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MANUAL OF METHODS IN AQUATIC ENVIRONMENT RESEARCH

Part 7 – Selected Bioassays for the Mediterranean

with the cooperation of the
United Nations Environment Programme

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

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UNITED NATIONS ENVIRONMENT PROGRAMME

MANUAL OF METHODS IN AQUATIC ENVIRONMENT RESEARCH

Part 7. Selected bioassays for the Mediterranean (Tests used by the FAO(GFCM)/UNEP Joint Coordinated Project on Pollution in the Mediterranean)

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PREPARATION OF THIS MANUAL

This Manual was prepared as part of a cooperative project of the United Nations Environment Programme entitled:

FAO(GFCM)/UNEP Joint Coordinated Project on Pollution in the Mediterranean

with the Food and Agriculture Organization of the United Nations as cooperating agency.

DEFINITION OF MARINE POLLUTION

Pollution of the marine environment means: "The introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) which results in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities".

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PREFACE

Under the Mediterranean Action Plan, developed by the United Nations Environment Programme and endorsed by the Intergovernmental Meeting on the Protection of the Mediterranean (Barcelona, 28 January - 4 February 1975), the Co-ordinated Mediterranean Pollution Monitoring and Research Programme was established. As part of this programme, an FAO(GFCM)/UNEP Joint Co-ordinated Project on Pollution in the Mediterranean was initiated. The project, among other subjects, aims at organizing a cooperative programme on research on the effects of different pollutants on marine organisms and their populations.

The Operational Document, which serves as the programmatic basis for the collaboration of Mediterranean laboratories in the above-mentioned project, was developed at the Expert Consultation on the Joint Co-ordinated Project on Pollution in the Mediterranean, convened by FAO(GFCM) in Rome, 23 June - 4 July 1975. This document specifies the organisms and pollutants to be monitored. It also states that effects of these pollutants on the organisms should be studied by means of bioassays.

At the request of the Mid-term Expert Consultation on the Joint FAO(GFCM)/UNEP Co-ordinated Project on Pollution in the Mediterranean, which reviewed the progress of the programme, and was held in Dubrovnik, 2-13 May 1977, this Manual was prepared to give detailed guidance on bioassay procedures to be used. Its aim is to present, as accurately and simply as possible, a few types of bioassays which could be directly used by all the Mediterranean laboratories working on the pilot project: "Research on the effects of pollutants on marine organisms and their populations (MED POL IV)". The main purpose of this Manual is to describe some methods which would allow the assessment of the effects of pollutants, mainly chemical ones, on marine organisms. It endeavours to be strictly practical and references of a theoretical nature will only be used to recall details it is considered the users already know. It would like to help the laboratories by serving as a guide for selection of species, preparation and stardardizing procedures for the implementation of bioassays. The pollutants chosen (and their concentrations) are, of course, examples only. In this way, the comparability of results obtained by the different laboratories is increased.

It is also recommended to refer to the "Manual of Methods in Aquatic Environment Research - Part IV: Bases for selecting biological tests to evaluate marine pollution" (FAO Fisheries Technical Paper (164), FIRI/T164, Rome 1977).

It is expected that experience gained during the use of this manual will lead to comments and proposals for improvements which should be sent to FAO. Such comments and improvements will form the basis for the preparation of reference methods for studies on pollution in the Mediterranean.

The author wishes to express special thanks to Mr. R. Establier, Institute of Fishery Research, Cadiz, for his collaboration during the drafting of the Manual. He also wishes to thank Mr. L. Saliba, University of Malta, for his constructive comments.

The views expressed in the Manual are those of the author and do not necessarily represent the views of either FAO or UNEP.

SUMMARY

Under the FAO(GFCM)/UNEP Joint Co-ordinated Project on Pollution in the Mediterranean, a series of selected bioassays have been developed. After a brief reminder of the sources and effects of marine pollution, the aims of the tests and the different procedures are presented which must be followed to obtain significant data. Short and long-term tests on different organisms (polychaetes, molluscs, crustaceans, fish) are described in detail and the test procedures which must be followed step by step are set out. Two statistical methods for assessing the results are proposed.

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1. BRIEF REMINDER OF THE SOURCES AND EFFECTS OF MARINE POLLUTION

1.1 Sources[1/]

- Fixed point sources

Discharge always occurs at the same point (example: urban domestic wastes, industrial wastes). There are continuous and intermittent discharges.

- Variable location sources

The continuous or nearly continuous discharges do not differ basically from the above. The only difference is that the discharge point varies from time to time over a geographical area whose size may vary. Intermittent, occasional or accidental discharges may occur in any place or at any time, since neither the time nor place is foreseeable.

- Diffuse sources

Rivers, run-off water, atmospheric fallout play an active part in pollution of the environment.

1.2 Effects

If the undesirable direct, continuous or sporadic, effects are well known and do not require further development or special comment, emphasis must be placed, however, on the indirect effects. A large number of products, substances or physical phenomena which result from human activity can, even though they have no apparent direct effect, seriously modify the marine environment and through phenomena of interference have totally undesirable, even disastrous, effects on the environment.

2. REASONS FOR AND PURPOSES OF TESTS

The apparent multiplicity of tests (or bioassays) which exist is due to the following facts: requirements concerning the quality of water, the desired level of protection of the environment, the large number of organisms (species) and ecological systems (communities, ecosystems) which exist, the different types of discharges and the pollutants discharged - just to mention a few examples.

The result is a very wide range of measured responses which may cover lethal, as well as sub-lethal, phenomena and concern ecosystems (natural, man-made, those set up in the laboratory, etc...) and intracellular enzyme reactions.

If literature abounds with descriptions of a multitude of test procedures this is because the problems posed are many and require different lines of approach.

1/ The contracting parties to the Convention for the Protection of the Mediterranean against Pollution (Barcelona Convention), undertook to promote measures concerning the protection of the marine environment in the Mediterranean area against all types and sources of pollution, such as:

- Pollution caused by dumping by ships and aircraft;
- pollution by ships;
- pollution resulting from the exploration and exploitation of the continental shelf, the sea-bed and its sub-soil;
- land-based pollution.

2.1 Screening Tests

Screening tests are done to obtain indications of the concentrations of the tested substances which are potentially harmful to marine life. They may be used to provide an increasing (or decreasing) order of toxicity of the substances (e.g. to aid the choice of oil spill dispersants and detergents).

A few "standard" test species are selected and their wide geographic distribution will enable data obtained in different regions (or laboratories) to be compared. These are usually lethal toxicity tests (but they may sometimes be sub-lethal).

If these tests are well "standardized" and carefully implemented, they will provide plenty of information.

At the strictly sub-lethal level, they produce qualitative rather than quantitative information on alterations of a histological and teratological or biochemical nature.

2.2 Legal Tests[2/]

These are the same as those above, but they are defined and used on a legal plane (governmental or intergovernmental).

2.3 Tests to Establish Water Quality and Effluent Monitoring Criteria

These tests are usually not standardized and are used in local situations which involve a variety of discharges (or a complex mixture) from both a qualitative and quantitative point of view. The purpose of these tests is to determine the "safe" levels with reference to ecological consequences. They are frequently carried out in situ.

2.4 Tests to Protect Higher Trophic Levels

These accumulation tests are mainly used to determine the possible effect on higher trophic levels of substances that accumulate in the tissues of marine organisms. They take into consideration the transfers which occur through food chains, as well as the possible effects on human health, since the human being is the ultimate consumer, situated at the top of the food chain.

Tests allowing for an estimate of the ecological effects of various types of pollution may also be considered together with tests designed to protect higher trophic levels.

2.5 Tests for Biostimulation

The aim of these tests is to identify the problems of accelerated eutrophication (distrophy). Either laboratory tests of field studies may be used to assess stimulation of algal growth.

2.6 Miscellaneous Tests

Effluent or discharge area monitoring do not fall within the scope of this Manual.

Organoleptic tests, which are used to determine the acceptability of marine products for human consumption and which require experienced human 'tasters' are not taken into account in this Manual either.

2/ The tests described in this Manual can in no way be considered as tests recommended for legal ends. The final aim, however, is, on the basis of the experience acquired in the Mediterranean (during their implementation), to suggest that these tests be used by the coastal states as reference methods for baseline studies on pollution in the Mediterranean in fulfillment of their obligations to the Barcelona Convention.

3. TYPES OF TEST PROCEDURES

3.1 General Data

The term 'bioassay' is used in this document according to the definition given in chapter 4 of FAO (1977):

> 'Bioassay signifies a test in which a living tissue, organism or group of organisms is used as a reagent for the determination of the potency of any physiologically active substance of unknown activity'.

This term, 'bioassay', has a very wide usage, embracing almost any kind of test with organisms.

Tests giving direct or indirect responses may be used.

Bioassays in the strict sense of the word, as they are considered in this Manual, measure direct responses. Indirect response tests will be mentioned as a matter of interest.

