FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

UNITED NATIONS ENVIRONMENT PROGRAMME

EURO-MEDITERRANEAN CENTRE ON MARINE CONTAMINATION HAZARDS (COUNCIL OF EUROPE)

REPORT OF THE WORKING GROUP ON BIOMONITORING IN THE MEDITERRANEAN AND THE BLACK SEA

(Valletta, Malta, 10-11 November 1992)

Organised in the framework of the Long-term Programme
for Pollution Monitoring and Research in the
Mediterranean (MED POL - Phase II)

Athens, December 1992

TABLE OF CONTENTS

Introduction

The FAO/UNEP/IOC Workshop on the Biological Effects of Pollutants on Marine Organisms which was jointly organised with the Euro-Mediterranean Centre on Marine Contamination Hazards (Malta, 10-14 September 1991) recommended the establishment of a Working Group which would formulate a pilot biomonitoring exercise to be implemented by selected Mediterranean institutions in their respective areas. FAO/UNEP and the Euro-Mediterranean Centre undertook the initiative to establish such a Working Group and invited it to meet at the Foundation for International Studies in Valletta, Malta, on 10 and 11 November 1992. Names and addresses of all participants to the present meeting can be found in Annex I. The agenda followed by the Group appears as Annex II.

The meeting was opened by Mr. A. Micallef, Director of the Euro-Mediterranean Centre on Marine Contamination Hazards and by Mr. G.P. Gabrielides, Senior Fishery Officer (Marine Pollution), on behalf of FAO and UNEP (Co-ordinating Unit for the Mediterranean Action Plan). Mr. Micallef explained the role of his Centre which is a Council of Europe centre established in the framework of the Open Partial Agreement for the protection and prevention of the marine environment against pollution hazards. They both indicated that the present meeting is a follow-up activity of the FAO/UNEP/IOC Workshop on the biological effects of pollutants on marine organisms which was jointly organised with the Centre and took place at the same place in September 1991.

1. Background and scope

Mr. Gabrielides informed the Group that the ultimate aim is to introduce biomonitoring in parallel with chemical monitoring in the framework of the MED POL programme. He said that a number of biological effects techniques were identified during the 1991 workshop some of which were considered as potential candidates for biomonitoring. The aim of the Working Group is to formulate a biomonitoring programme to be implemented on a pilot scale. An important issue would be to suggest the most appropriate biomarkers since not all biomarkers are adaptable or useful for all types of contaminants within the Mediterranean.

The Group could also recommend potential participants and deal with the needs for establishing such an exercise in the Mediterranean and Black Sea regions. Experts represented major Mediterranean scientific institutions at different levels but were present in their personal capacity and should give an unbiased advice on the selection of the most appropriate biomonitoring techniques for the Mediterranean region.

2. Objectives of the biomonitoring pilot programme

The major objective of the programme is to enable a small core of institutions within the Mediterranean region to use a selected number of biomonitoring techniques corresponding to priority contaminants already determined in the chemical monitoring. The aim will not be to test the various techniques available but to put into operation established ones in order to develop in the future a fully-operational Mediterranean-wide biomonitoring programme using the achievements of the pilot exercise.

The chemical contaminants determined were heavy metals such as mercury, cadmium, copper, lead and arsenic and organics such as PCBs, DDTs and PAHs. The selection of biomonitoring techniques should also take into account the chemical analyses that participating laboratories can undertake. Other criteria could include the advantages and disadvantages of the techniques, their cost and complexity and the type of species that can be used. The ultimate number of chosen techniques should be very limited so that they can be implemented as soon as possible.

3. Identification of suitable biomarkers of environmental contamination

The Working Group discussed the criteria for selecting suitable biomarkers to be used in the pilot exercise. The basic criteria agreed upon were that the techniques should be well-established, simple for routine use and should not require expensive and sophisticated equipment. The Group listed, in a random order, general and specific biomarkers known to be in use, for further consideration by the Group:

General stress indices

- Scope for Growth (SFG)
- "Stress on Stress" response
- lysosomal membrane stability and/or histopathological changes
- community structure modifications
- the Microtox test
- cytogenetics (micronuclei estimation) and DNA alterations
- acute toxicity tests on early life stages
- serum enzymes and protein parameters
- adenylate energy utilization

Specific stress indices

- Mixed-function oxidases especially EROD
- Phase II (conjugating) enzymes; GST
- Acetylcholinesterase (AChE)
- Metallothioneins
- Imposex

General stress indices

The Scope for Growth index is sensitive to both organic and inorganic contaminants. It also has a high ecological significance and if the dose vs response relationship is available for specific contaminants then one can partition the effects in such a way as to indicate the possible insulting agent. Another advantage is the possibility of transporting exposed test organisms to be tested for their SFG in laboratories that are relatively far away from the testing site. However, the major drawbacks are the relatively difficult and time-consuming procedure which should be carried out with skill and the high capital expenditure. In addition, SFG intercalibration is very difficult to achieve and its use is restricted only to mussels which cannot be found in every part of the Mediterranean. It was pointed out that the simpler lysosomal membrane stability technique was found to be directly correlated with an SFG index along a pollution gradient.

