representing mortality values <20% and >80% frequently deviate by a large amount from the fitted line.

Values of LT16, LT50 and LT84 (see 9.2.3) are shown on this diagram.

NOTE: A sample of logarithmic-probability graph paper is included at the end of this manual. It may be photocopied for use by investigators who have difficulty in obtaining paper from commercial sources.

When plotting data on a probability scale, the zero and 100% values cannot be plotted. One of two conventions may be employed:

a) Plot the actual cumulative percentage values obtained in the experiment, ignoring the 100% value, or

b) Reduce each percentage value by half the percentage value of one animal, and plot the corrected values. Thus, if there are ten animals in each vessel, each animal represents 10% of the sample and the corrected value is obtained by subtracting 5% from each value to be plotted. Thus cumulative percentage mortalities are plotted as 5%, 15%, 25%, instead of 10%, 20%, 30% etc., and the 100% value is corrected to 95%.

NOTE: Although the difference between the two methods is, in practice, small, method (b) is statistically correct. It provides a more accurate estimate of the true (population) median value. Note that as the sample size increases, the magnitude of the correction decreases. For example, if the sample size is 20, the correction is only 2.5% instead of 5%. This method also alows all the data points to be plotted on the graph.

9.2.2. Fit a straight line, by eye, to the points, paying particular attention to points in the middle section of the graph.