3.1.1 Direct response tests

(a) Toxicity tests

The purpose of toxicity tests is to determine the concentration which is just sufficient to produce, after a given time of exposure, a certain response, indicative of toxicity (death, immobilization, impairment of reproduction, etc.). These tests will also serve to determine the speed and degree of response. They may be static or continuous-flow.

A static bioassay is performed without continuous renewal of solutions tested. They may or may not be periodically renewed. Renewal is necessary with some toxicants which change considerably. Static bioassays may be considered superior to continuous-flow tests when it is necessary to have the test medium 'age' for detection and measurement of latent toxicity. They are also useful in the evaluation of sporadic or accidental discharges with high initial concentrations.

In a continuous-flow bioassay the test solutions are continuously or nearly continuously added to the water flow as to easily maintain constant concentrations of active toxicants.

(b) Repellence tests

These tests are used to measure the repellence or avoidance reactions of animals to a pollutant. They can also measure attraction to the pollutant. Their purpose is to determine anomalies and changes in the animals' behaviour. It is difficult to establish predictions from this data, and a number of precautions must be taken when so doing.

(c) Tests for bioaccumulation and trophic accumulation

These tests are necessary to measure the effects of pollutants which accumulate in the marine environment in general and in plant and animal tissues in particular. Even if the accumulation is not toxic at the beginning of the trophic chain, it may become toxic, to the point of causing death, as we go up the trophic chain of predators (or consumers).

Concentrations in water must be correlated not only with the responses of the organisms but also with measurements of accumulated pollutants in the tissues. Account must also be taken, as regards prey/predator relationships, of the risk for the latter of ingesting prey that is already contaminated.

(d) Biostimulation tests

These tests with algae detect and measure the likelihood of domestic wastes and chemical pollutants stimulating (or reducing) multiplication and growth of algae. Eutrophication and algal blooms are taken into consideration, as are their effects (beneficial or adverse) on the receiving waters.

(e) Ecological surveys

Field observations may be considered as an ultimate bio-evaluation to ascertain whether lethal or sub-lethal laboratory tests are of any real significance in the field. Natural communities are monitors with time of the effects of discharges (and overall pollution).

The investigations take account of the analysis of the communities, in accordance with criteria traditionally used under the circumstances and observation of the evolution of these communities can provide valuable information on the possible influence of pollution (or pollutants) on the communities observed.

Alternatively, small artificial ecosystems may be set up, or small natural ecosystems may be manipulated. These methods of bio-evaluation are, however, time-consuming and complex.

3.1.2 Indirect response tests

These tests are not covered in this Manual.

Special precautions must be taken with organoleptic tests. Human 'tasters' are used as 'bioassay organisms'. These tests must be carried out as part of food studies.

Indirect effects may be obtained with biostimulation tests: the appearance of toxins in fish after 'red tides', deoxygenation of algal blooms.

Field studies and checks can show the indirect actions of the modification of the substrate, of variations in dissolved oxygen content, etc.

3.2 Test Procedures

3.2.1 Selection of organisms

The choice of organism should depend on the aim of the test (and on the type of test finally selected), with attention being paid to a certain number of criteria pertaining to the test conditions and to the very specific character of the organisms.

General conditions for selection must take into consideration:

- the availability of the organisms;
- their resistance to laboratory conditions (if possible, their facility to adapt to or withstand stress);
- their size in relation to experimental set-up;
- biology (which must be known): life span, development cycle, feeding habits, behaviour, etc.;

- geographical distribution in the region considered (Mediterranean).

Conditions relating to the organisms themselves must also be taken into account:

- the taxonomic group of the organism (and its representativity for that group);
- the trophic level;
- the ecological significance (especially its value as indicator of the state of the environment, the extent, nature or absence of pollution, etc.);
- the development stage at which the organism can be used;
- the economic value (fish, crustacea, molluscs).

In general the mandatory or recommended species have been carefully chosen by the FAO(GFCM)/UNEP Expert Consultation held in Rome during 23 June - 4 July 1975. However, it is the 'compulsory' species which give most difficulty. (Difficult, or unfeasible, to collect *Mytilus galloprovincialis* or *Parapenaeus longirostris* locally).

The species selected for the bioassays described in this Manual are the following:

Capitella capitata (Fabricius), 1780 - (Fauvel, 1927)

Mytilus galloprovincialis (Lamarck), 1819 - (Fischer, 1973)

Tisbe bulbisetosa Volkmann-Rocco, 1972 - (Volkmann-Rocco, 1972)

Idotea balthica basteri Andouin, 1827 - (Tinturier-Hamelin, 1963)

Palaemon elegans Rathke, 1837 - (Zariquiey Alvarez, 1968)

Mugil auratus Risso, 1810 - (Fischer, 1973)

3.2.2 Collecting and keeping the selected species alive

The possibility of cultivating many species in the laboratory needs to be mentioned. In such cases, the entire life cycle of the species must be controlled.

Algae cultures are relatively frequent and many laboratories run permanent cultures of many strains of different species.

Invertebrate culture (and especially fish culture) seems to be more complicated and, above all, less frequently done. Various species of polychaetes belonging to the *Capitella*, *Scolelepis*, *Ctenodrilus*, *Nereis* genuses, or crustacea (*Jaera*, *Idotea*, *Tisbe*), etc., can however be raised in the laboratory. Starting with a very small number of organisms and preferably from a single couple (even a single organism, *Ctenodrilus serratus*), veritable clones are obtained. These are very useful and can easily be drawn from.

Collection from the natural environment is nevertheless still the rule. It is done with traditional gear as used for plankton and benthos or by commercial fishing methods. The main problem consists in collecting these organisms with the utmost care so that they may be in as good a condition as possible. Care taken during collection must extend through transport to the laboratory. Any contact with the atmosphere must be avoided and the animals must be given all the water (and oxygenation) they need by renewing the water as often as possible. Any considerable rise in temperature must be avoided during transport.

In the laboratory, the animals must be transferred to tanks or containers of suitable size as they must not be over-crowded. Changes in quality, and especially temperature, of the water, must be gradual until conditions as close as possible to the natural environment are reached, which will be kept throughout the holding period. These conditions will prevail for at least seven days and will differ according to the species and the care (feeding, oxygenation, etc.) to be given to the animals. On arrival in the laboratory, all the animals which show signs of being in an unsatisfactory condition, of being injured or parasited, will be removed. It is useful to check, in a sample of the test population, that the animals have not been contaminated prior to being collected. This applies especially for the substances to which they will be subjected during the tests. This could be done by prolonging the holding period.

At the end of the holding period the animals must be transferred to normal holding tanks and they must be gradually acclimated to future test conditions. The time this will take depends on the type of organism and the extent of the changes in the quality of the water. The holding tanks must be cleaned frequently (every day or every two days). It would be preferable to use a continuous flow of water, or change the water often.

The animals must be healthy and must be kept in a clean, controlled and stable environment.

3.2.3 Laboratory

The laboratory must be air-conditioned. In view of the large quantities of water being used, air-conditioning of the type used in offices is usually unsuitable. In order to avoid as much as possible variations in temperature, basement premises are often suitable. A stable temperature is absolutely necessary. It may be advisable to maintain the temperature all through the year slightly lower than the average for the water in the collection area. It might be necessary to conduct bioassays at selected temperatures and the temperature of the room could be adjusted to this level. Variations of temperature in the test room must be as small as possible and must be stretched gradually over long intervals. The temperature must be frequently checked (if possible with a recorder) at different points in the room and in the different test or holding media.

If tests are to be carried out at a temperature which differs from the room temperature or, more so, if tests are to be carried out at different temperatures, certain test media must be cooled or heated. The use of a 'double-boiler' system is advisable to obtain water at a perfectly stable temperature. It may be necessary to use a cryostat and a thermostat simultaneously. When continuous-flow systems are used, heating or cooling of the water at the tank outlet before it mixes with the pollutant may be necessary, especially when the flow is considerable.

Seawater input could be through PVC pipes, which must first of all be proved to be harmless. This rule is valid for all materials used in the tests.

Aeration of the test rooms, especially the small ones, must be satisfactory. Neon bulbs should be used preferably for lighting. A device for simulating the type and periods of 'day light' and 'darkness' corresponding to the rhythm of the seasons (or any other rhythm) must be provided.

The different air-conditioning, aeration and lighting systems must be automatic as far as possible and a suitable alarm system must warn of any break-down.

Test media conditions (especially temperature, lighting) must be strictly controlled.

3.2.4 Test medium

In principle, the test media must meet a certain number of criteria as regards temperature, dissolved oxygen concentration, salinity, pH, etc., which correspond to those of the natural environment. They must, therefore, change as the natural environment does. It must always be possible to bring about changes to the test medium, or at least to set up test conditions which meet the different variable parameters of the water quality in the natural environment.

The first problem that arises concerns the properties of sea water. Either natural or artificial sea water may be used.

In spite of its cost, artificial sea water is attractive since it can be readily obtained at the desired salinity and, in principle, pure. However, it presents some disadvantages, such as the difficulty to dissolve certain salts or the presence of unexpected chelating agents.

