

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

ORGANISATION DES NATIONS UNIES POUR L'ALIMENTATION ET L'AGRICULTURE

ORGANIZACION DE LAS NACIONES UNIDAS PARA LA AGRICULTURA Y LA ALIMENTACION FIR/MEDPOL/TOX/8
31 OCTOBER 1988

REPORT OF THE FAO/UNEP CONSULTATION MEETING
ON THE TOXICITY OF SELECTED SUBSTANCES
TO MARINE ORGANISMS

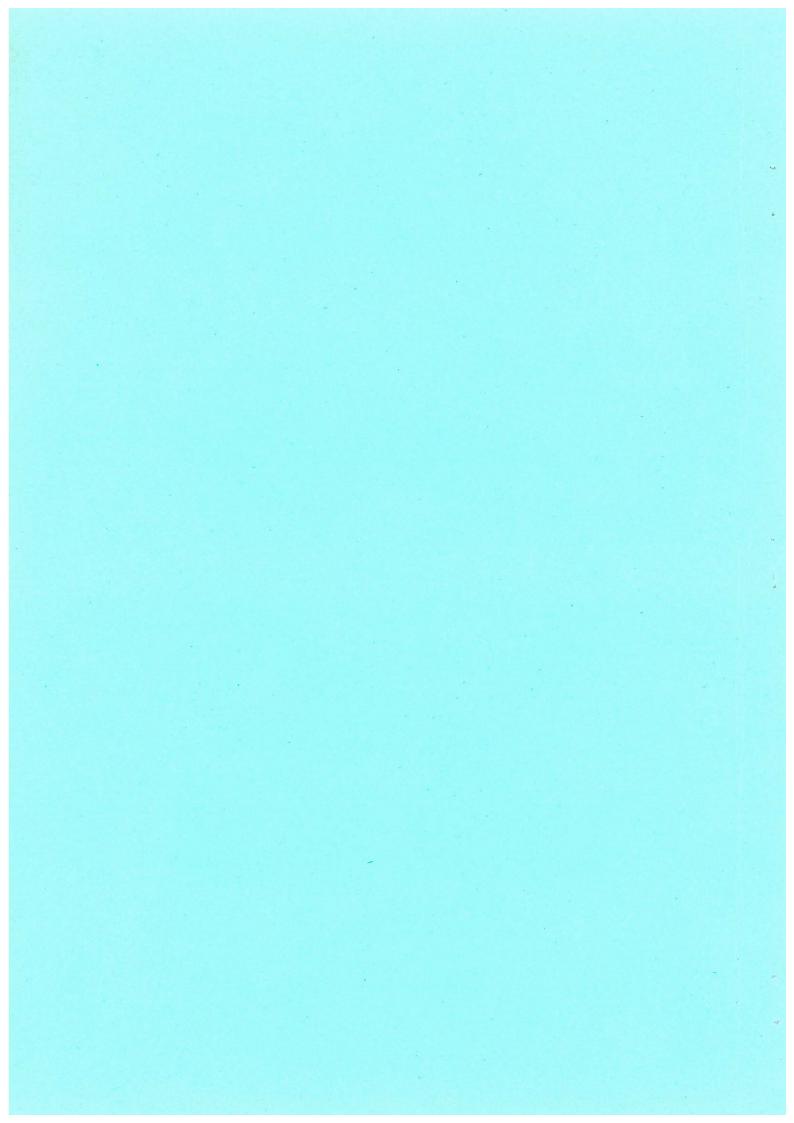
Villefranche-sur-mer, France, 10-14 October 1988

(Organized within the framework of the MED POL - Phase II programme)

In cooperation with:



UNEP





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Introduction

- The Long-term Programme for Pollution Monitoring and Research in the Mediterranean Sea (MED POL - Phase II), which constitutes the scientific and technical component of the Mediterranean Action Plan, is basically divided into two groups of activities, namely monitoring and research.
- One of the main objectives of the research component and in fact of the whole MED POL programme is to generate the required information for the technical implementation of the protocols to the Barcelona Convention and especially the one dealing with land-based sources.
- 3. The research component is divided into twelve topics one of which (research activity "G") is concerned with the toxicity, persistence, bioaccumulation, mutagenicity and carcinogenicity of selected substances listed in the annexes of the protocols.
- 4. The present Consultation Meeting on the Toxicity of Selected Substances to Marine Organisms was jointly convened by FAO and UNEP in the framework of activity "G". Following a kind invitation by INSERM Unité 303 the meeting took place in Villefranche-sur-mer, France, from 10-14 October 1988. It was attended by 29 participants from 11 Mediterranean countries and the United Kingdom and by representatives of the Food and Agriculture Organization of the United Nations and of the International Atomic Energy Agency. The list of participants is given in Annex I.

Agenda item no. 1: Opening of the meeting

- 5. The meeting was opened by Mr. G.P. Gabrielides, FAO Senior Fishery Officer (Marine Pollution) on behalf of the Food and Agriculture Organization of the United Nations and the Coordinating Unit for the Mediterranean Action Plan, and by Ms S. Puiseux-Dao, Professor of Toxicology and Cellular Biology at the University of Paris VII and Director of INSERM Unité 303 on behalf of the host Institution.
- 6. After welcoming the participants and thanking the local organizers for accepting to host the meeting, Mr. Gabrielides outlined the importance of toxicological research in the framework of the MED POL programme and reminded the participants of the ultimate aim of the Mediterranean Action Plan which is the protection of the Mediterranean sea from pollution. The toxicity of substances to marine organisms constitutes a basic criterion for the type of pollution control measures to be recommended to the Contracting Parties for action.

7. Ms Puiseux-Dao welcomed the participants and expressed her satisfaction that the meeting was hosted by her Institute. She considered that the development of sensitive bioassays for testing environmental toxicology in the Mediterranean should be a major concern of the meeting. Organism sensitivity to toxic substances is very variable and depends mainly on entry barriers and metabolizing capabilities. Genetic potential and diversity as well as reproduction potential should be maintained in organisms and ecosystems; therefore toxicity tests should be directed to processes linked to these biological characteristics but must be adapted to Mediterranean conditions.