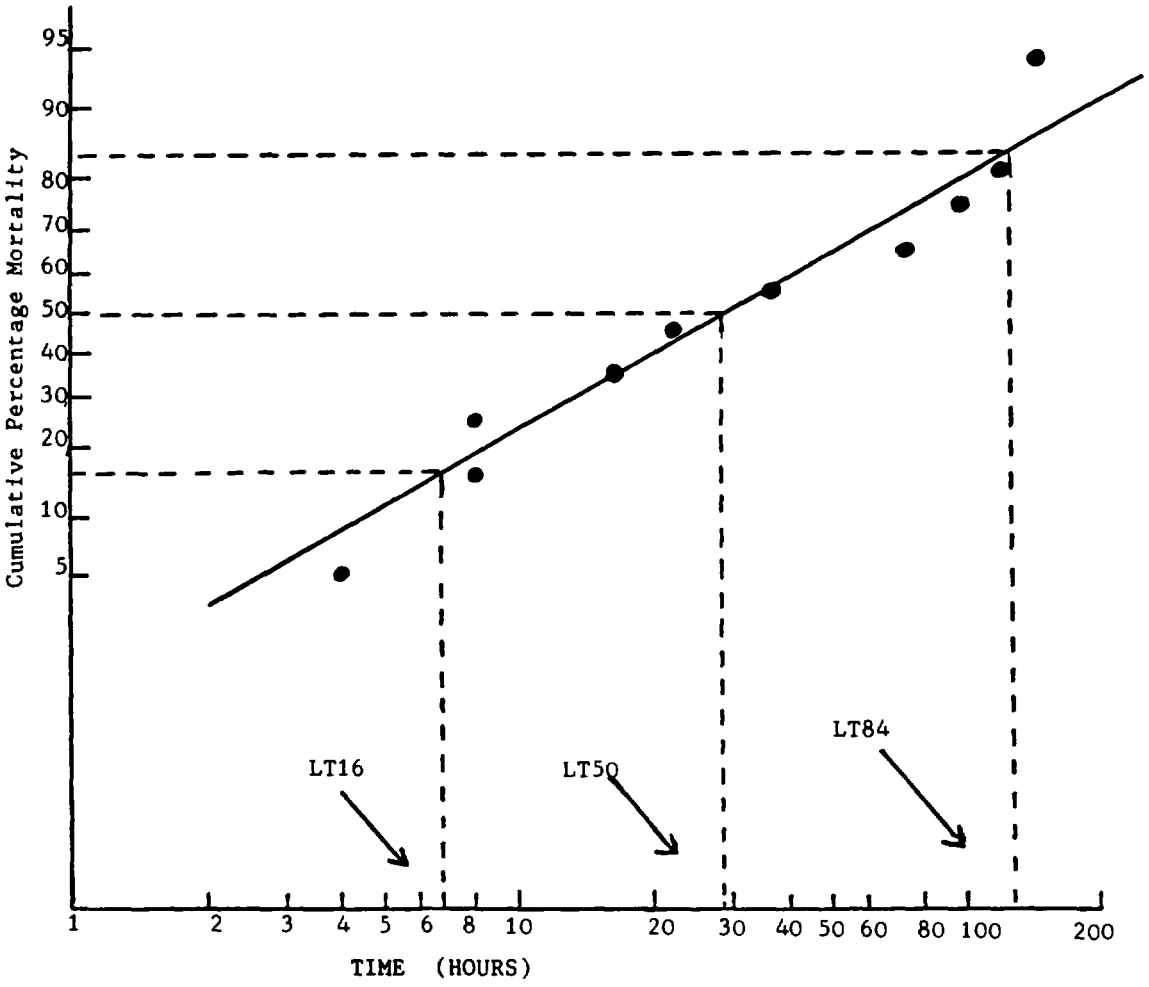


FIGURE 1: Hypothetical plot of cumulative percentage mortality (probability scale) against elapsed time (logarithmic scale). In this example, the data for the 20 mg l^{-1} concentration in Table 1 have been plotted, using the method described in 9.2.1 paragraph (b).

NOTE: Points representing very low (< 20%) or very high (> 80%) mortality values frequently show large deviations from the fitted line.

9.2.3. Read off from the fitted line values for LT16, LT50 and LT84 (see Fig. 1.).

NOTE: Where not all of the animals have died, the fitted line may be extrapolated to obtain the LT84 value.

9.2.4. Calculate the slope function S:

$$S = \frac{\frac{LT50}{LT16} + \frac{LT84}{LT50}}{2} .$$

9.2.5 Where not all the animals in a particular test concentration have died, omit this step and proceed to 9.2.8.

If all the animals in the test vessel have died, calculate f:

$$f = S^{\,1.96/\sqrt{N}}$$

An alternative formula is

$$f = \text{antilog}\left(\frac{1.96 \log S}{\sqrt{N}} .\right)$$

NOTE: These formulae are mathematically equivalent.

9.2.6 Calculate the upper and lower 95% confidence limits of the LT50.

Upper confidence limit = LT50 x f

Lower confidence limit = LT50 / f.

9.2.7 Repeat this process for each experimental vessel in which more than half of the animals died.

9.2.8 Where not all the animals have died, it is necessary to calculate a value for "N", where "N" lies between N (the number of animals in the test vessel) and the number of animals which actually died. This is done by using the nomograph given in Fig. 2.

Place a straight edge between N, the number of animals in the test vessel, and x, the percentage of animals which died. Read off the value of "N" where the straight edge intersects the "n" scale.

Calculate f, using one of the formulae given in section 9.2.5, substituting "N" for N. Now proceed as described in 9.2.6 and 9.2.7.

NOTE: It does not matter if "N" is not a whole number.

9.3 **Construction of the toxicity curve**

9.3.1 Tabulate the LT50 values and their confidence limits.

9.3.2 Plot a graph of LT50 and its confidence limits against concentration of test substance on log/log scales as in Fig. 3.

NOTE: This is most easily done on commercially-available log/log graph paper. If this is not available, plot the data on arithmetical paper by taking logarithms of the values to be plotted. Note that transforming both sets of data does not alter the shape of the toxicity curve. If the range of concentrations tested is not wide, it is often possible to plot the points on arithmetic scales.

9.3.3 Fit a line by eye to the points on the graph (Fig. 3).

In this example, a lethal threshold concentration is apparent, i.e. the curve has become asymptotic to the time axis. This does not happen in all cases, however, particularly when the experiment is of short duration. Also, although toxicity curves frequently take the form shown, in many cases a straight line can be equally well fitted to the points, especially when the range of concentrations of test substance tested is relatively narrow.

9.4 **Estimation of LC50 values (median lethal concentration)**

NOTE: Median lethal concentration (LC50) values are meaningless unless the exposure time is specified (e.g. 96 hour LC50).