Natural sea water is often much more easily obtainable, costs little and is available in almost unlimited quantities. Unfortunately, many laboratories are supplied with sea water via tubes which draw up the water in areas which are contaminated or polluted by substances which are being investigated or which may have synergistic or antagonistic effects.

Under such conditions the water cannot be used for bioassays. It must be taken from the open sea, far from any source of pollution. This increases costs and causes storage problems.

The use of artificial sea water allows for easy adjustment of the required salinity. The salinity must, however, be checked once the salts have dissolved and when this water is stored or used several days after it has been prepared, periodic checks must be carried out.

The salinity of natural sea water must also be checked and recorded systematically (including during tests, when there is even only a slight risk of evaporation). To reduce the salinity of sea water, it must simply be diluted with distilled or totally demineralized water. To increase the salinity, moderate heating, with slow evaporation, may be used, or evaporation by fast and prolonged boiling, which gives a highly saline liquid. Addition of distilled water suffices to reduce the salinity to the desired level. Sea water becomes concentrated by successive deposits of salts and the quality of dissolved salts will, of course, not be the same as that encountered at normal salinity. In this case, if the salinity is measured with a salinity measuring bridge (electrolytical conductivity measurement), considerable differences from the readings obtained by means of the traditional chemical determination method may be found. This may be remedied in part by mixing a highly concentrated sea water (e.g. 100‰) with sea water at normal salinity. Salinity measured by means of the chemical method is also extrapolated (by calculation) from the chlorinity determination.

Other characteristics of the quality of the environment may be changed, either voluntarily or otherwise and must therefore be checked. pH and dissolved oxygen fall into this category.

Aeration is generally used to maintain the concentration of dissolved oxygen at normal level. There are devices which enable oxygen concentration to be checked automatically.

The sea water must be very pure. Salinity, temperature, concentration of dissolved oxygen and the pH of the test medium must be regularly checked.

3.2.5 Test equipment

3.2.5.1 Static tests

The shapes and sizes of the vessels used vary considerably. They may be made of glass, plastic, fibreglass or even wood coated with plastic.

Indeed, the first criterion to be taken into consideration is the adequacy of the vessel: size of vessel/size of organism. The second criterion concerns the material the vessel is made of. Vessels must not themselves be toxic. In some cases they must be weathered prior to being used (e.g. they must be soaked for several weeks in sea water which is frequently renewed so that they may release the soluble substances which could be toxic; fibro-cement tanks are among them).

Care must also be taken to prevent the toxic products to be tested from being adsorbed on the walls. If adsorption cannot be avoided or controlled, allowance must be made for this, especially by renewing the solutions more frequently. Glass or plexiglass vessels are the most reliable from this point of view, but, unfortunately, they are costly and fragile. Increasing use is being made of polystyrene vessels. For tests with algae (and particularly for biostimulation tests), tubes, Erlenmeyer flasks, culture flasks and Petri dishes are used.

For tests with animals, Petri dishes of different sizes, which are easy to manipulate and which allow for examination with a low-power stereomicroscope (or binocular lens), are placed in beakers, jars or dishes. These vessels must be carefully covered (even corked) so as to prevent evaporation of the solutions as far as possible. One specimen per vessel is preferable, in view of the tendency of many species to agglomerate, become tangled and to cannibalism. In addition, dead or dying specimens quickly alter the environment and artificially contaminate the other specimens. In any case, the number of specimens per vessel will depend on the size of the vessel.

It is often necessary to provide means or systems to ensure survival or, more simply, to isolate the specimens. Distribution of specimens in the different test series must be in accordance with strict statistical criteria to obtain reliable results: the sub-samples which are subjected to several tests must be as homogeneous as possible as regards size, sex, age (or physiological stage).

Renewal of the media - The renewal of the media is a first necessity during static tests to reduce damage which may be caused by the changes which occur (variation of salinity, dissolved oxygen, pH, changes in the pollutant, excretions of the organisms' metabolism, etc.).

For tests which exceed the usual 96 hours, this renewal is absolutely necessary, unless the volume of the solution containing the animals is very large in relation to the number and size of the animals. It must be done with the utmost care and presents great difficulties with small animals (e.g. zooplankton or larval stages), since filtration using fine-meshed gauze is not easily and safely done.

The organisms may be transferred from one vessel to another using a pipette, or may be caught with pincers, small nets or any other device unlikely to cause the loss of the animals or to injure them.

If the same vessel is used for the whole of the bioassay, only part of the volume of the medium (from 1/3 to 3/4) need be replaced.

Renewal must be done at least twice a week, but even every second day or daily. Care must be taken to remove (usually by means of a pipette) any debris, food remains, excretions, etc., from the vessel. This is most often desirable even for short-term tests which do not require renewal of the medium.

Feeding - For long-term (or medium-term) bioassays and for certain bioassays on larvae, even though they last only a few days, the organisms must be fed.

The food varies with the organism. There is generally a tendency to overfeed the organisms; it is obvious, however, that the unabsorbed or wasted food cannot but alter the test environment.

Nutrients are provided for algae. Zooplankton and larvae are usually fed with planktonic algae. Their mass cultivation in the laboratory is now a technique that has been totally mastered.

3.2.5.2 Continuous-flow tests

Continuous-flow tests are highly desirable for chemical pollutants which have a high oxygen demand or are unstable or volatile. They are also ideal for large species or for those with a high metabolic rate.

Continuous-flow tests require dosing pumps and various other equipment for administration of pollutants or substances to be tested to the dilution water before it reaches the vessels in which the tests are conducted. Satisfactory mixing of pollutants with sea water may prove difficult, especially when it is automatic and continuous. Furthermore, it is sometimes necessary to provide various stirring systems to keep the solution homogeneous (but this is also valid for static tests).

The basic arrangement for a continuous-flow bioassay system comprises a large vessel containing sea water and a smaller vessel containing the pollutant (or a very concentrated solution of the pollutant). Any device (from the simplest to the most sophisticated) may be used to mix them in the proportions defined beforehand to obtain a solution for tests at desired concentrations which would then be brought to the vessels where the actual tests are carried out.

The materials and utensils used must be as inert as possible. They must never be made of a substance that is to be tested.

3.2.6 Introduction of pollutants

The introduction of pollutants often requires prior preparation. For continuous-flow tests, a certain number of operations are carried out automatically, but it may nevertheless be necessary to carry out certain operations prior to the pollutant being introduced into the special tank.

For static tests, pollutant solutions must be prepared in advance, so that the required quantities may be added to the sea water to obtain the different concentrations desired. It must be borne in mind that the pollutants may be unstable. All solutions intended for the same series of tests must be made from the same parent solutions.

It is difficult to dissolve some pollutants, such as petroleum and oil, uniformly in the test solution. Constant agitation is required. The same applies for tests with insoluble solid particles, however small.

The manner in which tests are conducted and pollutants introduced, mixed or maintained in homogeneous suspension and renewed depends on the various changes which may occur in the solution to be tested and in the test medium during the test. In order to reflect the frequent concentration changes of pollutants in effluents, it is often required to simulate those variations experimentally, i.e. alter temporally the concentration of a pollutant in the course of the same experiment. Of course, these variations are more easily obtained with a continuous-flow system which allows for the irregular introduction of the pollutant into the test medium.

It must be noted that it may be necessary to use solvents (preferably non-toxic or only slightly toxic) to disperse or dissolve the pollutant. The toxicity of the solvent must therefore be tested or two controls must be prepared, one with no additive and the other with the highest used concentration of the additive (solvent).

When the solution is highly toxic, the measured quantity of the pollutant must not be added directly to the test vessel. It may be diluted in a fraction of the volume of sea water from the vessel which will be later added to the vessel. Likewise, when a large number of vessels is used, it may be useful to fill them with only half of the necessary water, to add the animals, then to complete the medium by adding the same volume of a solution, the concentration of which is double that required.

Table I may be useful in determining the volume of the suitable parent solutions (or dilutions) to be added.

Table I

Dilutions in relation to concentrations of test solutions

Concentration of test solution			Volume of parent solution to be added to sea water per litre of test solution (ml)				
%	mg/l	µg/l	100 g/l	10 g/l	1 g/l	0.1 g/l	0.001 g/l
1.0	10 000		100				
0.56	5 600		56				
0.32	3 200		32				
0.18	1 800		18				
0.10	1 000		10	100			
0.056	560		5.6	56			
0.032	320		3.2	32			
0.018	180		1.8	18			
0.010	100		1.0	10	100		
0.0056	56			5.6	56		
0.0032	32			3.2	32		
0.0018	18			1.8	18		
0.0010	10			1.0	10	100	
0.00056	5.6				5.6	56	
0.00032	3.2				3.2	32	
0.00018	1.8				1.8	18	
0.00010	1.0	1 000			1.0	10	100
0.000056	0.56	560				5.6	56
0.000032	0.32	320				3.2	32
0.000018	0.18	180				1.8	18
0.000010	0.10	100				1.0	10
0.0000056	0.056	56					5.6
0.0000032	0.032	32					3.2
0.0000018	0.018	18					1.8
0.0000010	0.010	10					1.0

Source: American Public Health Association, American Water Works Association, Water Pollution Control Association, © 1976, with kind permission of the publisher

4. THE TESTS

4.1 Short-term Test with *Capitella capitata*

- Species: *Capitella capitata*

- Pollutant: Cadmium chloride

- Initial solution: 1 000 mg/l of cadmium in deionized water

- Preparation of initial solution (parent solution): Dissolve 1.63 g of cadmium chloride in 1 l of deionized water. If possible, check the concentration by atomic absorption spectrophotometry.