Agenda item no. 2: Background and scope

- 8. Mr. Gabrielides, in introducing this agenda item, referred to the previous toxicity meeting in the framework of activity "G" (Rovinj, 1984) and described the activities that took place since then. Since one of the recommendations of that meeting stressed the need for standard toxicity testing procedures with marine organisms for screening purposes, FAO developed and published a manual on "Short-term static bioassays" in the series Manual of Methods in Aquatic Environment Research (FAO Fish.Tech.Pap. 247).
- 9. At the end of 1987, UNEP in cooperation with FAO and IAEA issued in the series "Reference Methods for Marine Pollution Studies" the following three draft reference methods for acute toxicity testing.
 - R.M. 43 "Test of the acute lethal toxicity of pollutants to marine fish and invertebrates".
 - R.M. 44 "Estimation of the toxicity of pollutants to marine phytoplanktonic and zooplanktonic organisms".
 - R.M. 45 "Comparative toxicity test of water-soluble fractions of oils and oil dispersants to marine organisms".
- 10. An inter-laboratory toxicity testing exercise was initiated in the beginning of 1988 to evaluate the three draft reference methods and to identify any aspects of the methods which might need improvement.
- 11. Another activity was to commission a consultant from outside the Mediterranean region, expert in the field of toxicity, to evaluate the final reports on projects implemented in the framework of activity G.
- 12. The purpose of the Consultation meeting was therefore to:
 - a) review the results of the inter-laboratory toxicity testing exercise with marine organisms,
 - b) review and, if necessary, amend the three draft reference methods on acute toxicity (R.M. 43, 44 and 45),

- c) review the research work already carried out within the framework of research activity G relevant to toxicity, persistence and bioaccumulation,
- d) formulate the framework for future work within the activity as regards the above topics.

Agenda item no. 3: Election of officers

13. The meeting unanimously elected Ms S. Puiseux-Dao, Professor of Toxicology and Cellular Biology at the University of Paris VII and Director of INSERM Unité 303 as Chairperson, Mr. S. Papoutsoglou, Professor of Applied Hydrobiology at the Agricultural University of Athens as Vice-chairperson and Mr. V. Axiak, Professor of Biology at the University of Malta as Rapporteur. Mr. G.P. Gabrielides, FAO Senior Fishery Officer (Marine Pollution) acted as Technical Secretary of the meeting.

Agenda item no. 4: Adoption of the agenda

14. The provisional agenda shown in Annex II was unanimously adopted. Following a request by the Technical Secretary, agenda item 8 was discussed before agenda item 6.

Agenda item no. 5: Organization of work

15. The meeting agreed to work in plenary only but if necessary drafting groups could be formed for specific tasks assigned by the Chairperson. The working hours were agreed to be 09:30-13:00 hrs for the morning session and 15:00-18:00 hrs for the afternoon session.

Agenda item no. 6: Review of the results of the inter-laboratory toxicity testing exercise

- 16. The Technical Secretary informed the meeting that all National Coordinators for the MED POL were contacted in December 1987 and asked to identify laboratories which might be interested in participating in the exercise. Only 5 Co-ordinators replied, nominating a total of 12 laboratories. In February 1988, another 14 laboratories were contacted directly. Specific instructions were given for the evaluation of two out of the three methods (Nos 43 and 45). These instructions, and the accompanying documentation, are given in Annex III.
- 17. Mr. P.D. Abel, Lecturer at Sunderland Polytechnic, U.K., who acted as technical coordinator of the exercise, introduced document FIR/MEDPOL/TOX/6. He stressed the fact that the limited response of participating laboratories made evaluation difficult. Despite this, certain general conclusions could be drawn and these are the following:

18. Response to the inter-laboratory toxicity testing exercise

Out of 26 laboratories invited to participate, only five submitted results for central evaluation before the consultation meeting. Two more laboratories submitted results at the meeting itself. In addition one laboratory outside the Mediterranean submitted results for central evaluation which were useful for some purposes of comparison although they did not form part of the exercise as the test species and experimental conditions were very different. A summary of the response of participating laboratories is given in Table I.

Table I

Summary of the response of participating laboratories to the inter-laboratory toxicity testing exercise. X indicates that results were submitted for evaluation.

Reference Laboratory Method A B C D E* F							F	G
43	Zn Cu Hg	X X X	x	X X X	X X	X X X	Х	
44		Х			x	#		Х
45		Х				Х		

^{*} Laboratory outside the Mediterranean

19. Experimental conditions - RM 43

All laboratories used <u>Palaemon</u> <u>elegans</u>, of mean weight ranging from approximately 0.18 g to 2.0 g. The temperature range was from 17°C to 23°C, pH from 8.05 to 8.26, and salinity from 30 to 38. In such a small sample of results, it is impossible to establish whether the environmental conditions had any consistent effect on the results. The volume of test solution used ranged from 10-24 litres. All but one laboratory renewed the test solutions every 24 hours.

Only two laboratories measured the test concentrations during the experiments. The results indicated that in some cases, the concentrations of the test substance decrease rapidly but there is no consistent pattern. For example, in one laboratory measured mercury concentrations stayed close to their nominal values, while in another they fell rapidly to about one quarter of their nominal values. Similar findings were reported for copper. This indicates that measurement of test substance concentations during the test is extremely important.

20. Analysis of the results - RM 43

Nearly all laboratories analyzed the results correctly. One laboratory made a very minor error. Results from one laboratory included major errors but it was discovered that this was due to an ambiguity in the reference method, i.e. a mathematical equation which had not been laid out clearly. Each laboratory was provided with the evaluator's comments and the results of the ensuing discussions will be incorporated into the revised version of the method.

21. Comparison of results between laboratories - RM 43

A full comparison of results could not be made because of their limited number and late submission. However some simple comparisons were used to assess the performance of the reference methods.

- Absolute values of 96h LC50 - Table II shows a summary.

Table II

Summary of 96h LC50 values obtained for <u>P. elegans</u> exposed to Cu, Zn and Hg. (95% confidence limits in parentheses). All values expressed in mg/l.

