REGIONAL SEAS

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Comparative toxicity test of water-accommodated fractions of oils and oil dispersants to marine organisms

Reference Methods For Marine Pollution Studies No.45

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:

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

PREFACE cont'd.

(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 45 was prepared in co-operation with the Food and Agriculture Organization o the United Nations (FAO) and the International Atomic Energy Agency (IAEA) It includes comments received from a number of scientists who reviewed th method and particularly the conclusions of the FAO/UNEP Consultation meetin on the toxicity of selected substances to marine organisms held i Villefranche-sur-mer, France, 13-14 October, 1988. The assistance of al those who contributed to the preparation of this reference method i gratefully acknowledged.

CONTENTS

1. SCOPE AND FIELD OF APPLICATION

This reference method describes a simple procedure for comparing the toxicity of oil, oil dispersants, and mixtures thereof, to marine animals. It allows the toxicity of different dispersants to be rapidly compared to that of oil, or of a mixture of oil and oil dispersant. It is designed for routine monitoring and screening purposes and is not appropriate as a research method.

NOTE: The physical and chemical properties of oil and oil dispersants create many difficulties in the measurements of their toxicity to marine organisms. Strictly speaking, their toxicity can only be accurately estimated using complex procedures and apparatus. (A relatively simple apparatus for preparing oil/water or oil/water/oil dispersant emulsions is described in Appendix B). Simpler methods can provide useful information, provided their limitations are clearly understood and taken into consideration in the assessment and application of their results. Some of the special considerations relating to the measurement of the toxicity of oil and oil dispersants are described in Appendix A. The Appendix also explains the rationale and limitations of the method described here. (Appendix A should be carefully read before this method is attempted.)

2. REFERENCES

REISH, D.J. AND OSHIDA, P.D. (1986) Manual of Methods for Marine 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. AND PARRISH, P.R. (1982) Manual of Methods in Marine Environment Research. Part 6. Toxicity tests. FAO. Fish. Tech. Pap. (No.185) 23pp., Rome, FAO.

3. PRINCIPLES

Aqueous extracts of oil, of oil dispersants or of mixtures of oil and oil dispersant are made. Samples of 10 test animals are exposed to each of a series of dilutions of this extract. The concentrations of the extracts which kill half of the test animals within 24 hours are estimated. A simple test of significance is used to indicate the relative toxicity of the substances tested. If a standard oil or standard oil dispersant is available, the toxicity of an oil dispersant can be compared to that of the standards.

NOTE: In some countries, experiments on living animals are subject to certain legal restrictions. Investigators should ensure that they hold any necessary licenses 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 35 salinity. If other salinities are required, adjust the amount of distilled water pro rata, e.g. for sea water of 37 salinity, dissolve the above chemicals with distilled water to 946 g.

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.

4.2 Oil

If the oil is required for a relatively small number of tests, proceed as in 4.2.1. If the oil is to be used as a standard or reference material in a longer programme of testing, proceed as described in 4.2.2.

NOTE: In some circumstances it may be necessary to establish a standard or reference oil, whose toxicity can be compared to that of other oils or to that of oil dispersants, in a long-term programme of toxicity testing. There is no internationally-agreed standard or reference oil for toxicity testing purposes. Further, oils from the same source and of the same specification do vary in their chemical constitution from time to time. Therefore, if it is desired to establish a standard or reference oil, each laboratory must obtain a large quantity of suitable oil, such that sufficient oil is available to complete the anticipated programme. In practice, a quantity as large as 1000 litres may be possible. Light crude oil, or light fuel oil are useful as standards, but the choice of standard oil depends upon the precise purpose of the testing programme.

4.2.1 Generally a sample of about 1 litre of oil will be sufficient. Place the sample in a suitable test container. The container must not be made of plastic, fibreglass or other synthetic material. It should preferably be opaque. Fill the container completely and seal it tightly, ensuring that no air is left inside the container. The container should not be unsealed until immediately before use. If the oil has to be stored before use, store it in a cool, dark place.

4.2.2 Ensure that the stock of reference oil is thoroughly mixed, by a means which does not require exposure to air. Divide the stock into aliquots of 0.5 or 1.0 litre in containers as described in 4.2.1. Seal the containers and store in a cool, dark place. During this process, avoid as far as possible exposing the oil to air.

NOTE: Handling and storage of large quantities of oil should be carried out with due regard to safety precautions. In some countries there are specific laws or regulations which must be followed, and investigators should ensure that they comply with these.

4.3 Oil dispersant

If the oil dispersant is required for a relatively small number of tests, proceed as in 4.3.1. If the oil dispersant is to be used as a standard or reference material in a longer programme of testing, proceed as described in 4.3.2.

NOTE: Although oil dispersants are generally more precisely formulated than oils, their exact chemical composition can vary from time to time, as many manufacturers alter the specification of the dispersant periodically. Also, many manufacturers do not, in practice, precisely define or control the composition of their products; and frequently a particular dispersant may be withdrawn from supply for commercial reasons. It is therefore advisable to establish a stock of standard or reference oil dispersant.