- Concentrations to be used: 1.0; 3.2; 5.6; 10.0 and 32.0 mg/l; gradual bisection of intervals on a logarithmic scale. If the toxicity of the pollutant is unknown, prepare a preliminary (or screening) test with wide concentration intervals, e.g. 1 000; 500; 100; 50; 25; 10; 5; 1; 0.5; 0.25; 0.10 mg/l (some of the lower concentrations may be eliminated, etc.), plus a control batch. On completion of this test, the concentrations for the actual bioassay will be selected.

- Preparation of concentrations: Concentrations for bioassays will be prepared in 1 l Erlenmeyer flasks, from a 1 000 mg/l cadmium parent solution. The cadmium test solutions will be prepared as follows:

Final cadmium test concentration (mg/l)	Parent solution at 1 000 mg/l	
32.0	32.0	
10.0	10.0	
5.6	5.6	Adjust to 1 l with sea water
3.2	3.2	
1.0	1.0	

  Provide a control with pure sea water (without cadmium chloride).

- Sea water source: Artificial or natural sea water. The natural sea water must be taken from a non-polluted area and must be filtered through a membrane of 0.45 µ. Adjust the salinity, if necessary.

- Temperature: Must be recorded daily, must be constant and not exceed 20°C, if possible.

- Light: 12 hour day/night cycle

- Food: No food

- Test vessels: Plastic (polystyrene), disposable Petri dishes, diameter 100 mm, height 20 mm. As far as possible, all the Petri dishes will be new and will have been rinsed with deionized water or filtered sea water (not tap water). The respective concentration will be marked on each dish.

- Procedure:

  (1) Place *Capitella* taken from the acclimated stock in large glass dishes which have been washed in diluted nitric acid and rinsed. If possible,

let the worm come out of its tube spontaneously, by e.g. tapping one extremity of the tube, so as to reduce handling.

(2) Transfer the worm to a Petri dish containing sea water. Several worms may be put in the same Petri dish.

(3) Examine each worm through a low-power stereomicroscope. Remove all injured worms and egg-carrying females.

(4) Use 20 worms per concentration. With one worm per Petri dish, there will be 20 dishes per concentration. Random distribution of the specimens in the Petri dishes is desirable. Fill the dishes with 50 ml of the solution at the chosen concentration (50 ml sea water for the controls).

(5) Duration of tests: 96 hours without renewal of test medium.

(6) Observations (using low-power stereomicroscope) after 24, 48, 72 and 96 hours. *Capitella* is considered dead when it fails to move when lightly touched. If the worm is severed, the specimen will be considered dead if the front part is dead. No account will be taken of the state of the hind part. Data are recorded on suitable charts. It is also useful to record the presence or absence of tube and faecal pellets.

4.2 Long-term Test with *Capitella capitata*

- Species: *Capitella capitata*
- Pollutant: cadmium chloride
- Initial solution: 1 000 mg/l of cadmium in deionized water
- Preparation of initial solution (parent solution): Dissolve 1.63 g cadmium chloride in 1 l deionized water. If possible, check the concentration by atomic absorption spetrophotometry.
- Concentrations to be used: 0.1; 0.32; 0.56; 1.0 and 3.2 mg/l; gradual bisection of intervals on a logarithmic scale
- Preparation of concentrations: Test concentrations will be prepared in 1 l-Erlenmeyer flasks from the 1 000 mg/l cadmium parent solution. New media will be prepared at each renewal throughout the tests (i.e. twice a week). The cadmium test solutions will be prepared as follows:

Final cadmium test concentration (mg/l)	ml parent solution at 1 000 mg/l	
3.2	3.2	
1.0	1.0	
0.56	0.56	Adjust to 1 litre with sea water
0.32	0.32	
0.10	0.10	

Provide a control with pure sea water (without cadmium chloride).

- Source of sea water: Artificial or natural sea water. Natural sea water must be taken from an area which has not been contaminated by pollution and must be filtered through a 0.45-μ membrane. Adjust the salinity, if necessary.

- Temperature: The temperature must be recorded daily. It must be constant and must not exceed 20°C, if possible.

- Light: 12 hour day/night cycle

- Food: During holding prior to the test (1 week ± 1 day) the *Capitella* will be fed with Tetramin (about 0.20 g/day per vessel of 4 to 5 l). During the tests, *Capitella capitata* will be fed with previously dried, then rehydrated *Enteromorpha*. The preparation of the food will be explained later.

- Test vessels: Plastic (polystyrene) disposable Petri dishes, diameter 10 mm, height 20 mm. As far as possible, all Petri dishes will be new and will have been rinsed with deionized water or filtered sea water (not tap water).

- Procedure:

  (1) Place the *Capitella* taken from the acclimated stock in large glass dishes which have been cleaned with diluted nitric acid and rinsed. If possible, let the worm come out of its tube spontaneously, by e.g. tapping one extremity of the tube, so as to reduce handling.

  (2) Transfer the worm to a Petri dish containing sea water. Several worms may be put into the same Petri dish.

  (3) Examine each worm through a low-power stereomicroscope. Remove all injured worms and ovigorous females.

  (4) Use 42 worms per concentration. With 3 worms per Petri dish, there will be 14 dishes per concentration. The worms must be distributed at random over the Petri dishes. Fill all the Petri dishes (including those of the control batch) with 25 ml sea water, distribute the worms at random, then add 25 ml of the cadmium solution at double the final concentration required (25 ml sea water for the control batch). Agitate to secure thorough mixture.

Final cadmium concentration (mg/l)	Add 25 ml of cadmium solution at mg/l
3.2	6.4
1.0	2.0
0.56	1.12
0.32	0.64
0.1	0.2

  Add food (*Enteromorpha*, see (6)) to each Petri dish.

  (5) The test will last 28 days. The test medium will be renewed twice a week. The Petri dishes will not be changed. Prepare 1 000 ml of each concentration required (3.2; 1.0; 0.56; 0.32 and 0.1 mg/l). Carefully empty the dishes taking care not to lose the worms. Remove excess food with pincers, but do not disturb a worm in an agglomerate of debris.

Fill the Petri dish with 50 ml of the suitable cadmium solution and add food (see (6)). The specimens will be examined after 24, 48, 72 and 96 hours, then twice a week, when the test medium will be renewed (see (6)). All observations will be conducted using a binocular lens. *Capitella* is considered dead if failing to move when lightly touched. Should the worm be severed, the specimen will be considered dead if the front part is dead. No account will be taken of the state of the hind part. Dead worms are removed from all Petri dishes during examination. Data are recorded on suitable charts. It is also useful to record the presence or absence of pieces of tube and faecal pellets.

Day		Duration	
Monday	1		Beginning of test
Tuesday	2	24 h	1st observation
Wednesday	3	48 h	2nd observation
Thursday	4	72 h	3rd observation
Friday	5	96 h	4th observation; change medium and food
Monday	8	7th day)	
Friday	12	11th day)	
Monday	15	14th day)	Observations must be carried out and
Friday	19	18th day)	medium and food changed on these days
Monday	22	21st day)	
Friday	26	25th day)	
Monday	29	29th day)	

(6) Food: Instructions to obtain enough food for each change of test medium, i.e. 5 concentrations plus the control (concentration nil): Weigh 1.42 g dried *Enteromorpha*. Place it in a grinder with 75 ml sea water. Grind until *Enteromorpha* is reduced to small pieces of 2 to 3 mm and until there are no tufts. Dry *Enteromorpha* briefly on gauze. Re-weigh when damp and divide into 6 equal portions. Fill 6 jars, 5 with 15 ml of each test concentration and one with sea water for the control. Add *Enteromorpha* to each jar and mix. Using a pipette, put 1 ml of the appropriate food into each Petri dish. To administer as precisely as possible the same quantity of food in each dish, pipette 1 ml only each time and shake the jar each time before refilling the pipette. Use a 10-ml plastic pipette after having cut off the tapered end. *Enteromorpha* must be collected from low-contamination areas.