Laboratory	Results							
	Нд	Cu	Zn					
A	0.053(0.036-0.077)	19(9.2-40)	23(13-40)					
В	0.013(0.006-0.027)							
С	0.116(0.101-0.134)	4.34(1.09-17.3)	>30					
D	0.029(0.013-0.062)	21(11-39)	16					
F			21(13.5-33)					

The results show generally good agreement between the different laboratories, values for Cu and Zn being not significantly different when results between laboratories are compared. Mercury results show less good agreement, but the range of variation shown is not unexpected, particularly in view of the simple nature of the experimental procedure.

- Ranking of Hg, Cu and Zn toxicity - the three metals were chosen for the exercise partly because the expected result is that mercury will be much more toxic than copper or zinc; copper and zinc toxicity is, however, more difficult to distinguish and in the present case, the test organism is a decapod crustacean which normally metabolises significant quantities of copper.

Results from all laboratories (including Laboratory E) showed that the ranking of toxicity of the three metals was as expected. Mercury was found to be significantly more toxic than Cu or Zn, but only in some cases was the expected (smaller) difference between Cu and Zn detected.

22. Reference Method 44

Table III summarises the results from three laboratories which determined the 96h EC50 of several pollutants to phytoplankton species.

Table III

(All figures expressed as mg/l).

Laboratory	Species	96h EC50				
A	<u>Phaeodactylum</u> tricornutum	Cu 0.62 Zn 5.0 Hg 0.0043				
ם	Phaeodactylum tricornutum	Malathic				
G	<u>Skeletonema</u> costatum	LAS (synthetic detergent)	0.1 0.12 0.25	3 trials		

The results suggest that the method is very promising. Laboratory A tested Cu, Zn and Hg and the results indicate that the method is more sensitive than animal test methods, and also clearly distinguishes between the toxicity of the three methods. Laboratory G tested one pollutant three times, and showed that the method produces satisfactorily consistent results.

23. Reference Method 45

Results from 2 laboratories which attempted to evaluate RM 45 agreed closely that the method successfully distinguishes between the toxicity of different oil and oil products, oil dispersants, and mixtures of oil and oil dispersants. The opinion was expressed that insufficient data was presented to indicate whether the rationale and the methodology to test the toxicity of oil and dispersant mixtures were valid.

24. Results from Laboratory E

Laboratory E is not a MED POL participant, but carried out evaluations of RM's 43 and 45 for purposes of comparison. This laboratory used amphipod crustaceans of the genus Gammarus and Marinogammarus at a temperature of 15°C.

The method 43 ranked the toxicity of Hg, Cu and Zn in the expected manner. The method also ranked the toxicity of oil products and oil/dispersant mixtures satisfactorily. The results were centrally evaluated and have been used to assist in the evaluation of the Reference Methods.

25. <u>Comments from participants</u>

Many valuable comments were received from participating laboratories concerning the difficulties they encountered in testing the methods and any suggested modifications. All these comments formed the basis for a sincere and fruitful discussion.

26. Conclusions

- The poor response of the laboratories invited to participate in the exercise was disappointing. Few laboratories were able or willing to undertake this exercise for a variety of reasons. At present in the case of an emergency (such as a major pollution incident), few laboratories would be able to respond by giving specific advice within a short time. This point should be brought to the attention of National Coordinators and Directors of Laboratories;
- On the basis of the limited data available, it appears that RM 43 is generally satisfactory, subject to minor revisions. RM 44 produced some very promising results and further evaluation should be encouraged. RM 45 needs further evaluation.
- There is need to improve the availability of chemical analytical facilities for use in conjunction with toxicological work.

Agenda item no. 7: Review of draft reference methods no. 43, 44 and 45

- 27. In the light of the results of the inter-laboratory testing exercise and subsequent discussions, the meeting recommended a number of modifications to be made to the existing drafts of reference methods 43, 44 and 45.
- 28. The main points raised, as far as RM 43 is concerned, were the use of artificial sea water, the use of the word "poison", and the line of good fit. Detailed recommendations for the revision of this method are shown in Annex IV.
- 29. The main points raised, as far as RM 44 is concerned, were the preparation of test cultures, the light conditions, the use of optical density as a means of drawing a rough growth curve and the analysis of data. Detailed recommendation for the revision of this method are shown in Annex IV.
- 30. The main points raised, as far as RM 45 is concerned, were the introduction of a new annex on mechanically dispersed crude oils and the use of the term "water accommodated fractions". Detailed recommendations for the revision of this method appear in Annex IV.

Agenda item no. 8: Review of research work already carried out within the framework of activity G and relevant to toxicity, persistence and bioaccumulation

- 31. The Technical Secretary informed the meeting that it has been decided to review and evaluate MED POL activities during the biennium 1988/89 with a view to re-orientate and refocus the activities to the objectives of the Mediterranean Action Plan. In the framework of these activities a consultant was commissioned to review and evaluate the final reports on projects implemented within activity G and received in the MED Unit before spring of 1988. The evaluation report was distributed as document FIR/MEDPOL/TOX/7.
- 32. The above document was introduced by Mr. R. Lloyd of MAFF Laboratory at Burnham-on-Crouch, U.K. who was responsible for its preparation. He outlined the main points of the document giving emphasis to the general conclusions and recommendations.
- 33. The main points raised during the discussion concerned the submission of final reports on MED POL projects and especially the requested format, the time limit of submission, their publication and their evaluation. Scepticism was expressed whether the publication of final reports in the MAP Technical Reports Series before their publication in scientific journals might constitute a copyright problem. At the end of the discussion the meeting expressed its gratitude to Mr. Lloyd for his contribution in the success of the meeting and decided overwhelmingly to adopt his recommendations and incorporate them in the recommendations of the meeting.

Agenda item no. 9: Recommendations

34. The meeting discussed extensively the future framework of activity G with an aim in achieving the objectives of MED POL-Phase II. It was finally decided to recommend certain guidelines for future work and to identify specific examples of research topics.