4.3.1 Obtain a sample of the oil dispersant and treat it exactly as described in 4.2.1.

4.3.2 Treat the stock of reference oil dispersant exactly as described in 4.2.2.

4.4 Analytical reagents (optional)

Reagents for the measurement of petroleum hydrocarbon concentrations in water-accommodated fractions of oil, oil dispersant or oil/oil dispersant mixtures as described in section 8.5.

5. APPARATUS

5.1 Apparatus for preparation of the aqueous extract of oil or oil dispersants

5.1.1 Magnetic stirrers.

5.1.2 1 litre and 5 litre conical flasks.

5.1.3 Glass tubing.

5.1.4 Salinometer or salinity probe of adequate accuracy.

5.2 The test vessels

These should be made of glass, and of appropriate size (see 7.1.). Vessels made of plastic, fibre-glass, or other synthetic materials are not suitable.

5.3 Logarithmic-probability graph paper

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

5.5 (Optional) Apparatus for the measurement of the concentrations of petroleum hydrocarbons in water-accommodated fractions of oil, oil dispersant or oil/oil dispersant mixtures, as described in section 8.5.

6. THE EXPERIMENTAL ANIMALS

6.1 Any available fish or macroinvertebrate 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 be of similar size. For the purposes of this method, small organisms are preferred, but species which are known to be resistant to pollutants, or to react slowly to them, should obviously be avoided.

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

6.2 Acclimation to laboratory conditions

NOTE: Laboratories which are routinely engaged in toxicity testing, or in which a series of toxicity experiments is to take place, should establish and maintain a stock of acclimated test animals. In some circumstances it may be necessary to conduct experiments at short notice using unacclimated animals. In these cases care must be taken to ensure that the experimental conditions are similar to those of the environment from which the animals were taken; particular attention must be paid to the incidence of mortalities among the control animals; and the fact that unacclimated animals have been used should be reported and taken into account when the significance of the results is assessed.

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 temperature change does not exceed 1°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-intensity illumination. Avoid unpredictable alterations in lighting conditions, e.g. sudden switching on and off of 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 which prevailed 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 sudden variations in the level of illumination. 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 the experiment.

7. THE EXPERIMENTAL CONDITIONS

NOTE: In this reference method, the experimental conditions required have been simplified as far as possible. Investigators should be aware, however, of the large influence of the experimental conditions on the result of the experiment. Consult the references given in section 2.

7.1 **Volume of test solution**

Each experimental vessel should contain at least 1 litre of test solution per gram of animal tissue, and preferably more.

NOTE: Since the test solutions must be prepared as described in section 8, the advantages of choosing small animals for the experiment are clear. See also 7.2.

7.2 **Aeration**

Since aeration promotes the evaporation of volatile substances, it is preferable that the vessels are not aerated.

NOTE: Since the experiment lasts only 24 hours, small animals can be satisfactorily maintained if the volume of test solution is sufficient.

7.3 **Temperature**

The temperature of the experiment should be the same as the acclimation temperature (see 6.2).

7.4 **Salinity**

It is advisable to check that the salinity of the test solution does not differ ($\pm$ 1) from that of normal sea water.

7.5 **pH**

It is advisable to check that the pH of the test solutions does not differ ($\pm$ 0.05) from that of normal sea water.

7.6 **Illumination**

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

8. THE EXPERIMENTAL PROCEDURE

8.1 Estimate the total volume of test solution required.

NOTE: The volume of test solution required depends upon the size of the test vessels, and the toxicity of the material to be tested (i.e. the extent to which the test solution has to be diluted). Some preliminary experimentation will sometimes be required to establish the volume of test solution required.

8.2 Prepare the required volume of test solution. To prepare a test solution of oil or of oil dispersant, proceed as described in 8.2.2. To prepare a test solution of a mixture of oil and oil dispersant, proceed as described in 8.2.1.

8.2.1 Place 900ml of sea water, 100ml of oil and 10 ml of oil dispersant in a 1-litre flask. Do not place a stopper on the flask. Proceed as described in 8.2.3.

8.2.2 Place 900ml of sea water and 100ml of oil or oil dispersant in a 1-litre flask. Do not place a stopper on the flask.

8.2.3 Put a Teflon-covered magnetic bar in the flask and place the flask on a magnetic stirrer. Adjust the speed of the stirrer so that the vortex in the flask does not exceed more than 25% of the distance from the top of the fluid to the bottom of the flask. Stir the mixture for 20 hours.

8.2.4 After 20 hours turn off the stirrer and allow the mixture to sit undisturbed for 30 minutes. Two layers of fluid exist, the water-accommodated fraction in the lower level.

8.2.5 Siphon the water-accommodated fraction through glass tubing into a one-litre Erlenmeyer flask. Place a stopper on the flask until required for the bioassay.

NOTE: This procedure prepares about 900ml of test solution. More solution may be required e.g. if the material is not very toxic or if the total volume of the test containers is large. Quantities of 2 or 3 litres can be prepared in a single container of suitable size. Larger volumes are best made by using several similar containers and stirrers simultaneously. The water-accommodated fractions should be mixed in a single, large container before use, to ensure homogeneity.