9.4.1 Select the exposure time for which the LC50 value is required. In the following example it will be assumed that 96 hours is the specified time.

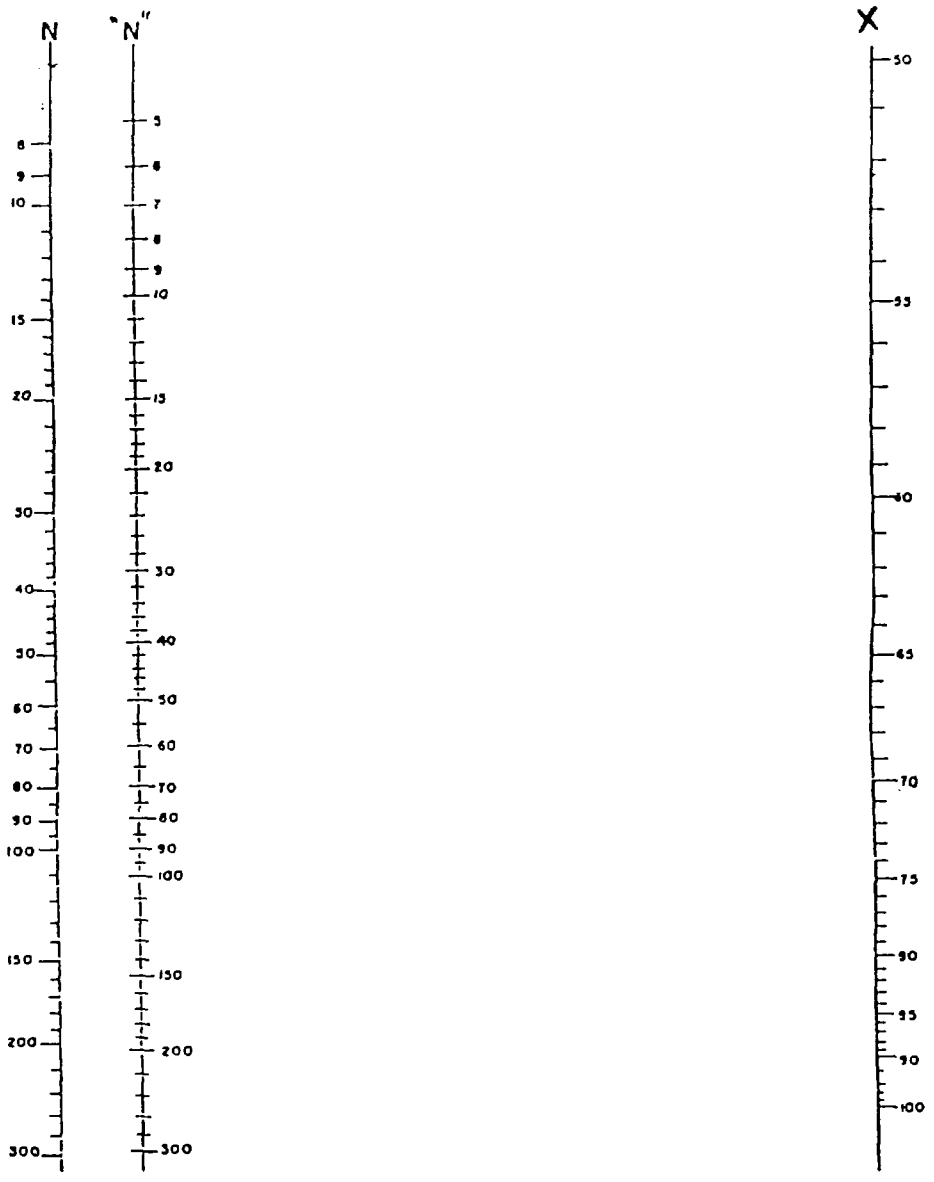


FIGURE 2: Nomograph for determining "N" where not all the animals exposed to a test concentration have died before the experiment is terminated. The correct use of the nomograph is described in 9.2.8.