35. <u>Guidelines</u>

- a) Priority should be given to the investigation of biological responses to sublethal levels of pollutants which may be studied both in the laboratory and in the field;
- b) Emphasis should be given to the development of field bioassays/biomonitoring to measure the biological quality of water and to identify signs of incipient damage to living marine resources;
- c) Test species to be used, are to be representative of the marine communities within the region and which are of economic and/or of ecological importance;
- d) The scope of the investigations should be enlarged so as to include studies of the biological responses of early developmental stages of the test species;
- e) Greater emphasis should be given to investigations of significant polluting discharges, incorporating the identification of dominant toxic substances and the combined effects of minor pollutants;
- f) Greater emphasis should be given to investigations of known contaminated areas, using a combination of laboratory and field experiments.

36. Specific examples

- a) Effects at the molecular and subcellular level: including effects on metal binding proteins, enzymes (activity and isoforms), biological response modifiers (hormones, factors, neurotransmitters) and other physiologically significant proteins; effects on general peptides and proteins resolved by electrophoretic and chromatographic techniques;
- b) Effects at the cellular level with particular emphasis on cytotoxicological investigations on gills, digestive glands, liver, gonads and kidneys in exposed organisms;

- c) Effects at the whole individual level; in particular, physiological responses including growth rate, bioenergetic and reproductive performances of the test species. Other possible biological responses to be investigated include ciliary activities and ionic regulations;
- d) Kinetics of uptake, bioaccumulation, persistence and loss of pollutants by individuals and of transfer of pollutants across different trophic levels.
- 37. The following general recommendations were also made:
 - a) Ecotoxicological research is essentially of a multidisciplinary nature and must necessarily involve collaboration between different departments within the same institutes and preferably also between different institutes of the region. This collaboration should be evident in the research contract proposals to be submitted in the future. In this respect, National Coordinators should be requested to ensure full collaboration between monitoring and research components, including the use of equipment;
 - b) In order to ensure the necessary standard of research as well as its applicability to pollution control, better project supervision and training in applied pollution science are required;
 - c) The general quality of final research reports should be improved to the standard required for an international scientific journal. In particular, such reports should include a full discussion and interpretation of the data presented so as to show how such information could be used for pollution prevention and control;
 - d) Research centres participating in MED POL Phase II activities must realize the importance of inter-laboratory toxicity testing exercises and are to consider such exercises as an essential component of their research contract activities;
 - e) Research centres should be encouraged to contribute towards the reviewing and improvement of reference methods in acute and sublethal toxicity tests.

Agenda item no. 10: Any other matter

38. In the absence of any other matter for discussion the Chairperson decided to allow a number of participants, if they so wished, to present their work.

Agenda item no. 11: Adoption of the report

39. The present report was adopted by the meeting on Friday, October the 14th 1988.

Agenda item no. 12: Closure of the meeting

40. In his closing remarks the Technical Secretary reiterated the fact that all research activities implemented in the framework of MED POL - Phase II should comply with its objectives. He expressed satisfaction for the results of the meeting and appreciation for the constructive spirit in which it was conducted. He also thanked everybody who contributed in the success of the meeting. After the customary exchange of courtesies, the meeting was closed by the Chairperson.

ANNEX I

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ANNEX II

PROVISIONAL AGENDA

- 1. Opening of the meeting
- 2. Background and scope
- 3. Election of officers
- 4. Adoption of the agenda
- 5. Organisation of the meeting
- Review of the results of the inter-laboratory toxicity testing exercise
- 7. Review of draft reference methods nos. 43, 44 and 45
- Review of research work already carried out within the framework of activity G and relevant to toxicity, persistence and bioaccumulation
- 9. Recommendations for future work
- 10. Any other matter
- 11. Adoption of the report
- 12. Closure of the meeting

ANNEX III

Inter-laboratory acute toxicity testing exercise with marine organisms

1. At the FAO/UNEP Meeting on the Toxicity and Bioaccumulation of Selected Substances in Marine Organisms (Rovinj, Yugoslavia, 5-9 November 1984) it was apparent that one of the main problems was the lack of standard methodology. One of the recommendations of the meeting was that:

"there is a need for standarisation of toxicity testing procedures with marine organisms for screening purposes, involving substances such as oil dispersants and some others, where the results may be of legal or administrative, rather than strictly biological significance. FAO should produce a document describing in detail recommended methods for screening tests, similar to that produced by the OECD for freshwater organisms in the Guidelines for Testing of Chemicals".

- 2. In response to this recommendation, FAO has prepared Part 10 of the series "Manual of Methods in Aquatic Environment Research" entitled "Short-term static bioassays" by Reish and Oshida. In addition three draft reference methods have been prepared in the UNEP Regional Seas Series "Reference Methods for Marine Pollution Studies". These are:
 - R.M. 43 UNEP/FAO/IAEA "Test of the acute lethal toxicity of pollutants to marine fish and invertebrates".
 - R.M. 44 UNEP/FAO/IAEA "Estimation of the toxicity of pollutants to marine phytoplanktonic and zooplanktonic organisms".
 - R.M. 45 UNEP/FAO/IAEA "Comparative toxicity test of water-soluble fractions of oils and oil dispersants to marine organisms".
- and to identify any aspects of the methods which require improvement. Participants will be required to carry out certain experiments according to the prescribed procedures. They will then submit their results and raw experimental data for central analysis and evaluation. The overall results of the test will be discussed at a meeting to be convened in October 1988. Participating laboratories will be invited to send delegates to this meeting to present their results and participate in the discussion. Subsequently, the reference methods will be revised as necessary and made availabe for genelar use.

- 4. Specific instructions are given here for the evaluation of two of the methods. These are Reference Method for Marine Pollution Studies No. 43 (Draft), and Reference Method for Marine Pollution Studies No. 45 (Draft). Copies of these documents are enclosed. Participants are advised to read the documents carefully, in conjunction with Appendix A and Appendix B (enclosed) which give specific instructions about the tests to be carried out, the species to be tested, the experimental conditions, and the format in which the results must be reported. Participants are also advised to note the paragraphs below.
- 5. Modifications to the specified procedure are <u>not</u> permitted. Since this is an inter-laboratory calibration exercise, it is important that all participants carry out the experiments in exactly the same way. Participants who wish to suggest modifications may do so, giving reasons, in the space provided on the report form. Participants may, if they wish, perform experiments with modifications and report the results, but only if they <u>also</u> carry out the experiments using the unmodified procedure. Where the specified method permits a choice (e.g. size of test vessel, temperature), the choice selected must be stated, though reasons are not required to be given.
- 6. Participants <u>must</u> provide all the information requested on the report form. A separate report form must be provided for each test. (Please make your own photocopies of the forms attached). Tables and graphs must be clearly identified and attached to the appropriate report form.
- 7. Further advice or information should be sought, if required, from the Co-ordinating Unit for the Mediterranean Action Plan, P.O. Box 18019, Vas. Konstantinou 48, Athens 116 10, Greece.