NOTE: If the oil or oil dispersant has been drawn from a stock of standard material as described in 4.2, discard any remainder. It should not be used for further experiments after exposure to air.

8.3 Prepare a series of dilutions of the water-accommodated fraction in sea water. Five or six concentrations, plus a control, are normally required. The concentrations selected should follow a roughly logarithmic series, e.g. 1%, 2%, 5%, 10%, 20%, 50%. More closely-spaced concentrations are often useful, e.g. 1%, 2%, 5%, 10%, 13%, 20% 30%.

NOTE: Some preliminary experimentation is often necessary to establish the appropriate range of concentrations. The aim should be to ensure that in the highest concentrations, most of the animals die within 24 hours, and in the lowest concentration, most of the animals survive.

8.4 Place the required quantity of each diluted test solution into each of the experimental vessels.

8.5 Measurement of petroleum hydrocarbon concentrations in water-accommodated fractions (optional).

It is advantageous in many circumstances to measure the actual concentrations of petroleum hydrocarbons in the aqueous extracts and dilutions thereof. Firstly, if this is done the results can be expressed in terms of the actual concentration of petroleum hydrocarbons to which the animals are exposed, i.e. as LC50 rather than "LC50" (see Appendix A).

Secondly, the behaviour of petroleum hydrocarbons in water is complex; and in some circumstances a given dilution of the aqueous extract with clean water may not actually contain the expected concentration of water-accommodated petroleum hydrocarbon. Therefore it is useful to be able to measure the concentration of petroleum hydrocarbon in the aqueous extracts and dilutions thereof.

A standard method for doing this is not currently available, but investigators who have access to suitable facilities are recommended to experiment with the following general procedure.

A given volume of test mixture (e.g. 50 ml) is extracted twice with a quantity (e.g. 10 ml) of dichloromethane (Spectrosol grade or equivalent), by shaking for 5 minutes in a separating funnel. After each extraction, the contents of the funnel are allowed to settle for 5 minutes. The two volumes of dichloromethane are then combined and dried over anhydrous sodium sulphate. The dichloromethane extracts are then analysed on a spectrofluorometer. (The extracts may sometimes require concentration or dilution with pure dichloromethane). The spectrofluorometer must be standardised using a series of standard known concentrations of the test oil in dichloromethane. The excitation and emission wavelengths to be used vary according to the test oil, and have to be identified by performing excitation and emission scans on the oil itself in dichloromethane. In a typical case, 310 nm and 360 nm may be used as excitation and emission wavelengths respectively. The levels of petroleum hydrocarbon in the test mixtures are then reported in terms of the test oil equivalents.

8.6 Place 10 animals in each experimental vessel. The experiment has now begun. Note the time.

8.7 Leave the experiment undisturbed for 24 hours. After 24 hours, count and record the number of animals in each vessel which have died.

NOTE: Considerable turbidity may occur in some experiments. Special care is required when counting the animals.

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 cases; such criteria must be clearly defined.

9. DATA ANALYSIS

9.1 Construct a table showing percentage mortality after 24 hours in each dilution, as in the example given in Table 1.

NOTE: There should be no control mortalities. Control mortalities invalidate the experiment, and it must be repeated.

9.2 Using the logarithmic-probability paper, plot a graph of percentage mortality after 24 hours (probability scale) against the dilution of the test material (logarithmic scale) as in the example shown in Fig. 1. Ignore zero and 100 per cent values.

9.3 Examine the points on the line. There must be at least three points on the line, including at least one point above 50% and one below 50%. Zero and 100% values must not be included. If this is not the case, the experiment has failed and must be repeated using a different range of dilutions.

9.4 Fit a line, by eye, to the points on the graph.

9.5 Test the goodness of fit of the line according to the following procedure.

9.5.1 Tabulate the observed mortality values and the expected mortality values for each point on the graph.

NOTE: The observed mortality values are those which were recorded during the experiment. The expected values are read from the fitted line as shown in Fig. 1.

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

9.5.3 Determine the total chi^2 and multiply by the number of animals in each test vessel (normally 10).

Table 1: An example, using hypothetical data, of the data analysis described in section 9 (the graph is shown in Fig. 1)

Dilution of water-accommodated fraction(%)	No. of animals	Observed % mortality	Expected % mortality	Contribution to chi^2
Control	10	0		(Not plotted)
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		(not plotted)
		Total value of chi2 =		0.042

Since the number of animals tested per dilution is 10, the chi^2 value of the line in Fig. 1 is (0.042 x 10) = 0.42. The number of degrees of freedom is (6-2) = (see 9.5.4). Using Table 2, it can be seen that the critical 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.