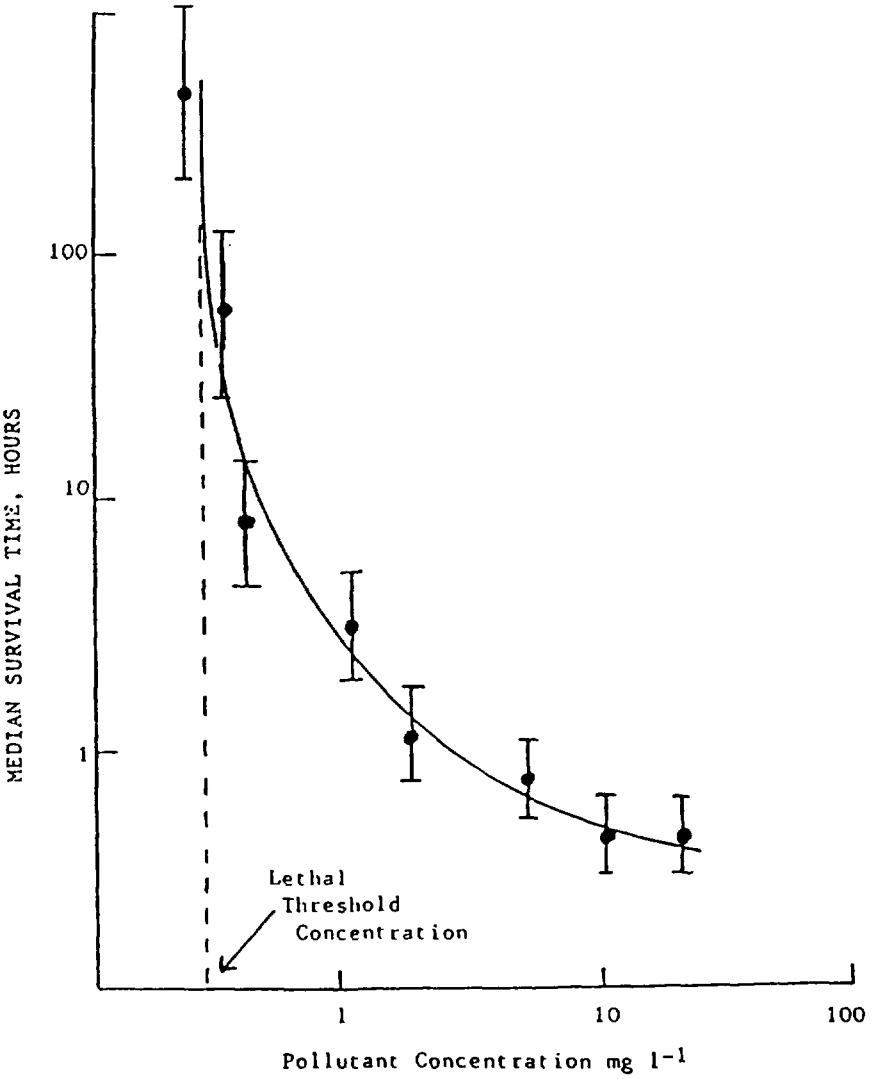


FIGURE 3: An example of a toxicity curve. Median survival times and their 95% confidence limits are plotted against concentration of test substance. Note that both axes of the graph are constructed on logarithmic scales.

9.4.2 Extract the required data from the table of results (see Table 1). In this case, it is necessary to know the percentage mortality which occurred after 96 hours in each test vessel.

9.4.3 Using logarithmic-probability graph paper, plot a graph of percentage mortality (probability scale) against concentration of test substance (logarithmic scale) as shown in Fig. 4. Ignore zero and 100% mortality.

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.

9.4.4 Fit a line, by eye, to the points on the graph and test its goodness of fit by the procedure described in 9.5.

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 9.5. If the line is not a good fit, further lines must be drawn and tested until a line of good fit is obtained.

9.4.5 If the line is a good fit, read off values for LC16, LC50 and LC84 as shown in Fig. 4. Calculate S, the slope function of the line, according to the formula:

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

9.4.6 Determine the value of N. This value is defined as the number of animals tested at concentrations whose <u>expected</u> effects were between 16% and 84% mortality.