APPENDIX A. Evaluation of Reference Method No. 43

UNEP/FAO/IAEA Test of the acute lethal toxicity of pollutants to marine fish and invertebrates. Reference Methods for Marine Pollution Studies No. 43 (Draft), UNEP 1987.

- Objective of the test. To determine the lethal toxicity of mercury (as HgCl2), copper (as CuSO4) and zinc (as ZnSO4) to the selected test species. For each metal, the toxicity curve (see sections 9.2 and 9.3 of the Reference Method) and the 96h LC50 (see section 9.4 of the Reference Method) must be determined.
- Choice of test species. Ideally, all participants should use the same test species and we therefore recommend the shrimp Palaemon elegans which is widely available in the Mediterranean region. If not easily available in your area, any other species of the same family will do. If another species is going to be used please contact the FAO Project Office, Coordinating Unit for the Mediterranean Action Plan, P.O. Box 18019, Athens 116 10, Greece for advice.

You <u>must</u> however, use the same species for all tests in this series.

Please refer to and follow the recommendations in section 6 of the Reference Method.

- 3. The experimental conditions. Participants are free to select any appropriate experimental conditions within the limitations described in section 7 of the Reference Method. The information required about the experimental conditions is listed on the report form.
- The test solutions. See section 4.2 of the Reference Method. Particular care is necessary in making up stock poison solutions to the correct strength. The information below may be useful:
 - 1 gram of Zn is contained in 4.398g of ZnSO4.7H2O

 - 1 gram of Zn is contained in 2.745g of ZnSO₄.H₂O
 1 gram of Cu is contained in 3.929g of CuSO₄.5H₂O
 - 1 gram of Cu is contained in 2.512g of anhydrous CuSO4
 - 1 gram of Hg is contained in 1.353g of anhydrous HgCl2
- 5. Reporting the data. The data required must be entered on the report form. One report form must be completed for each test. Participants are recommended to study the report form carefully before commencing the test. Incomplete reports will not be accepted.
- Preliminary experiments. It is often useful to carry out simple. preliminary experiments to determine the range of poison concentrations which are effective. No report is required of preliminary experiments.
- 7. It is desirable, but not essential to measure the poison concentrations at intervals during the test (see section 7.7 in the Reference Method).

REPORT FORM for evaluation of Reference Method No. 43

You	must	complete	one	report	form	for	<u>each</u>	experiment.
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- 1. Name of Investigator:
- Address of laboratory:

Telephone:

Telex:

- 3. Date experiment began:
- 4. Substance tested:
- 5. Give precise details of how the stock poison solution was formulated:
- 6. Test species (give Latin generic and specific name, and the <u>family</u> to which the test species belongs).
- 7. Source of test animals:
- 8. Period of acclimation:
- 9. Weight of animals at start of experiment.

Mean = Standard deviation = No. of animals weighed =

10. Source and nature of dilution water:

1	1	C a	٦	÷	n	÷	ty	,
4	4	20	4	4	Τī	_	L y	

pH:

Temperature:

(If these varied significantly during the experiment, give the data as a separate sheet of paper).

- 12. Was poison concentration measured during the test? Yes/No.
- 13. If so, by what method?
- 14. At what times were the poison solutions renewed?
- 15. What was the volume of test solution in each experimental vessel?
- 16. If the poison solutions were measured during the experiment, enter in the table below the <u>actual</u> poison concentrations.

Time in hours from start	Nominal poison concentration mg l-1								
			_						
7-5-5-1									
				_					
					_				
					2				
	•				_				
					*				

Mortality data

- 17. How many animals were used in each poison concentration?
- 18. Enter the mortality data in the table below (see section 9.1 in the Reference Method) (No. of animals <u>dead</u>).

Time in hours	Poison concentration (mg l ⁻¹)								
from start of experiment								Control	
			ш						
			:						
						s.			
						_			

- 19. Calculate median survival time (LT50) for each concentration (see section 9.2 in Reference Method).
 - (a) Attach copies of <u>each</u> graph of cumulative percentage mortality against time, <u>clearly marked with the poison tested and the concentration</u>.
 - (b) Enter the values of LT16, LT50, LT84, S, f, upper confidence limit and lower confidence limit in the table below:

Poison concentration mg l-1	LT16	LT50	LT84	S	£	Upper Conf. limit	Lower Conf. limit
*							
					1		

20. Construct a toxicity curve (see section 9.3 in the Reference Method).

Attach a copy of the toxicity curve to this report.

21. Estimate the 96h LC50 and its 95% confidence limits (see section 9.4 in the Reference Method).

(Note: If the experiment was terminated before 96 hours due to high control mortality, calculate the LC50 value for a shorter time, e.g. 48h, 72h).

Attach a copy of the relevant graph (see section 9.4.3 in the Reference Method).

Enter your values for:

96h LC50

Upper 95% confidence

limit

Lower 95% confidence

limit

- 22. Is this line a line of good fit, or a line of best fit? (See section 9.5.6 in the Reference Method).
 - (A line of best fit is the line of minimum chi²).
- 23. Do you wish to comment on any unusual features of your results, or any difficulties you experienced in following this method? (You may continue on a separate sheet of paper if you wish).

24. Do you have any comments about this test method, or do you wish to suggest any modifications? Please make your comments here, giving reasons. (You may continue on a separate sheet if you wish).

CHECK LIST:

You must attach to this report:

- a) Graphs of cumulative percentage mortality against elapsed time for each poison concentration.
- b) Your toxicity curve
- c) A graph of cumulative percentage mortality at 96h against poison concentration.