4.3 Short-term Mortality Test with Mussels, *Mytilus galloprovincialis*

- Species: *Mytilus galloprovincialis* (specimen size between 2.5 and 4 cm)
- Pollutant: Cadmium chloride
- Initial solution: 1 000 mg/l cadmium in deionized water
- Preparation of initial solution (parent solution): Dissolve 16.3 g cadmium chloride in 10 l of deionized water. If possible, check the concentration by atomic absorption spetrophotometry.
- Concentrations to be used: 1.0; 3.2; 5.6; 10.0 and 32.0 mg/l. Progressive bisection of intervals on a logarithmic scale, plus a control. If the toxicity of the substance is unknown, a preliminary (or screening) test must be carried out using wide concentration differences: 1 000; 500; 250; 100; 50; 25; 10; 5; 2.5; 1.0; 0.5; 0.25 and 0.10 mg/l, plus a control.
- Preparation of concentrations: Concentrations for bioassays are prepared in 10-l demi-johns from a 1000 mg/l cadmium solution. The cadmium solution will be

prepared as follows:

Final cadmium concentration (mg/l)	ml parent solution at 1 000 mg/l	
32.0	320.0	
10.0	100.0	
5.6	56.0	Adjust to 10 litres with sea water
3.2	32.0	
1.0	10.0	

Provide a control with pure sea water (without cadmium chloride).

- Source of sea water: Natural or artificial sea water. Natural sea water must be drawn up from an uncontaminated area and be filtered through 0.45 µ membranes. Adjust the salinity, if necessary.

- Temperature: Must be recorded daily, must be constant and must not exceed 20°C, if possible.

- Light: 12 hour day/night cycle

- Food: No food

- Vessels: Preferably 3 l-glass (or polyethylene) vessels. The vessels will be rinsed with diluted nitric acid, then with deionized water or filtered sea water. Provide a cork or cover to fit the vessel. Mark the respective concentration on each vessel.

- Procedure:

  (1) Cautiously brush mussels taken from the acclimated stock. Care must be taken not to damage byssus.

  (2) Transfer the mussels to large glass containers that have been cleaned in diluted nitric acid and rinsed with deionized water or filtered sea water. Adjust salinity, if necessary. A large number of mussels may be placed into the same container.

  (3) Check that the mussel shells are tightly closed and cannot be opened by hand. Prick the edge of the mantle of those that are open lightly and briefly with a tapered glass tube. Keep only the specimens which close immediately.

  (4) Use 20 mussels per concentration: 5 specimens per test vessel will give 4 vessels per concentration. The mussels must be distributed in the vessels at random. Fill the vessels with 1 l of the solution at the desired concentration (1 l filtered sea water for the control). Provide gentle aeration.

  (5) Duration of test: 96 hours (may be extended to 144 hours). There will be no renewal of the test medium if the test does not exceed 96 hours.

(6) Observatiors will be carried out after 24, 48, 72, 96 hours (120 and 144 hours if necessary). *Mytilus* will be considered dead when there is no reaction to stimulation with a tapered glass tube of the mantle fringe of the open mussels. The first prick will be light and will be followed by another, more pronounced, if the animal does not react. Any specimen considered dead will be removed immediately. Checks made at 12-hour intervals will be sufficient. Animals which die between checks will be considered to have died after 24, 72 or 96 hours. Data will be recorded on suitable charts.

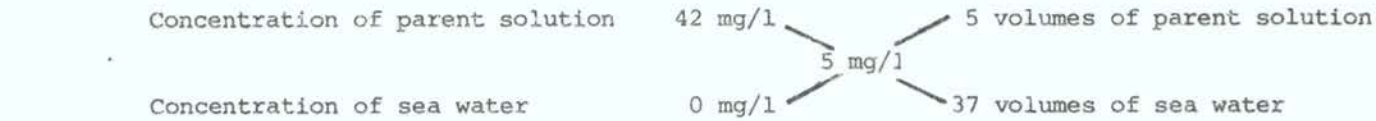
4.4 Short-term mortality test with *Tisbe bulbisetosa*

- Species: *Tisbe bulbisetosa*, crustacean, harpacticoid copepod, approximately 1 mm length.

- Pollutant: Kuwait oil

- Preparation of parent solution: 1 ml of Kuwait oil and 0.2 ml of Corexit 7664 [1/] are added to 1 000 ml of filtered sea water of 35 ± 1°/oo salinity and stirred for 5 hours with a magnetic stirrer at ambient temperature (about 20°C). The speed of the stirrer is adjusted to that the vortex does not exceed 25% of the distance between the surface of the liquid and the bottom of the vessel. The suspension will then be poured into a decantation flask with a glass stopper and left to rest for 5 to 10 days so that part of the oil may stratify on the surface (Dalla Venezia and Fossato, 1977).

- Concentrations to be used: The desired concentrations are prepared by diluting the parent solution with sea water. If the analysis shows that the hydrocarbon concentration of the parent solution is 42 mg/l and if a concentration of 5 mg/l is required, the volumes of suspension and sea water to be mixed could be calculated as follows:



In addition, two controls must be used: A solution of 0.2 mg/l Corexit 7664 in sea water and sea water.

- Source of sea water: Natural sea water, collected from the open sea, far from sources of pollution and stored in vessels fitted with a continuous flow filtering system using charcoal and glass-wool filters as well as sterilizing UV lamps. Before being used the water will be membrane filtered through a Jena glass funnel, fitted additionally with two sheets of common filter paper (i.e., Rapida A or Opale 3002).

- Temperature: The animals will be stored in thermostated chambers at 18 ± 1°C.

- Light: 12 hour day/night cycle

- Food: Every two days, 0.5 ml *Dunaliella* suspension and small pieces of boiled wheat, for each 20 ml dish.

- Test vessels: 20 ml and 100 ml culture dishes, covered with glass discs of adequate size.

1/ Corexit 7664, a low toxicity, tensioactive sustance, is added here to facilitate suspension of the oil in sea water and to make it more stable over time.

- Procedure:

(1) Samples of the genus *Tisbe* can be collected with a fine meshed net among shallow water algae and placed in small vessels with sea water. A few hours afterwards, as oxygen diminishes, *Tisbe* migrates to the surface and can be easily gathered by means of a glass pipette, transferred to another glass vessel and then separated by species. The vessels, having a 3 l capacity contain sea water and are stored in thermostated chambers. It is important to add a thallus of fresh *Ulva* to each vessel to ensure oxygenation of the water. An adequate quantity of food is administred once a week. Since *Tisbe* has a short life-cycle, of between 15 and 20 days according to the species, the sampled specimens will soon generate mono-specific stocks, which will keep increasing in number and will supply as many individuals as are necessary for the tests, all already acclimated.

(2) Thirty to forty ovigorous females are taken from the stock and each placed in a 20 ml vessel with sea water, a piece of *Ulva*, a fragment of boiled wheat and, if necessary, 0.5 ml of *Dunaliella* suspension.

(3) The animals will be observed daily. When the nauplii have hatched from the eggs, the females will be removed from the dishes and the date of birth of the nauplii will be recorded on the lid.

(4) The nauplii will be checked at least every two days until the 8th to the 10th day. They will then be ready for mating.

(5) For mating the offspring of 30 dishes will be treated as follows: Divide the dishes in 2 groups of 15. Assemble the females of the first group and the males of the second group and vice versa. Place 30 specimens in each 100 ml vessel for mating. During mating, females and males remain "tail to tail" together for one or more days.

(6) In the experiments, ovigorous females after mating will be used (this procedure is necessary, to obtain females that are all at the same physiological stage). The males used for mating have all reached the same stage of maturity and can also be used in the tests.

(7) At this stage, ten 20 ml dishes are prepared for each concentration to be tested and for each control (two). The vessels of each group are numbered from 1 to 10 with different colours. Add a fragment of *Ulva* and a little piece of boiled wheat to each dish. To secure that the *Tisbe* in the different treatments are distributed at random, all the vessels bearing the same number but containing different solutions will be lined up and placed in numerical order. 1 *Tisbe* will be placed in each dish in the line. The operation will be repeated until there are 5 specimens in each vessel.

(8) Duration of the test: One week (or more depending on the resistance of the animals). The animals will be observed after 24, 48, 72, 96 hours, etc.

(9) The dishes are checked daily. All dead specimens are counted and removed. If 50 specimens are used for each treatment, as indicated above, the cumulative mortality calculation expressed in percentages can be done quickly every day.

4.5 Long-term test with *Tisbe bulbisetosa*

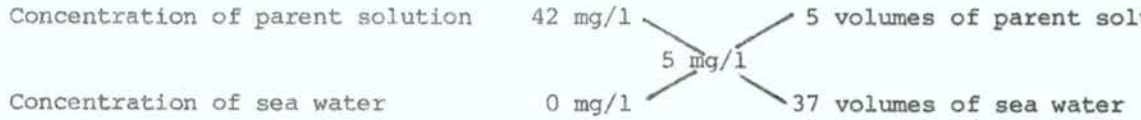
- Species: *Tisbe bulbisetosa*, crustacean, harpacticoid copepod, approximately 1 mm length

- Pollutant: Kuwait oil

- Preparation of the parent solution: 1 ml of Kuwait oil and 0.2 ml of Corexit 7664 (see footnote 1/ on page 16) are added to 1 000 ml filtered sea water of 35 ± 1°/oo salinity and are stirred for 5 hours with a magnetic stirrer at ambient temperature (about 20°C). The speed of the stirrer is adjusted so that the vortex does not exceed 25% of the distance between the surface of the liquid and the bottom of the vessel. The suspension will then be poured into a decantation flask with a glass stopper and left without agitation for 5 to 10 days so that part of the oil may stratify on the surface (Dalla Venezia and Fossato, 1977).