NOTE: In Fig. 4, there are five points on the graph. The <u>expected</u> mortality at concentration of test substance 1 mg l^{-1} is less than 16%. The <u>expected</u> 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.

9.4.7 Calculate f, where

$$f = \text{antilog}\left(\frac{2.77 \log S}{\sqrt{N}}\right) = S^{2.77/\sqrt{N}}$$

9.4.8 Calculate the upper and lower 95% confidence limits of the LC50. The LC50 value is read from the line (see Fig. 4), provided the line is a good fit (see 9.5).

Upper confidence limit = LC50 x f

Lower confidence limit = LC50 / f .

9.5 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 the method described in 9.4. In such cases other methods of data analysis are required, or modification of the experimental procedure should be considered.

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

9.5.2 Using the nomograph in Fig. 5, determine the contribution to chi^2 of each point on the graph (see Table 2).

9.5.3 Determine the sum of the individual contributions to chi^2. Multiply this by the number of animals tested per concentration, i.e. if the animals were tested in each concentration, multiply the total chi^2 value by 10 (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.

9.5.4 Determine the number of degrees of freedom: this is given by N-2, where N is the number of concentrations tested. Do not include in this calculation the controls or those concentrations in which no mortalities occurred.

9.5.5 Using Table 3, read the value of chi^2 for the appropriate number of degrees of freedom.

9.5.6 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.

NOTE: In a few cases, a line of good fit cannot be constructed and the LC50 value cannot be calculated by this method.

NOTE: Where the constructed line is of good fit, it is nevertheless good practice to construct and test several lines, to determine the line which has the minimum value of chi^2.