Please check that you have entered all the data required on the report form.

APPENDIX B. Evaluation of Reference Method No. 45

UNEP/FAO/IAEA Comparative toxicity test of water-soluble fractions of oils and oil dispersants to marine organisms. Reference Methods for Marine Pollution Studies No. 45 (Draft). UNEP 1987.

Objective of the test. To compare the toxicity to marine organisms of:

a) Three petroleum products (if possible)

- b) An oil dispersant, a petroleum product and a mixture of the two, using the procedure described in the Reference Method.
- Choice of test substances. Participants should obtain a supply of each of the following:
 - a) Petrol (gasoline) as used in motor vehicles

b) Diesel fuel as used in motor vehicles

c) Light or medium grade fuel oil, or a sample of crude oil

d) A commercially-available oil dispersant

NOTE: for the purposes of this exercise, the oil-dispersant chosen is not intrinsically important. However, wherever possible participants should select a dispersant which is widely available on the international market, such as Finasol, BP 1002 or BP 1100, in preference to locally-available dispersants. Participants are required to report data on the oil-dispersant chosen (see Report Form).

- Experiments to be carried out. Participants are required to carry out 3. two experiments:
 - a) To compare the toxicity of petrol, diesel fuel and fuel oil or crude oil to the test species
 - b) To compare the toxicity of oil dispersant, fuel oil or crude oil and a mixture of oil dispersant and fuel oil or crude oil to the test species

In each case the procedure described in the Reference Method must be followed exactly and the results reported as required in the Report Form.

The experimental procedure and conditions. The experimental conditions and the procedures to be carried out are clearly described in sections 6, 7 and 8 of the Reference Method. The procedures described must be followed exactly.

IMPORTANT NOTE: (Refer to section 9.5.5 of the Reference Method). the purposes of this evaluation, participants must submit, for each experiment, data and results from the first line of good fit, and also they must compute the line of best fit and submit data and results. This is to allow a comparison between results obtained from using lines of good fit, and those obtained from using lines of best fit. The line of best fit is the line which has the minimum value of chi2. (See Report Form).

- Reporting the data. For each experiment, a report form must be completed. Participants are recommended to study the report form before commencing the test. Incomplete reports will not be accepted.
- 5. Preliminary experiments. It is often useful to carry out simple, preliminary experiments to determine the range of concentrations which are effective. No report is required of preliminary experiments.

REPORT FORM for evaluation of Reference Method No. 45

You	must	complete	one	report	form	for	each	experiment.

1.	Name of Investigator:		
2.	Address of laboratory:		
	Telephone:	Telex:	
3.	Date experiment began:		
4.	Substances tested:		
	Petrol/Diesel/Fuel Oil		
	Dispersant/Fuel Oil/Dispersant-fuel	l oil mixture:	
	Other (specify)		

5. Details of dispersant used

Name of dispersant:

Manufacturer:

Approximate date of manufacture:

Please give as much additional information as you can, including if possible the formulation of the dispersant. If you have any technical data or publicity material supplied by the manufacturer, please enclose a copy.

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6.	Test species (give Latin generic and specific names, and the Family to which the test organism belongs).
7.	Source of test animals.
8.	Period of acclimation:
9.	Weight of animals at start of experiment:
	Mean = Standard deviation = No. of animals weighed =
10.	Source and nature of dilution water:
11.	Salinity:
	Temperature: pH:
	(If these varied significantly during the experiment, give the data on a separate sheet of paper).
12.	What was the volume of test solution in each experimental vessel?
13.	How many animals were used in each test concentration?

14. Enter the mortality data in the table below (number of animals dead after 24h).

Substance		Concentration of extract %							
tested									Control
						_			
	Appendix and the second			· · · · · · · · · · · · · · · · · · ·					
			2						

15. Prepare graphs of cumulative percentage mortality against dilutions of test material as described in section 9 of the reference method.

Prepare two sets of graphs.

(a) Showing the first line of good fit obtained (see section 9.5 in the Reference Method).

Attach copies of these graphs to this report form. Each graph must be clearly marked to allow it to be identified.

Enter the required data from each graph in the table below:

Test substance	"LC16"	"LC50"	"LC84"	Total chi² of line	S	f	Upper Conf. limit	Lower Conf. limit
								4
						-		
		5						

(b) Prepare a similar set of graphs showing the line of best fit (i.e. the line which has the minimum value of chi²). (See section 9.5 in the Reference Method).

Attach copies of these graphs to this report form. Each graph must be clearly marked to allow it to be identified.

Enter the required data from each graph in the table below:

Test substance	"LC16"	"LC50"	"LC84"	Total chi² of line	ಬ	f	Upper Conf. limit	Lower Conf. limit
								2

17. What conclusions do you draw from this experiment?

18. Do you wish to comment on any unusual features of your results, or any difficulties you experienced in following this method. (You may continue on a separate sheet if you wish).

19. Do you have any comments about this test method, or do you wish to suggest any modifications? Please make your comments here, giving reasons. (You may continue on a separate sheet if you wish).

CHECK LIST:

You must attach to this report:

- a) Any available information about the oil dispersant you have used.
- b) Graphs of cumulative percentage mortality against concentration of test material (see section 16 of the Report Form).

ANNEX IV

Proposed modifications to the existing draft reference methods

R.M. 43

 The word "poison" to be changed to "test substances" throughout the text.

Paragraph 4.1 - line 3:

Add a new sentence "It is strongly recommended that natural sea water be used wherever 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.

Paragraph 6.1:

Sentence beginning on line 3 should read "They should be obtained from an uncontaminated area, or from aquaculture, and as far as possible..." (continue to end).

Paragraph 7.3 - line 3 should be:
"...should not exceed ± 1% of that chosen." (NOT ± 1%).

Paragraph 8.3.4 - 1st sentence should read:

"Observations on mortality should be made at least at the following intervals (in hours):" (continue to end).

Paragraph 9.2.1 - line 17: 9.2.1 should be corrected to 9.2.1(b).