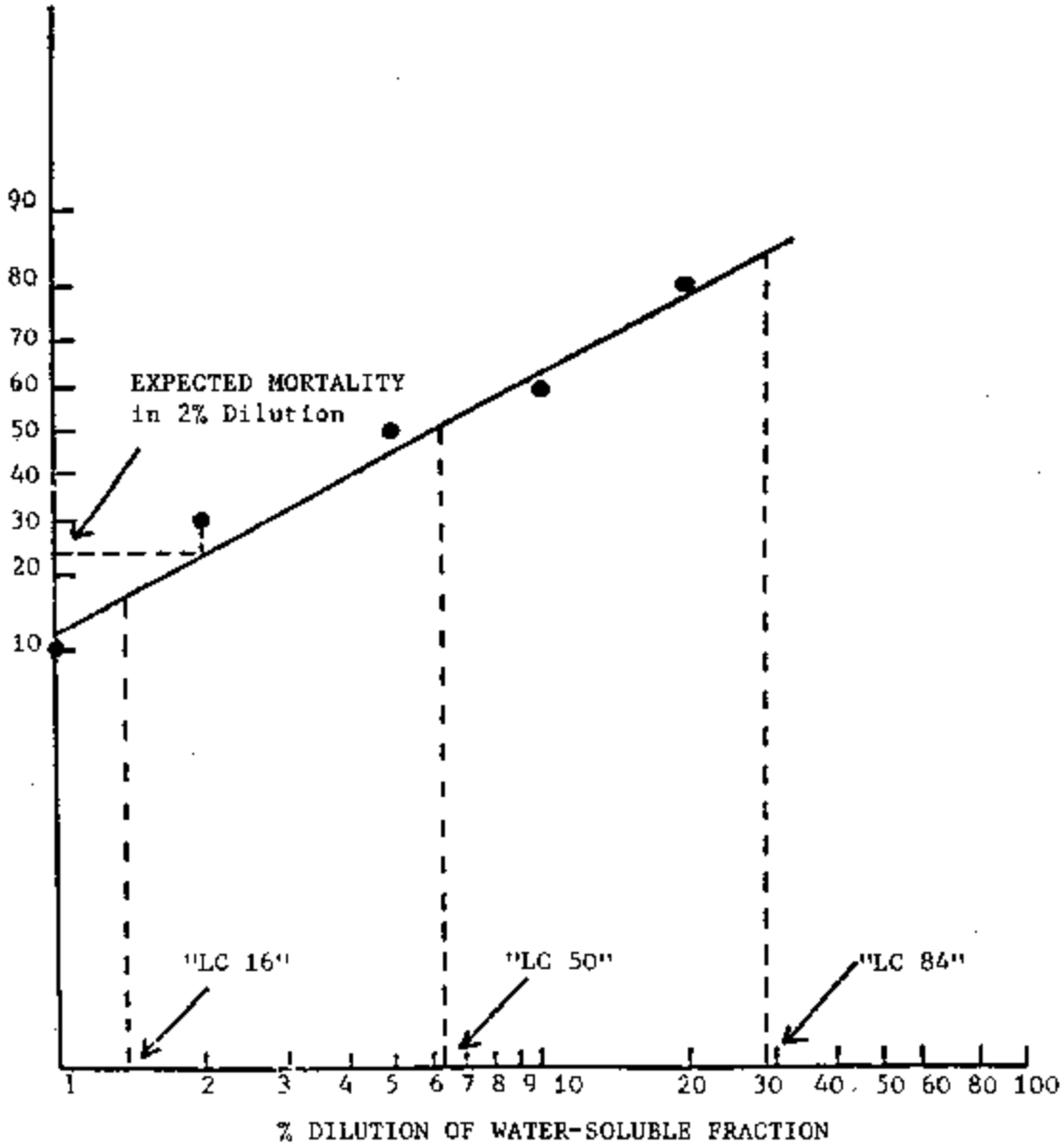


FIGURE 1: Estimation of the 24 hour "LC50" value as described in Section 9. In this example, the appropriate data from Table 1 have been plotted by the method described. Note that zero and 100% mortality values are not plotted. Values for "LC16", "LC50" and "LC84" are read from the line as shown in the 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 determined from the graph in the manner shown.