- Test concentrations (suspensions) to be used: Sub-lethal concentrations of hydrocarbons of less than 5 mg/l and 0.02 ml Corexit 7664 (see footnote 1/ on page 16).

- Preparation of concentrations: The concentrations are prepared by diluting the parent solution with sea water. If analysis shows that the hydrocarbon concentration of the parent solution is 42 mg/l and if a concentration of 5 mg/l is required, the volumes of suspension and sea water to be mixed could be calculated as follows:



In addition, 2 controls must be used: a solution at 0.2 mg/l Corexit 7664 in sea water and sea water.

- Source of sea water: Natural sea water, collected from the open sea, far from sources of pollution, stored in vessels fitted with a continuous-flow filtering system using charcoal and glass-wool filters as well as sterilizing UV lamps. Before being used, the water will be membrane filtered through a Jena glass funnel, fitted additionally with two sheets of common filter paper (i.e., Rapida A or Opale 3002).

- Temperature: The animals must be kept in thermostated rooms at 18 ± 1°C.

- Light: 12 hour day/night cycle

- Food: A piece of *Ulva* and every 2 days a few pieces of boiled wheat and *Dunaliella*, depending on the size of the test vessel.

- Test vessels: 100 and 200 ml dishes

- Procedure:

  (1) A certain number of ovigorous females taken from the acclimated stock are distributed at random in the 100 ml dishes containing sea water of 35 ± 1°/oo salinity with the different hydrocarbon concentrations and the Corexit 7664 solvent. A piece of *Ulva* is added to each vessel.

  (2) Every 10 to 12 days, the animals will be transferred by means of a glass pipette to other vessels with new suspensions, prepared at least 5 days previously. Considering the duration of the life cycle of *Tisbe*, after about 1 month of treatment, the experimental stock has been subjected to the pollutant for three generations. After 45 to 50 days, there are 4 generations and so on. The generation to be investigated is chosen after about 30 or 45 days at the most. The females are isolated for mating and after a few days they will produce their first egg sac and be transferred to another vessel with the same pollutant concentration.

(3) When the eggs in the sac are ripe, the ovigorous females from each concentration will be divided at random into 2 groups. Those in the first group will be killed and the eggs contained in the sac counted. Those in the second group will be isolated, separately, in 20 ml vessels until the hatching of the nauplii which subsequently are counted. The animals must not be fed, to avoid the little nauplii being confused with pieces of food and debris.

(4) Observations to be made:

(a) 1st group: the female is placed on a slide in a drop of glycerine. Under a low power stereomicroscope, the sac is opened with 2 platinum needles mounted on a glass tube, and the eggs are counted.

(b) 2nd group: as soon as the female has spawned the eggs, she can be removed from the vessel to facilitate counting of the nauplii that hatch from the eggs. They will be taken up with a pipette and counted one by one.

(5) The results will give:

(a) the average number of eggs produced in the first sac by females of a stock which has lived for n generations in a given pollutant,

(b) the average number of nauplii produced in the first sac by females of the same generation,

(c) the hatching percentage, that is the ratio between the number of nauplii and the number of eggs obtained under the same test conditions.

4.6 Short-term mortality test with the isopod *Idotea balthica basteri*

- Species: *Idotea balthica basteri*

- Pollutant: Cadmium chloride

- Initial solution: 1 000 mg/l cadmium in deionized water.

- Preparation of initial solution (parent solution): Dissolve 16.3 g of cadmium chloride in 10 l of deionized water. If possible check the concentration by atomic absorption spectrophotometry.

- Concentrations to be used: 1.0; 3.2; 5.6; 10.0 and 32.0 mg/l; progressive bisection of the intervals on a logarithmic scale, plus one control.
If the toxicity of the pollutant is unknown, a preliminary (or screening) test must be carried out using wide concentration differences, i.e. 1 000; 500; 250; 100; 50; 25; 10; 5; 0.25; 0.10 mg/l (some lower concentrations may be eliminated); plus a control batch. On completion of this test, the concentrations for the actual bioassay will be selected.

- Preparation of concentrations: The concentrations for the bioassays will be prepared in 10 litre glass demi-johns from the 1 000 mg/l cadmium parent solution. The cadmium dilutions must be prepared as follows:

Final cadmium concentration (mg/l)	ml parent solution at 1 000 mg/l	
32.0	320	
10.0	100	
5.6	56	Adjust to 10 litres with sea water
3.2	32	
1.0	10	

Provide a control with pure sea water (without cadmium chloride).

- Source of sea water: Artificial or natural sea water. Natural sea water must be taken from a zone which is uncontaminated and must be filtered through a 0.45µ membrane. If necessary, adjust the salinity.

- Temperature: Must be recorded daily, must be constant and must not exceed 20°C, if possible.

- Light: 12 hour day/night cycle

- Food: No food

- Test vessels: 500 ml glass jars with lids. All the jars will be rinsed with diluted nitric acid, then with deionized water or filtered sea water (not tap water). The respective concentration will be marked on each jar.

- Procedure:

  (1) Place the *Idotea* taken from the acclimated stocks into large glass beakers which have been previously washed in nitric acid and rinsed with deionized water or filtered sea water (not tap water). Add fragments of *Posidonia* leaves or pieces of dried *Codium*. Young adult animals will be chosen.

  (2) Transfer the isopod to a jar containing suitable sea water. If *Posidonia* or *Codium* are added, several isopods may be placed in the same jar.

  (3) Examine each animal under a low-power stereomicroscope. Remove the injured specimens and the incubating females.

  (4) Use 20 specimens of the same sex per concentration. One *Idotea* per jar will give 20 jars per concentration. Fill the jars with 450 ml of the chosen pollutant concentration (450 ml sea water for the control). Random distribution of the specimens in the vessels is desirable.

  (5) Duration of test: 96 hours

  (6) Observations will be carried out after 24, 48, 72 and 96 hours. The test medium will not be renewed.
  The *Idotea* will be considered dead when it fails to react to light touch of the pleopods with a mounted needle or with a tapered glass tube. If the animal does not react, to the first light touch, the second will be more pronounced.

4.7 Short-term mortality test with the shrimp *Palaemon elegans*

- Species: *Palaemon elegans* (size of specimens: about 2 to 3 cm)

- Pollutant: Cadmium chloride

- Initial solution: 1 000 mg/l cadmium in deionized water

- Preparation of initial solution (parent solution): Dissolve 16.3 g of cadmium chloride in 10 litres of deionized water. If possible, check the concentration by atomic absorption spectrophotometry.

- Concentrations to be used: 1.0; 3.2; 5.6; 10.0; 32.0 and 56.0 mg/l; progressive bisection on a logarithmic scale, plus a control. If the toxicity of the substance is totally unknown, a preliminary (or screening) test must be conducted before using wide concentration intervals: 1 000; 500; 250; 100; 50; 25; 10; 5; 2.5; 1.0; 0.5; 0.25; and 0.10 mg/l, plus a control.

- Preparation of concentrations: Concentrations for the bioassays will be prepared in 10 l-glass demi-johns from a 1 000 mg/l cadmium parent solution. The cadmium dilutions will be prepared as follows:

Final cadmium test concentration (mg/l)	ml parent solution at 1 000 mg/l	
56.0	560.0	
32.0	320.0	
10.0	100.0	Adjust to 10 litres with sea water
5.6	56.0	
3.2	32.0	
1.0	10.0	

This procedure has to be repeated at least 7 times to prepare the minimum volume of test concentration to fill the vessels. Particular care must be taken to secure uniform test media within one series.
Provide a control with pure sea water (no cadmium chloride).

- Source of sea water: Artificial or natural sea water. Natural sea water must be drawn up from an unpolluted zone and must be filtered through 0.45µ membranes. If necessary, adjust the salinity. The water must be well aerated to give maximum oxygen saturation.

- Temperature: Must be recorded daily, must be constant and must not exceed 20°C, if possible.

- Light: 12 hour day/night cycle

- Food: No food

- Vessels: 5 l glass or polystyrene vessels with lids. All the jars will be rinsed with diluted nitric acid, then with deionized water or filtered sea water (not tap water). The respective concentration will be marked on each jar.

- Procedure:

  (1) Place the *Palaemon* taken from acclimated stocks in large glass vessels which have previously been washed in nitric acid and rinsed.

  (2) Transfer each shrimp to a small glass vessel containing suitable sea water.