Paragraph 9.2.5:

The equation $f=s^{1.96}/\sqrt{N}$ is not laid out clearly. It should be:

Also, insert brackets in the second equation thus:

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

Figure 2.

Delete "N2" and insert ""N"" (i.e. the "N" should have apostrophes as in paragraph 9.2.8).

Also, insert an X on the diagram above the right-hand line labelled "% reacting".

Figure 3.

A revised Fig. 3 to be inserted.

Paragraph 9.4.7

This equation needs parentheses to be inserted and more accurate layout thus:

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

Paragraph 9.5.4 - should read:

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

Paragraph 9.5.6:

<u>Delete</u> the <u>second</u> NOTE, i.e. lines 7, 8 and 9. <u>Delete</u> the last sentence on line 4, and <u>insert</u> "Repeat this procedure until you obtain the line which has the <u>minimum</u> value of chi².

R.M. 44

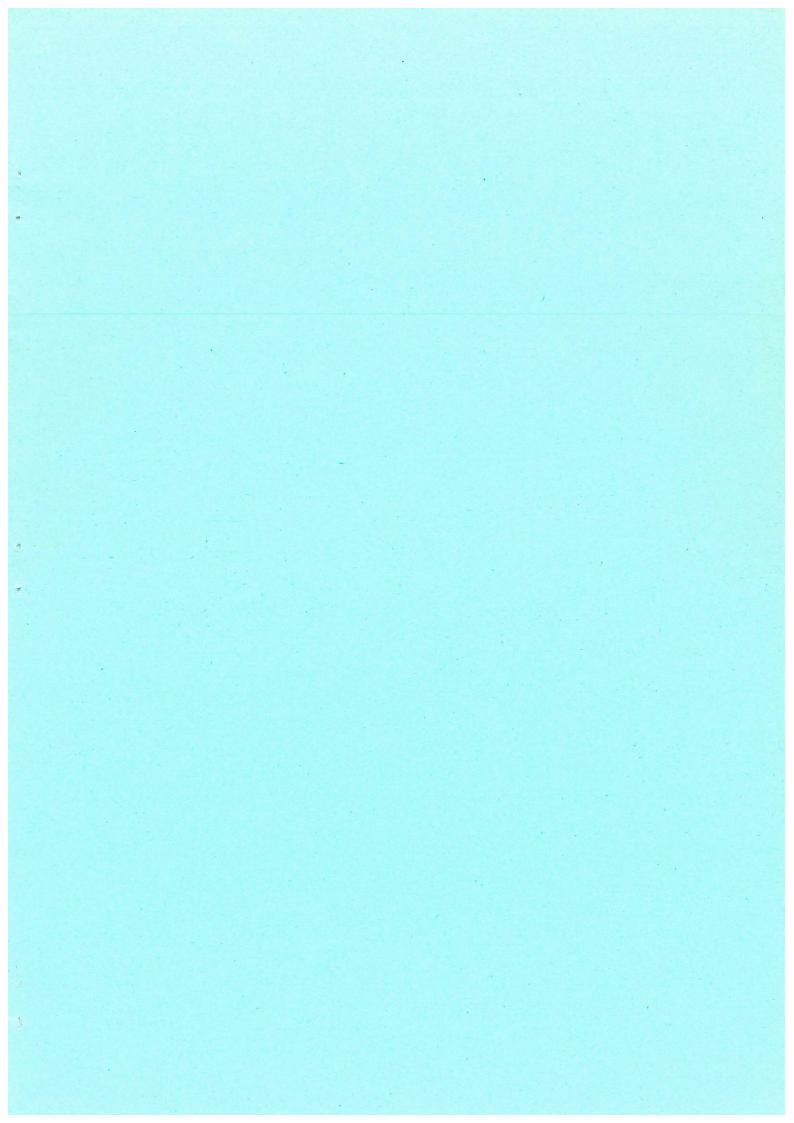
- 1. Correction of typographical errors
- 2. Minor changes (e.g. in the layout of formulae) to improve clarity.
- 3. Those sections of RM 44 which correspond to similar sections in RM 43 to be amended in a similar fashion.
- 4. Minor changes in the wording of paragraphs 8.3, 11.5, 11.8.3, 11.8.4 and 11.8.6 to improve clarity.
- 5. Section 6.2 reference to be made to the advisability of including silicate in the culture medium.
- 6. Section 6.2 recommendation that 0.22 um filter is better than 0.45 um.
- 7. Section 6.4 amend procedure to state that culture medium should <u>not</u> be autoclaved after addition of vitamins. Vitamins to be added aseptically <u>after</u> the medium has been autoclaved.
- 8. Section 6.5 (and related paragraphs). Light intensity of 2000 lux recommended as a starting condition, but may be increased as necessary according to the species used and the physical conditions of the room. Alternative means of agitation to orbital shaker to be referred to.