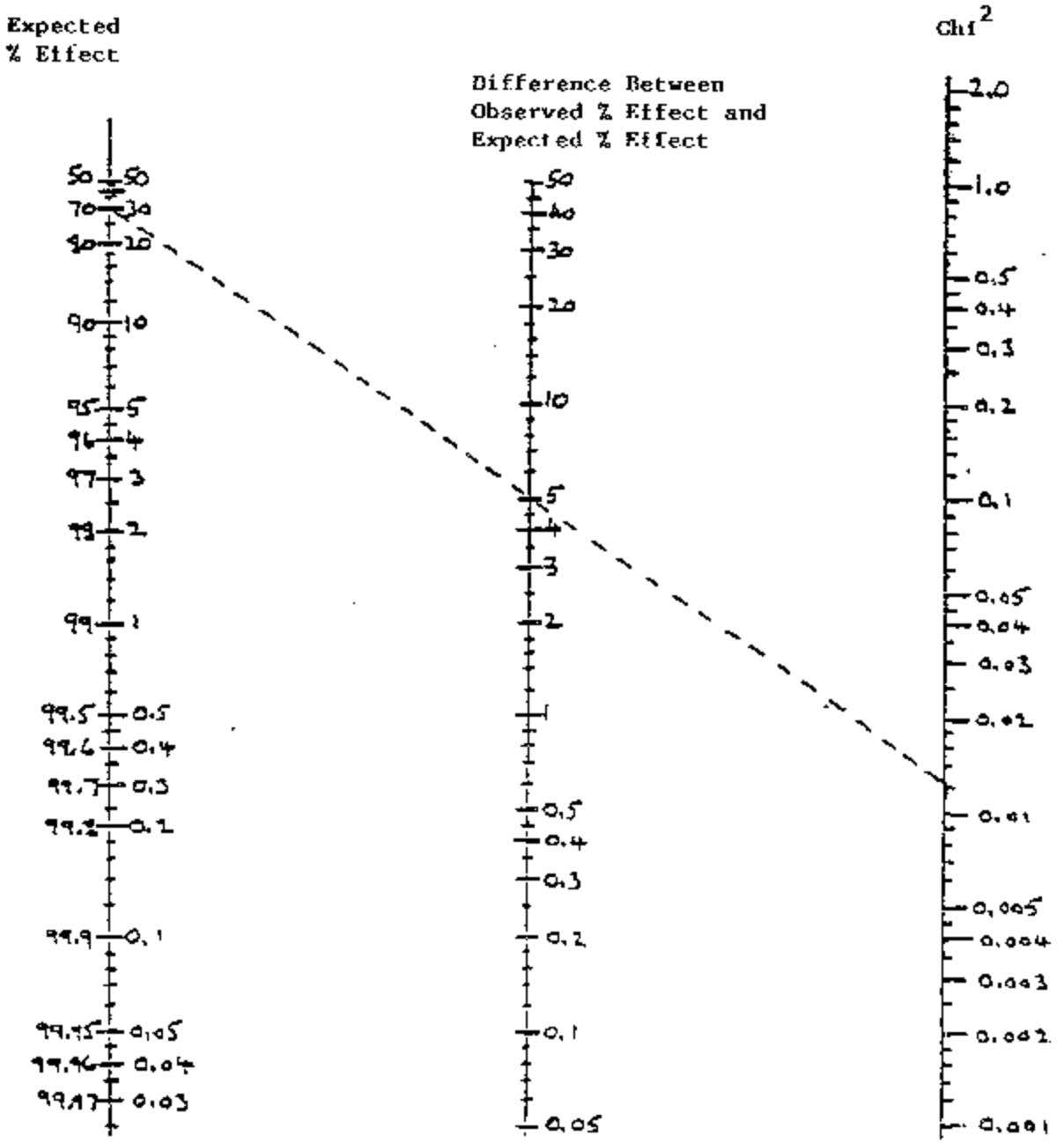


FIGURE 2: Nomograph for the determination of chi^2 (see 9.5.2). To use the nomograph, construct a straight line (or use a ruler) which connects the expected % effect value (on the left-hand axis) to the value of the difference between the observed and expected values (on the central scale). Project this 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 2: Values of chi^2 for p = 0.05. If the chi^2 value calculated from the line (e.g. Fig. 1) by the method described in section 9.5 is less 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.

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

9.5.4 Determine the number of degrees of freedom: This is given by N-2, where N is the number of dilutions tested. Do not include the control in this calculation.

9.5.5 If the chi^2 value of the line, as calculated in 9.5.4, is equal to or greater than the appropriate value given in Table 2, 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 some cases, a line of good fit cannot be obtained, and the analysis cannot proceed. Repeat the experiment using if necessary a different range of dilutions.

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.

9.6 Read off from the line values of "LC16", "LC50", "LC50" and "LC84" as shown in Fig. 1.

9.7 Calculate S, where

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

9.8 Determine N, where N is defined as the number of animals tested whose expected effects were between 16% and 84% mortality.

NOTE: In Fig. 1 and Table 1, four points on the graph represent test vessels where the expected mortality lies between 16 and 84 per cent. Therefore N = 4 x 10 = 40, since 10 animals were tested in each test vessel.

9.9 Calculate f, where

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

9.10 Calculate the upper and lower confidence limits of the 24 hour LC50.

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

9.11 To determine if one substance is more toxic than another (e.g. to see if one oil dispersant is more toxic than the reference material, or if dispersant is more or less toxic than oil), examine the data for the relevant pair of substances. If the confidence limits overlap, the substances do not differ significantly in toxicity. If the confidence limits do not overlap, one is significantly more toxic than the other.

NOTE: If a standard oil or standard oil dispersant is to be compared against another material, the standard and the new material must be tested simultaneously. Results of earlier tests of the standard material may not be validly compared with results of more recent tests.

NOTE: This simple test of significance is not sufficient where large numbers of comparisons must be made. Since the test is based on 95% confidence limits, on average one comparison out of twenty will result in a false conclusion. In the case, for example, where 6 oil dispersants are to be compared, the total number of comparisons to be made is 15, and there is a high probability that false conclusions will be drawn. In such cases more sophisticated tests of significance are required, such as the Student Newman-Keuls test. This test is described in detail by Reish & Oshida (see References, section 2).