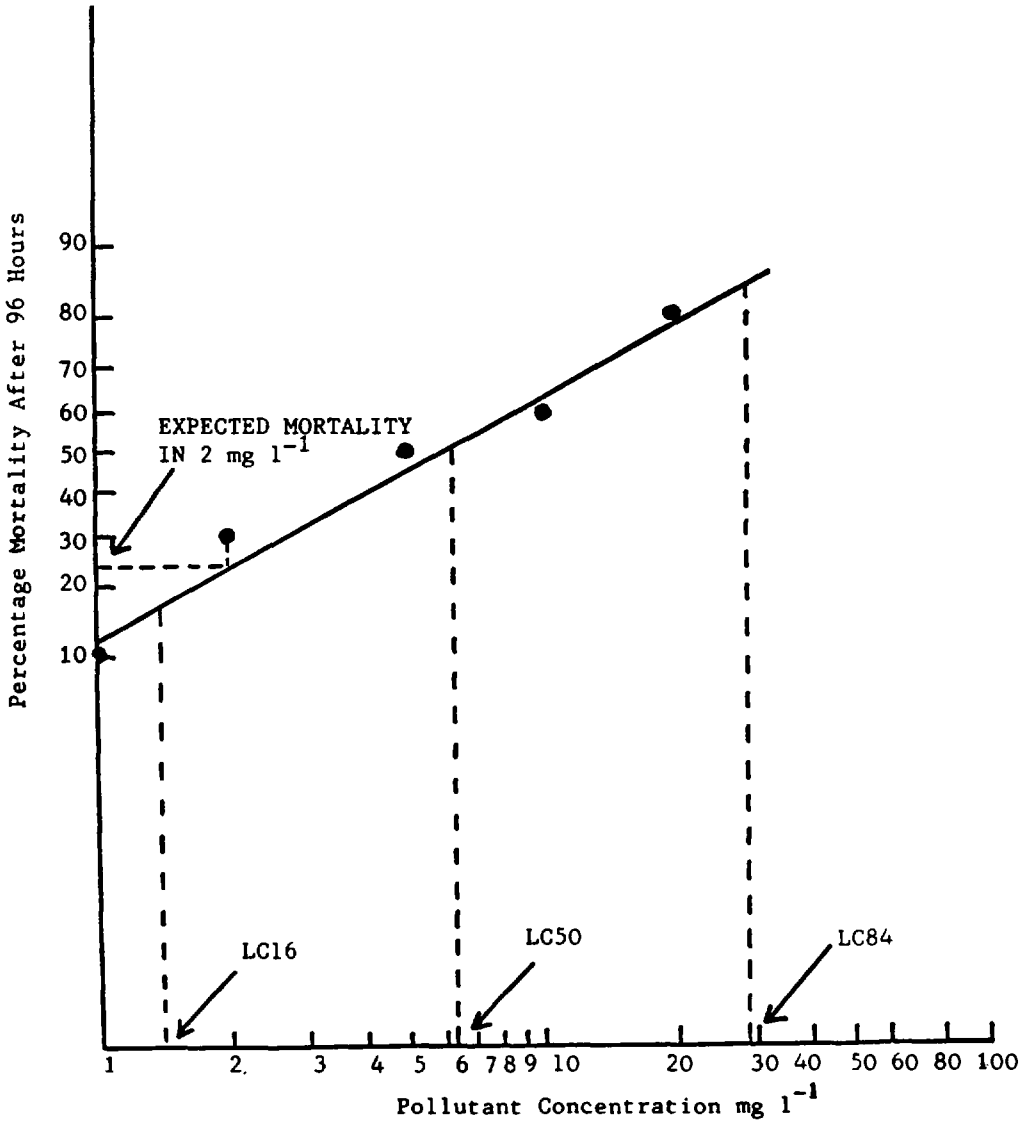


FIGURE 4: Estimation of the 96 hour LC50 value as described in 9.4. and 9.5. In this example, the data from Table 1 have been plotted by the method described. Note that zero and 100% mortality values are not plotted. Values for LC16, LC50 and LC84 are read from the line as shown in the diagram. To test the goodness of fit of the line (see 9.5), it is necessary to know the observed mortality values and the expected mortality values for each pollutant concentration plotted on the graph. The observed mortality values are those actually plotted. The expected mortality values are determined from the graph in the manner shown.

10. REPORTING THE RESULTS

10.1 Physical conditions

Report the following data:

10.1.1 The size and nature of the test vessels.

10.1.2 The location and lighting conditions in which the experiment was conducted.

Table 2: Data required to determine the goodness of fit of the line shown in Fig. 4

Concentration mg l^{-1}	No. of animals tested	Observed % mortality	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
			Total value of 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 (see Table 1) was 6, excluding the control, therefore the number of degrees of freedom is (6-2) = 4 (see 9.5.4). Using Table 3, it can be seen that the critical chi^2 value with 4 degrees of freedom is 9.49. Since 0.42 < 9.49, the line shown in Fig. 4 is a good fit.