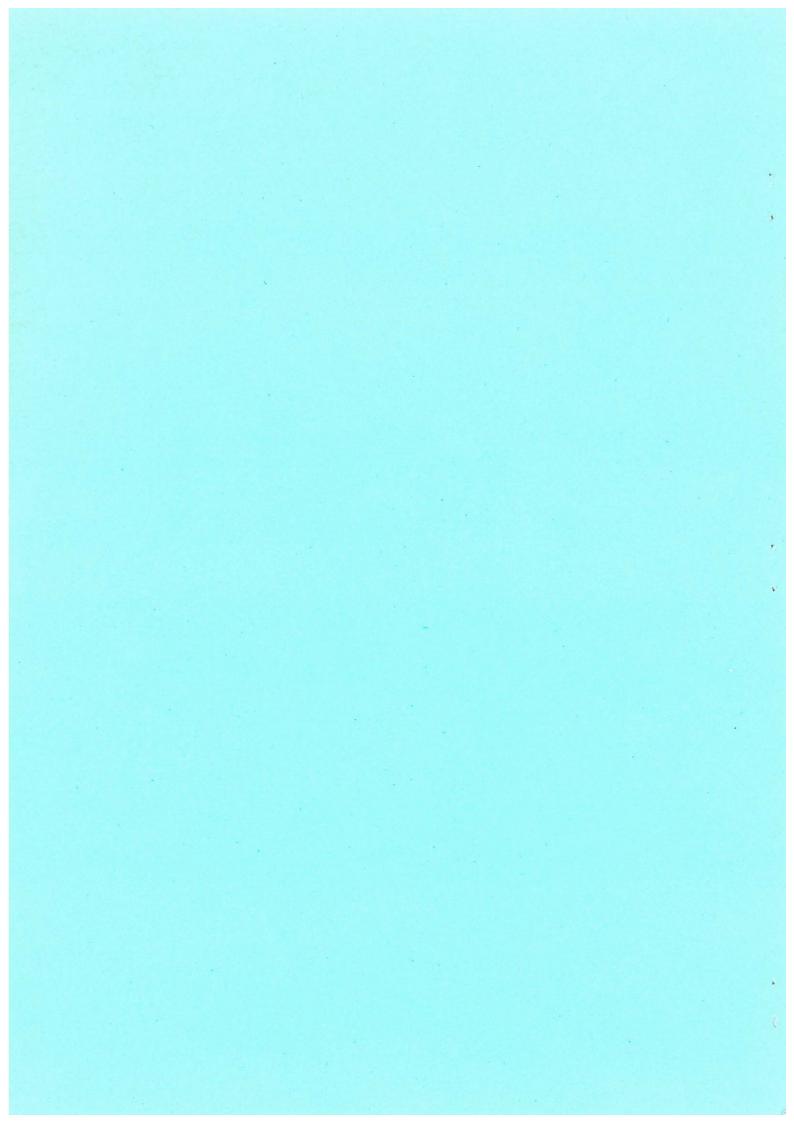
- Section 7. New paragraph to be inserted, referring to methods other than direct counting for measuring the algal population density.
- 10. Section 7.8.1 volume of Erlenmeyer flasks should preferably be 250 ml but could be smaller.
- 11. 7.8.2 and 7.8.3. Amend to the effect that inoculum is added after the medium.
- 12. 7.9.2. It should be made clear that the figure of 10,000 cells/ml is an example of the calculation, not a recommendation for the experimental procedure.
- 13. New section to be incorporated recommending (a) more frequent counting of cells in order to plot the growth curve, (b) the use, where appropriate, of experimental periods other than 96 hours.
- 14. Paragraph 7.14 greater detail concerning procedure for measuring chlorophyll-a is required.
- 15. Paragraph 8.3 amendment to direct the user to paragraph 11.4, not to section 11. The second NOTE to be amended to make the meaning more clear.
- 16. Section 11. Amendments to be incorporated to make clear how this method should be applied in phytoplankton tests (particularly the fact that N refers to the number of replicate test vessels at each concentration, not the number of cells in the culture flasks).
- 17. In view of the above amendments, appropriate amendments to be made to sections 1, 3, 4 and 5.
- 18. Sections and paragraphs will require to be re-numbered accordingly.

R.M. 45

- Correction of typographical errors.
- Minor changes (e.g. in the layout of formulae) to improve clarity.
- 3. Those sections of RM 45 which correspond to similar sections in RM 43 to be amended in a similar fashion.
- 4. The term "water-accommodated fractions" is preferred to "water-soluble fractions" and to be substituted accordingly throughout the text.

- 5. A new section to be incorporated offering guidance on methods for measuring the concentration of oil in the aqueous extract used in the method.
- 6. A new section to be incorporated describing suitable methods for the production of oil/water, dispersant/water, and oil/dispersant/water emulsions by standardised procedures.
- 7. In view of amendments (5) and (6), appropriate amendments to be made to sections 1, 3, 5, 8 and Appendix I.
- 8. All sections and paragraphs to be renumbered accordingly.





24. Do you have any comments about this test method, or do you wish to suggest any modifications? Please make your comments here, giving reasons. (You may continue on a separate sheet if you wish).

CHECK LIST:

You <u>must</u> attach to this report:

- a) Graphs of cumulative percentage mortality against elapsed time for each poison concentration.
- b) Your toxicity curve
- c) A graph of cumulative percentage mortality at 96h against poison concentration.

Please check that you have entered all the data required on the report form.

APPENDIX B. Evaluation of Reference Method No. 45

UNEP/FAO/IAEA Comparative toxicity test of water-soluble fractions of oils and oil dispersants to marine organisms. Reference Methods for Marine Pollution Studies No. 45 (Draft). UNEP 1987.

- 1. Objective of the test. To compare the toxicity to marine organisms of:
 - a) Three petroleum products (if possible)
 - b) An oil dispersant, a petroleum product and a mixture of the two, using the procedure described in the Reference Method.
- Choice of test substances. Participants should obtain a supply of each of the following:
 - a) Petrol (gasoline) as used in motor vehicles
 - b) Diesel fuel as used in motor vehicles
 - c) Light or medium grade fuel oil, or a sample of crude oil
 - d) A commercially-available oil dispersant

NOTE: for the purposes of this exercise, the oil-dispersant chosen is not intrinsically important. However, wherever possible participants should select a dispersant which is widely available on the international market, such as Finasol, BP 1002 or BP 1100, in preference to locally-available dispersants. Participants are required to report data on the oil-dispersant chosen (see Report Form).

- 3. Experiments to be carried out. Participants are required to carry out two experiments:
 - a) To compare the toxicity of petrol, diesel fuel and fuel oil or crude oil to the test species
 - b) To compare the toxicity of oil dispersant, fuel oil or crude oil and a mixture of oil dispersant and fuel oil or crude oil to the test species

In each case the procedure described in the Reference Method must be followed exactly and the results reported as required in the Report Form.

The experimental procedure and conditions. The experimental conditions and the procedures to be carried out are clearly described in sections 6, 7 and 8 of the Reference Method. The procedures described must be followed exactly.

IMPORTANT NOTE: (Refer to section 9.5.5 of the Reference Method). For the purposes of this evaluation, participants must submit, for each experiment, data and results from the first line of good fit, and also they must compute the line of best fit and submit data and results. This is to allow a comparison between results obtained from using lines of good fit, and those obtained from using lines of best fit. The line of best fit is the line which has the minimum value of chi^2 . (See Report Form).