  (3) Examine each animal under a binocular lens. Remove all injured specimens as well as incubating females.

  (4) Use 20 specimens per concentration. One *Palaemon* per vessel will give 20 vessels per concentration. Fill the vessels with 4 l of the solution at the chosen concentration (4 l sea water for controls). Random distribution of the specimens in the vessels is desirable. Check the dissolved oxygen concentration periodically. If necessary, provide gentle aeration for a short period, by means of an aerator, if dissolved oxygen concentration decreases by 25 to 30%.

  (5) Duration of test: 96 hours

(6) Observations will be carried out after 24, 48, 72 and 96 hours. The test medium will not be renewed.
Paleamon will be considered dead when it fails to react to a touch of the pleopods with a mounted needle or a tapered glass tube. If there is no reaction, to the first light touch, a second, more pronounced one will follow.

4.8 Short-term mortality test with the fish species *Mugil auratus*

- Species: *Mugil auratus*

- Pollutant: Cadmium chloride

- Initial solution: 1 000 mg/l cadmium in deionized water

- Preparation of initial solution (parent solution): Dissolve 16.3 g cadmium chloride in 10 litres deionized water. If possible, check the concentration by atomic absorption spectrophotometry.

- Concentrations to be used: 1.0; 3.2; 5.6; 10.0; 32.0 and 56.0 mg/l; progressive bisection of intervals on a logarithmic scale, plus a control. If the toxicity of the substance is totally unknown, a preliminary (or screening) test must be carried out beforehand, using wide concentration intervals: 1 000; 500; 250; 100; 50; 25; 10; 5; 2.5; 1.0; 0.5; and 0.10 mg/l, plus a control.

- Preparation of concentrations: Concentrations for the bioassays will be prepared in 10 l-glass demi-johns from a 1 000 mg/l solution of cadmium. The cadmium solutions will be prepared as follows:

Final test concentration (mg/l)	ml of parent solution at 1 000 mg/l	
56.0	560.0	
32.0	320.0	
10.0	100.0	Adjust to 10 litres with sea water
5.6	56.0	
3.2	32.0	
1.0	10.0	

Repeat procedure as often as necessary to fill all test vessels. Take care in preparation of test dilutions as to secure the same concentration within one series.
Provide a control with pure sea water (no cadmium chloride).

- Source of sea water: Artificial or natural sea water. Natural sea water must be taken from an unpolluted zone and filtered through 0.45µ membranes. If necessary, adjust the salinity. The water must be well-aerated to give maximum oxygen saturation.

- Temperature: Must be recorded daily, must be constant and must not exceed 20°C, if possible.

- Light: 12 hour day/night cycle

- Food: No food

- Vessels: 15 l polystyrene or glass vessel with lid. All the jars must be rinsed with diluted nitric acid, then with deionized water or filtered sea water (no tap water). The respective concentration will be marked on each jar.

- Procedure:

(1) Place the *Mugil* taken from acclimated stocks in large glass vessels which have been previously washed in nitric acid and rinsed.

(2) Transfer each fish to a small crystallizing dish containing suitable sea water.

(3) Examine each animal through low power stereomicroscope. Remove the injured animals.

(4) Use 20 specimens per concentration. One *Mugil* per vessel will give 20 vessels per concentration. Fill the vessels with 10 l of the solution at the chosen concentration (10 l sea water for control). Random distribution of the specimens in the vessels is desirable. Check dissolved oxygen concentration periodically. If necessary, provide gentle aeration for a short period, if dissolved oxygen concentration falls by between 25 and 30 percent.

(5) Duration of the test: 96 hours

(6) Observations will be carried out after 24, 48, 72 and 96 hours. The test medium will not be renewed.
Mugil will be considered dead when it fails to react to a touch of the gills and snout with a mounted needle or a tapered glass tube. If there is no reaction to the first light touch, the second one will be more pronounced.

5. EVALUATION AND PRESENTATION OF RESULTS

Numerous studies have been conducted on the evaluation of test results and they have resulted in various methods being developed.

We suggest that the statistical tests be limited to those listed, described and criticized in the "Manual of methods in aquatic environment research. Part 5 - Statistical Tests" (by F. Möller, FAO Fisheries Technical Paper No. 182, 131 p., Rome, 1979).

The results obtained can always be presented in table form, giving, for mortality bioassays, the cumulative percentages of deaths for each concentration at each observation time. It is nevertheless often preferable to present test results in codified form which is widely accepted (e.g. lethal time, lethal concentration).

In this Manual, it is suggested that the notion of lethal concentration 50 percent (LC_{50}) (and its derivatives lethal concentration 10 percent, lethal concentration 90 percent), be used. i.e. the pollutant concentration at which 50%, 10% or 90% respectively of the test animals die within a given period of time, frequently 96 hours.

The concentrations could be obtained precisely (in which case the confidence limits will be known) by means of the probit analysis (Bliss, 1935) and its derivatives.

The Bliss method applied to marine invertebrates (Stora, 1972) is recommended to express results obtained with different mortality bioassays. Details of this analysis are given in paragraph 5.1.

As regards the long-term test with *Tisbe bulbisetosa*, which is not a mortality test, it is suggested that the Kolmogoroff-Smirnov test be applied to estimate the significance of the results (cf. 5.2). Pages 40 and 41 and table A-7 on page 106 of Möller's publication should also be consulted (Möller, 1979).

5.1 The Bliss Method

The statistical treatment of data by Bliss' probit analysis (1935), modified by Bliss (1938) for tests using a small number of test animals, is particularly suitable for research on marine invertebrates.

It is based on the hypothesis that in the "dose-effect" relationship, effects which are proportional to the logarithms of concentrations, are normally distributed. If the percentages of mortality observed over a chosen period are plotted against the logarithms of the concentrations, a sigmoid curve, the integral of the Gauss curve is obtained. This curve does not lend itself well to calculation; when the percentages of mortality are transformed into probits, the sigmoid curve becomes linear.

The Bliss analysis which has a linear variation of probits of mortality in proportion to the logarithms of concentrations used, consists in establishing a regression in the form of $Y = \bar{y} + b\ (X - \bar{x})$ and presents numerous advantages. On one hand, it enables a small number of test animals to be used, since it is often difficult within an ecosystem to collect a large number of specimens of the same species. On the other hand, the number of animals used per concentration can vary, which makes Bliss' method particularly useful for studying the sensitivity of colony-forming species which cannot be easily divided into equal batches.

In spite of a holding period designed to minimize the effects of stress caused by removal from their natural environment, it is not unusual that a few specimens that are placed in artificial living conditions die in the control batches to which no pollutants have been added. A certain number of natural deaths of the animals can be allowed for in the determination of the regression line, without this causing the test data to be rejected.

The χ^2 test, used in the Bliss method checks the legitimacy of the linear adjustment. As Lazar and Lellouch (1969) point out, the purpose of this test is not to show that the regression of effect against dosage is linear, but only that the test data do not reject this hypothesis, which has the advantage of simplifying research on lethal concentrations. If the χ^2 test is significant, it is possible that the mortality of the specimens, in proportion to the concentrations of the pollutants, does not resemble a Gauss distribution. A significant deviation from the normal distribution as suggested by the χ^2 test may be related to the heterogeneity of the population tested, or simply to a manipulation error. Through the χ^2 calculation, we can assess whether the test is going well.

When the regression line has been calculated, the lethal concentration and its confidence interval can be obtained for any given percentage of deaths (except for 0 and 100 percent).

5.1.1 Calculation

(a) Transformation of concentrations

For concentrations lower than 1, depending on the case, one or several units are added to the calculated logarithm to avoid obtaining negative values, or the measurement unit is changed (from g to mg, from mg to µg).

(b) Transformation of number of deaths into percentages

Correction of mortality percentages allowing for natural mortality

Natural mortality can occur among the control specimens. If the natural mortality does not exceed 20%, Abbot's formula (1925) is used to correct pollutant mortality:

$$\% \text{ corrected mortality} = \frac{P_o - P_t}{100 - P_t}$$

P_o = Observed mortality in percent

P_t = Mortality among control specimens in percent

(c) Establishment of empirical probits

For each percentage of mortality, there is a corresponding probit value, termed empirical probit, read from a table.

(d) Plotting the provisional straight line

Empirical probits are plotted against the logarithms of the concentrations (X). The points are more or less in a straight line. The best straight line is fitted on the points obtained.

(e) Estimation of expected probits

The general formula of the straight line is $y' = a' + b'X$. The parameters for the specific form are obtained by taking the coordinates of 2 points on this line. The values of y', corresponding to the logarithms of concentrations calculated from the equation are termed expected probits. It must be pointed out that expected probits can be read directly from the provisional straight line as a function of the logarithms of the concentrations.

It is preferable to estimate the linear regression and derive the expected probits by programmable calculator instead of fitting the straight line by eye.

(f) Calculation of working probits

For complex theoretical reasons, the linear regression should be determined from expected probits. Working probits (y) are determined.