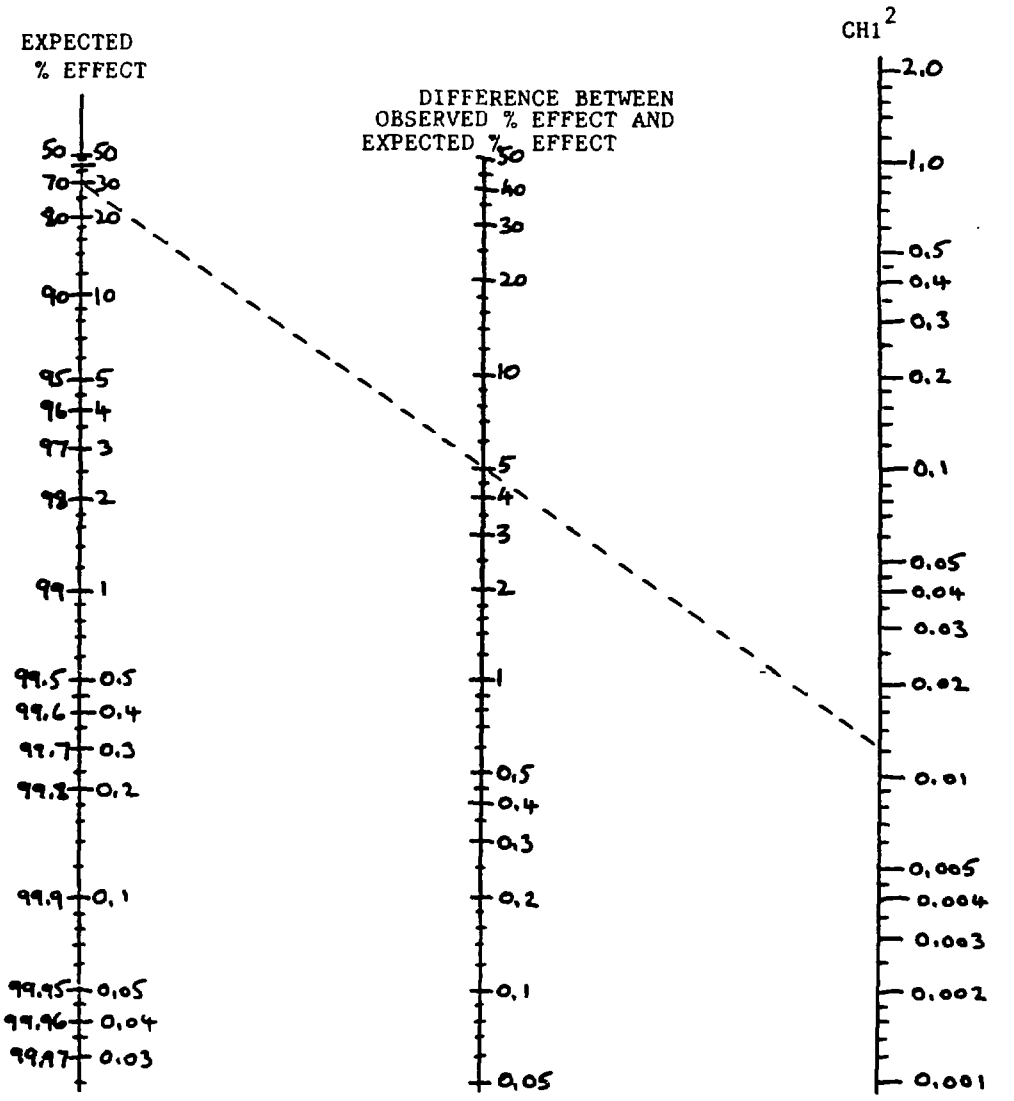


FIGURE 5. Nomograph for the determination of chi^2 (see 9.5.2.). To use the nomograph, construct a straight line (or use a ruler) which connects the expected % effect value (on the left-hand axis) to the value of the difference between the observed and expected values (on the central scale). Project 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 chi^2 value is 0.012. Remember that this chi^2 value must be multiplied by the number of animals tested per concentration (see 9.5.3), and added to the chi^2 values calculated in similar fashion for the other points on the graph (see 9.5.3).

Table 3: Values of chi^2 for p = 0.05. If the chi^2 value calculated from the line (e.g. Fig. 4) by the method described in section 9.5. is <u>less</u> than the value shown in this table for the appropriate number of degrees of freedom (see 9.5.4) the line is a good fit and the LC50 value and its 95% confidence limits may be calculated as described in section 9.4.

Degrees of freedom	Chi^2
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

10.1.3 The source, temperature, salinity and pH of the dilution water. Report mean values, the range of values, the standard deviations of the means, and the number of measurements made.

10.1.4 The frequency of renewal of the test solutions.

10.1.5 If measurements of actual concentrations of test substance have been made, report the mean value, the range of values, the standard deviations of the means, and the number of measurements made, for each test vessel.

10.2 The test organisms

Report the following data:

10.2.1 The source of the test organisms.

10.2.2 The full scientific (Latin) name of the organism.

10.2.3 The size and weight (means, standard deviations, and number of individuals measured) and, if known, the sex and age of the organisms.