APPENDIX A

Some comments on measuring the toxicity of oils and oil dispersants to marine animals:

The physical and chemical properties of oils and oil dispersants create particular difficulties for the measurement of toxicity to aquatic animals. Oil is a complex mixture of hydrocarbons whose precise composition is often unkoown. Most oil dispersants contain detergents, hydrocarbon solvents and other chemicals such as emulsifying agents and stabilising agents. Oil and oil dispersants have low solubility in water, but when added to water and agitated they form an emulsion. Such emulsions are generally not stable, i.e. their physical properties alter with time. Indeed the purpose of oil dispersants is to promote the formation and stabilisation of oil/water emulsions. Measuring the toxicity of these emulsions is not easy for several reasons, particularly if it is desired to express the toxicity in conventional terms such as median lethal concentrations (LC50) in units of weight per volume of water.

Firstly, it is found that the toxicity of these emulsions depends very greatly on the size of the droplets formed in the emulsion. This, in turn, depends upon the type and quantity of the energy applied in agitating the mixture to form the emulsion. Thus the results of a toxicity test are primarily influenced by the method of formation of the emulsion.

Secondly, since the emulsions are unstable, their toxicity will change with time.

Thirdly, oils and oil dispersants contain various fractions of volatile hydrocarbons (the "light" fraction) which tend to evaporate fairly quickly. These volatile components are generally of high toxicity. Again, this causes the toxicity of the emulsion to change markedly over short periods of time.

These three factors indicate that the accurate measurement of the toxicity of oils and oil dispersants requires complex apparatus and procedures. For example, it is required that the emulsion be agitated by a precisely-defined, standardised and carefully-controlled procedure. The agitation should continue throughout the experiment, in a controlled manner but without harming the test animals. Ideally the test solutions should be renewed frequently, preferably by automatic means, during the experiment. The apparatus required to meet these demands is difficult and expensive to install and maintain.

It is sometimes possible to obtain results with simple procedures. However unless specific precautions are taken to overcome the difficulties described above, and to allow for the special physical and chemical properties of oils and oil dispersants, the results obtained are of little real value. They are likely to differ very widely from their true values, and to be of little or no practical use. A relatively simple apparatus which

has been found useful in some laboratories is described in Appendix B. If used carefully and its results interpreted cautiously, it provides an alternative method for measuring the toxicity of oils and oil dispersants to the method described here involving the use of water-accommodated fractions.

The rationale underlying the use of water-accommodated fractions in toxicity tests of oil and oil dispersants is as follows. In devising a simple method, it is important to consider means by which the difficulties described above can be avoided, while ensuring that the results produced by the method are of practical use. Fortunately, oil and oil dispersants differ from most pollutants in the manner in which pollution actually occurs, and therefore in the type of toxicological information which is required about them.

Firstly, pollution by oil and oil dispersants nearly always results in high concentrations of pollutants being present in the water, but for a short time. Therefore a toxicity test of short duration is appropriate (in contrast, for other types of pollutant, tests of short duration are of very limited value).

Secondly, when pollution occurs the volatile components of oil and oil dispersants normally evaporate from the sea very quickly, and it is the toxicity of the residual matter which is of most importance. Therefore in this reference method, the more volatile components are removed before the experiment begins.

Thirdly, in practice it is not necessarily useful to know the toxicity of an oil dispersant expressed in conventional terms such as an LC50 value in milligrams per litre. Apart from the fact that it is difficult to estimate such a value accurately, for the reasons described, normally oil dispersants are only applied to the sea after severe pollution by oil has already occurred. In practice therefore, the most important datum required is whether the toxicity of the oil dispersant, or of a mixture of oil dispersant and the oil, is greater or less than that of the oil itself. It is also useful to compare the toxicities of different oil dispersants, so that the least toxic of the available dispersants can be selected.

In this reference method, a procedure is described whereby such comparisons may realistically be made. However, no attempt is made to determine LC50 values in the conventional sense, so the technical difficulties of doing so are avoided. (Investigators who wish to measure the toxicity of oils and/or oil dispersants by conventional means may attempt to use apparatus of the kind described in Appendix B, and to carry out the analysis of the data according to the methods described in Reference Method No. 43, listed in the references given in Section 2).

In this method (Reference Method 45), some stages of the data analysis are directly analogous to the calculation of conventional LC50 values. The use of the term LC50 is therefore retained for convenience, but is shown thus "LC50". It is calculated and expressed in terms of percentage

dilutions of the test solutions prepared as described in section 8.2. It is not equivalent to the conventional LC50 value of the original material under test, and its relation to that value is not known. The use of this method to determine the LC50, as conventionally understood, of oil or oil dispersants to aquatic animals is entirely erroneous.

APPENDIX B

A simple apparatus for determining the toxicity of oil, oil dispersants and mixtures thereof to aquatic animals

The apparatus described in this Appendix is relatively simple and inexpensive to construct, and allows investigators to measure the toxicity of oils, oil dispersants and mixtures thereof by conventional methods (rather than by measuring the toxicity of water-accommodated fractions as described in the Reference Method). It allows animals to be exposed to standardised emulsions of oil, oil dispersants or mixtures thereof in water.