If $y' \geq 5$ $\qquad y = (Y + \frac{Q}{Z}) - q\ (\frac{1}{Z})$

If $y' < 5$ $\qquad y = (Y - \frac{P}{Z}) + p\ (\frac{1}{Z})$

$(Y + \frac{Q}{Z})$, $(Y - \frac{P}{Z})$ and $(\frac{1}{Z})$ are parameters whose values are read from the Bliss tables on the basis of expected probits.
p represents the percentage of deaths, and q the percentage of live specimens (expressed with a decimal figure between 0 and unity, i.e. 20% = 0.2).

(g) Weighted probits

Each working probit is related to a weighting coefficient, $w = N\ (\frac{Z^2}{PQ})$, where N represents the number of animals tested,
$(\frac{Z^2}{PQ})$ is read from the tables on the basis of the expected probits.

(h) Calculation of the regression line

The regression line is determined from the logarithms of concentrations (X) and the working probits that have been assigned a weighting coefficient (w).

The equation of the linear regression is $Y = \bar{y} + b\ (X - \bar{x})$, where

$$\bar{y} = \frac{S(wy)}{S(w)} \quad ; \quad \bar{x} = \frac{S(wx)}{S(w)} \quad ; \quad b = \frac{S(wxy) - \bar{y}\ S(wx)}{S(wx^2) - \bar{x}\ S(wx)} \quad ; \quad S = \text{sum.}$$

The value of X is obtained with this equation:

$$X = \bar{x} + \frac{1}{b}\ (Y - \bar{y})$$

For 50% mortality Y = 5; thus the LC_{50} is estimated as $X = \log LC_{50} = \bar{x} + \frac{1}{b}(5 - \bar{y})$

(i) Linearity test for the regression

The agreement between the expected linearity and observed data is assessed by the χ^2 test:

$$\chi^2 = \{S(wy^2) - \bar{y}\ S(wy)\} - b\{S(wxy) - \bar{y}\ S(wx)\}$$

The value obtained is compared to that given in the χ^2 table (Möller 1979, p. 102, Table A5). To look up the critical value of the χ^2 distribution in this table, say at the 95% confidence level, you enter the column $\alpha = 0.05$ (equivalent to 5% of your observations outside the given threshold of your distribution) and read the value in the respective ν line. ν are the degrees of freedom, equal in this test to n - 2, where n is the number of concentrations tested. Provided that the χ^2 test does not invalidate the working hypothesis, i.e. if the calculated value does not exceed the critical value at the 95% confidence level, it may be concluded that the curve is linear and that the stock tested is homogeneous.

(j) Calculation of the variance and standard deviation of the LC_{50}

If the χ^2 test is positive, the variance (V) of LC_{50} is given by the following expression:

$$V(X) = \frac{V(b)\ (Y - a)^2 + V(a)\ b^2}{b^4} \qquad \text{where}$$

$$a = \bar{y} \quad ; \quad V(a) = \frac{1}{S(w)} \quad ; \quad V(b) = \frac{1}{S(wx^2) - \bar{x}\ S(wx)}$$

or it may be calculated directly from the transformed equation:

$$V(\log CL_{50}) = \left(\frac{1}{b}\right)^2 \left\{\left(\frac{1}{b}\right) \frac{(Y - \bar{y})^2}{S(wxy) - \bar{y}\ S(wx)} + \frac{1}{S(w)}\right\}$$

The standard deviation(s) of the LC_{50} is $\sigma = \sqrt{V(X)}$. Its confidence limits are $\pm\ 2\sigma$.

The upper and lower limit of the LC_{50} in logarithms are as follows:

$$\log \text{(upper limit)} = \log(LC_{50} + 2\sigma)$$
$$\log \text{(lower limit)} = \log(LC_{50} - 2\sigma)$$

(k) Calculation of LC_{90}

From the linear regression any lethal concentration can be determined. For example, for LC_{90}, the value of probit Y corresponding to 90% mortality is 6.2816.

$$\log LC_{90} = \bar{x} + \frac{1}{b} (6.2816 - \bar{y})$$

The variance calculation is done in the same way by replacing Y = 5 for 50% mortality, by Y = 6.2816 for 90% mortality.

(l) Numerical example

Table II gives a numerical example of the 48 hour LC_{50} calculation using the Bliss method.

TABLE II

48 hour LC_{50} - calculation using the Bliss method for *Scolelepis fuliginosa* in a non-ionic detergent oxyethylene nonyl phenol

mg/l	Mortality	%	log concentration	Empirical probits	Expected probits	Working probits	$\frac{Z^2}{PQ}$	w	wx	wy	wxy	wx^2	wy^2
4	0	0	0.60206	-	3.32	2.8420	0.2136	4.2720	2.5720	12.1410	7.3096	1.5485	34.5047
5	4	20	0.69897	4.1584	4.03	4.1673	0.4484	8.9680	6.2683	37.3723	26.1218	4.3813	155.7415
6	6	30	0.77815	4.4756	4.63	4.4803	0.6051	12.1020	9.4171	54.2205	42.1914	7.3279	242.9241
7	11	55	0.84510	5.1257	5.12	5.1255	0.6329	12.6580	10.6972	64.8785	54.8284	9.0402	332.5347
8	15	75	0.90309	5.6745	5.56	5.6701	0.5671	11.3420	10.2428	64.3102	58.0777	9.2501	364.6452
9	16	80	0.95424	5.8416	5.93	5.8371	0.4615	9.2300	8.8076	53.8764	51.4108	8.4045	314.4819
10	20	100	1	-	6.28	6.8506	0.3427	6.8540	6.8540	46.9540	46.9540	6.8540	321.6630
								S(w)	S(wx)	S(wy)	S(wxy)	$S(wx^2)$	$S(wy^2)$
								65.4260	54.8590	333.7529	286.8937	46.8064	1766.4951

Provisional curve: $y' = -1.14 + 7.42\ x$

$\bar{x} = 0.83848$

$\bar{y} = 5.10122$

$S(wx^2) - \bar{x}\ S(wx) = 0.8083$

$S(wxy) - \bar{y}\ S(wx) = 7.0459$

$S(wy^2) - y\ S(wy) = 63.9528$

$b = 8.7169$

$\chi^2 = 2.53$, the critical value at $\alpha = 0.05$ and 5 degrees of freedom is 11.07. Thus the linearity of the test data can be concluded and hence the LC_{50} and LC_{90} and their respective variance (V) and standard deviation may be calculated.

$\log LC_{50} = 0.82687$

$V(\log LC_{50}) = 0.000203$

$\log LC_{90} = 0.97383$

$V(\log LC_{90}) = 0.000446$

5.2 Kolmogoroff-Smirnov test

(a) The Kolmogoroff-Smirnov test may be used to estimate the significance of the results of long-term tests with *Tisbe bulbisetosa* subjected to a hydrocarbon as compared to a control.

The null hypothesis, supposing that the parameter under investigation has not significantly varied from one random sample to another, may be verified for continuous variables or for samples with more than 5 observations, by means of the Kolmogoroff-Smirnov test (Kolmogoroff, 1941; Smirnov, 1948). The test statistic used is the maximum difference between two cumulative distributions that is compared to the critical value calculated below as

$$\lambda\ (1 - \alpha)\ \sqrt{\frac{n_1 + n_2}{n_1 n_2}}$$

with $\lambda\ (1 - \alpha)$ given by Stange and Henning (1966), after Smirnov (1948)

$1 - \alpha$	0,95	0,975	0,99	0,995
$\lambda(1 - \alpha)$	1,36	1,48	1,63	1,73

If the maximum difference is greater than the critical value the two samples are considered significantly different, that is the null hypothesis is rejected.

(b) Numerical example for the Kolmogoroff-Smirnov test

Number of nauplii 1st sac	4.2 mg/l concentration hydrocarbons		Control sea water		Difference between cumulative frequencies 1/
	Number ♀♀	Cumulative frequencies F_1	Number ♀♀	Cumulative frequencies F_2	$\lvert F_1 - F_2 \rvert$
0-10	2	0.11	2	0.10	0.01
10-20	2	0.21	4	0.30	0.09
20-30	5	0.47	4	0.50	0.03
30-40	6	0.79	4	0.70	0.09
40-50	4	1.00	4	0.90	0.10 ←
50-60	-	1.00	1	0.95	0.05
60-70	-	1.00	1	1.00	0
	19		20		

$D_{19,20} = 0.10$ Maximum difference between cumulative frequencies.

95% critical value $= 1.36 \sqrt{\frac{19 + 20}{19 \times 20}} = 1.36 \sqrt{\frac{39}{380}}$

$= 1.36 \times 0,32 = 0.44$

1/ No account is taken of the sign, i.e only the absolute value is considered.

Since $0.10 < 0.44$, the samples are not significantly different from one another. Thus the copepods subjected to a concentration of 4.2 mg/l petroleum hydrocarbons did not display a significantly lower fertility than the controls.

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