10.2.4 The acclimation procedure.

10.2.5 The feeding regime employed before and during the experiment.

10.3 The results

Report the following:

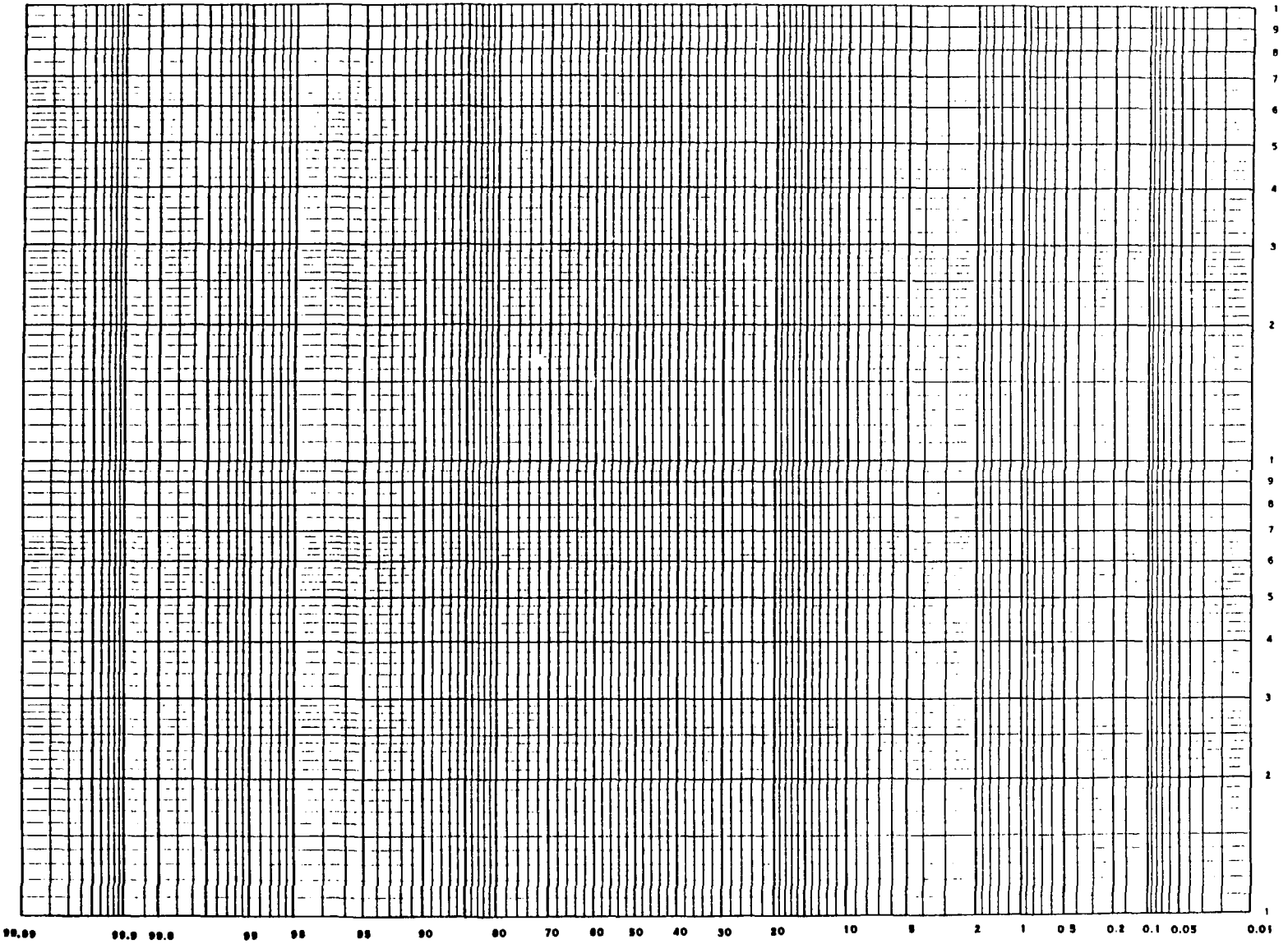
10.3.1 Median survival times their confidence limits and slope functions for each group of animals (see 9.2).

10.3.2. The toxicity curve (see 9.3).

10.3.3. The LC50 values slope functions and 95% confidence limits (see 9.4).

10.3.4. Any other relevant observations.

NOTE: In most experiments either median survival times (LT50) or median lethal concentrations are determined. However both may be estimated from the same experimental data and it is perfectly acceptable to use and report both methods of data analysis.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