The system consists of a collecting cylindrical chamber where emulsions are maintained by stirring paddles (see Figs. 3 and 4). The emulsion is then pumped to a header chamber which in turn feeds it to 8 small exposure funnels via short lengths of silicone tubing and calibrated plastic nibs (disposable plastic pipette tips as used in automatic dispensing pipettes). From these exposure funnels the emulsion returns to the central collecting chamber. The system may be very easily dismounted for cleaning after each run. It holds 10 litres of sea water and is dosed with a suitable volume of oil or oil/oil dispersant mixture.

Note that in the basic design, each exposure funnel receives the same concentration of oil or oil/oil dispersant mixture. Where it is required to expose animals simultaneously to each of a range of concentrations, a separate apparatus for each concentration will be required. In this case, and where results from different runs with the same apparatus are to be compared, certain features of the method must be rigorously standardised.

The reason for this is that a major source of variation in the results of toxicity experiments involving oil and/or oil dispersants is the degree of dispersion obtained in mixing the oil and water by mechanical means. In particular, attention must be paid to the size, shape, speed of rotation and precise positioning in the apparatus of the stirring paddles. These features must be exactly the same in every case, otherwise the results of the experiments are not valid.

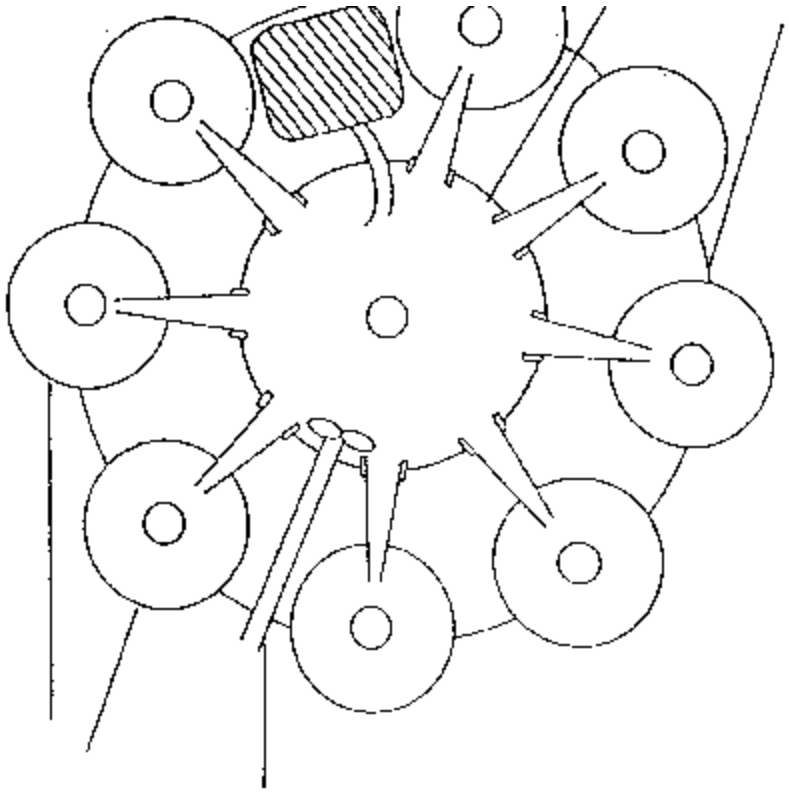


Fig. 3: (For description, see Annex B).

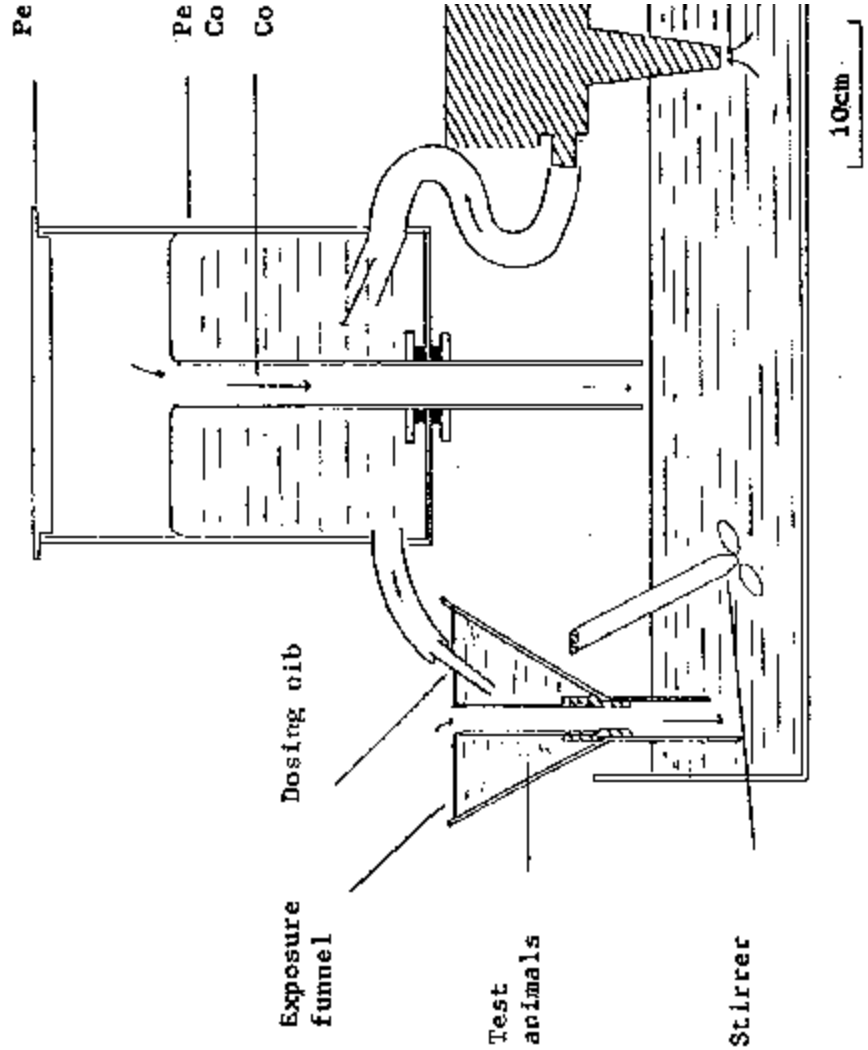
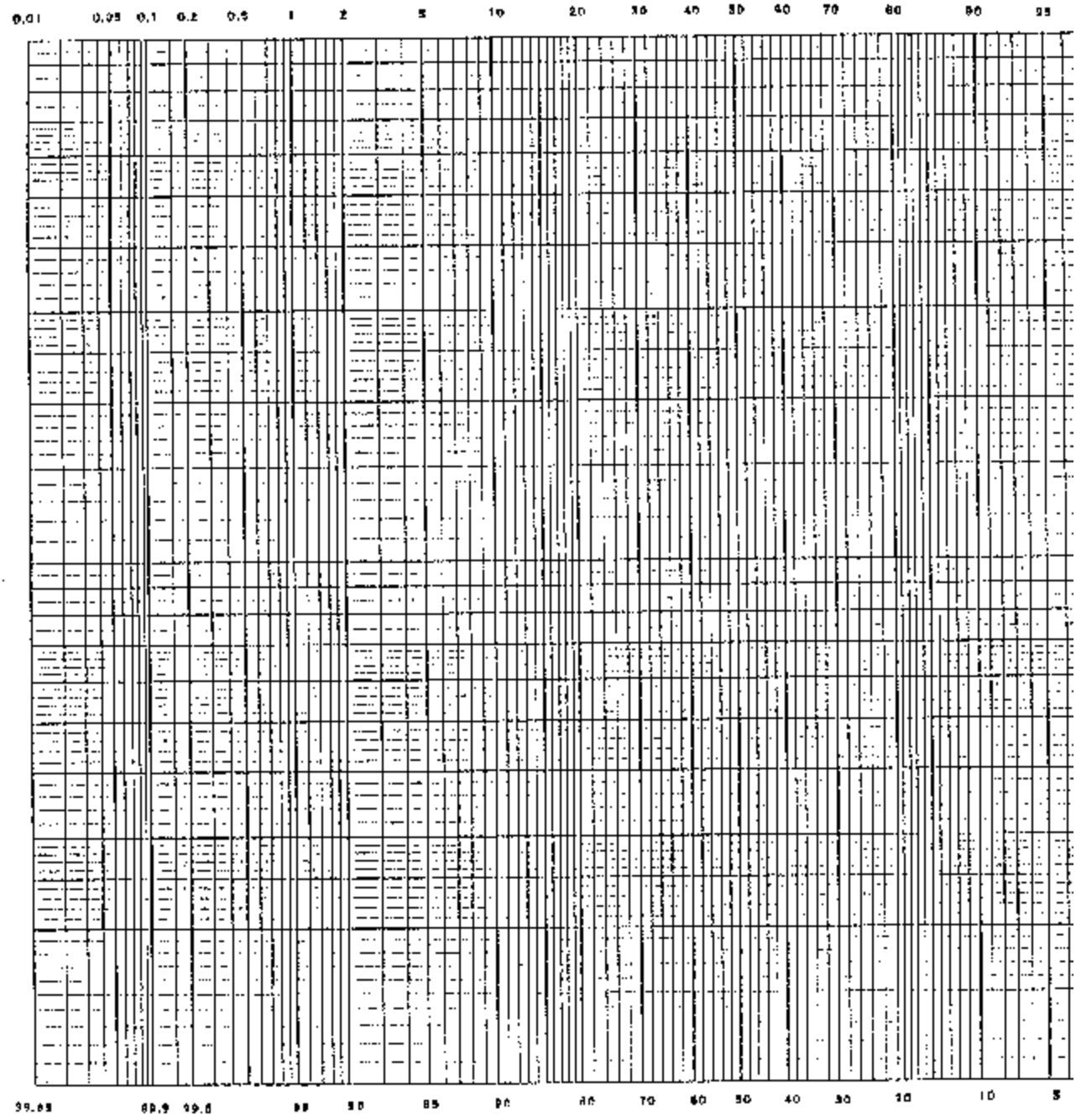


Fig. 4: (For description, see Annex B).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