The Group then considered the evaluation of lysosomal membrane stability as a result of contaminant exposure. This technique mainly requires a microscope and a cryostat, which is the only piece of equipment that can be considered as expensive. However, a cryostat can be commonly found in medical histopathological laboratories and can be thus easily utilized there. The technique offers the possibility of being integrated with histopathological examinations of the same tissue under investigation and therefore can give results that are very informative with respect to both early and late pollution responses.

The technique has been described by the GEEP workshops as very significant, simple, and easy to carry out and was shown that with further research it could work well with fish. In addition, the technique is sensitive both to organic and inorganic contaminants and is a more rapid response than DNA alteration. Moreover, this technique can be easily intercalibrated between the participating laboratories and developed for further analysis for a more clarified answer assessment of the response. Both mussels and fish can be used as test species for this technique, although the latter are much more preferred. However, investigations carried out during the mussels' reproductive season may give false positive results.

The "Stress on Stress" biological response was introduced by Dr. Viarengo. Essentially the test organism which is exposed to a specific contaminant(s) is subjected to a standardized stress (e.g. anoxia) and a corresponding response, such as death, is then assessed. The method is not as sensitive as the lysosomal membrane stability but it is very easy to perform and can be conducted at very low cost. This technique has been tested with mussels that were exposed to copper and a combination of Aroclor and PAHs. The technique can be modified to field situations by enclosing the test organisms in cages and placing them along a pollution gradient.

The Group agreed unanimously that community structure modifications is a too late a response in order to be considered with the early warning signals. Moreover, biotic effects are difficult to differentiate and may cause changes within the community that are not a consequence of pollution.

With regard to both the microtox test and embryotoxicity, the Working Group stressed that these two techniques have not yet been applied to pollution gradients in coastal marine environments but only to river effluent monitoring. Furthermore, it was also mentioned that embryotoxicity testing needs extensive standardization.

Cytogenetic tests have been applied for both fish and mussels. However, their sensitivity is questionable since the sensitivity of some of these methods is proportional to the size of the DNA in a way that fish are more sensitive than mussels simply because they have a greater amount of genetic material. Micronuclei seem significant in assessing past DNA damage. Another type of genetic studies include the determination of the extent of DNA alteration using the alkaline unwinding technique. The lower significance of DNA alteration as compared to lysosomal membrane stability was pointed out. The alkaline elution technique as modified recently gives good results. It was considered that the evaluation of micronuclei is very straightforward involving only direct observation using microscopy and it is less costly than carrying out alkaline elution. However, micronuclei represent mitotic alterations and that it is quite different from DNA alteration inferred by the DNA alkaline elution method.

As regards serum enzymes and protein parameters, it was pointed out that it is very difficult to correlate with human enzyme and protein parameters and thus specific kits and procedures meant for human clinical investigations should be adapted and standardized for the test species under investigation. Furthermore, it is very difficult to assess the determination of the normal parameters.

The adenylate energy utilization technique gave poor results when studied along a pollution gradient and thus the Group expressed strong reservations in including it in the pilot programme.

Specific stress indices

The discussion on the specific stress indices was very limited as nearly all of them were considered suitable by the Group. However, it was felt that AChE and imposex which are not addressed to priority MED POL contaminants should not be included in the programme. The Group discussed the potential of Phase II (conjugating) enzymes, GST index as a specific biomarker and mentioned that studies show a 50 to 80% increase in its concentration when compared to control experiments and felt that the technique could be chosen as a secondary index.

The importance of imposex in relation to organotin pollution was highlighted and the possibility of including it in the research component was considered.

At this stage, the Group discussed the number of general stress indicators and specific markers to be chosen for the pilot phase. One opinion was that the number should be kept to a minimum i.e., one general stress indicator and one specific biomarker to enable an easier and faster implementation of the programme. However, from a scientific point of view it was considered better to have more than one technique from each category. Since heavy metals and organohalogen compounds are priority contaminants, both Metallothioneins and MFOs should be incorporated in the pilot programme. As far as the global stressors are concerned the Group showed preference for lysosomal membrane stability testing and DNA alteration studies.

It was put forward that a valid biomonitoring programme should encompass a total of four techniques, two from each category, namely DNA alteration and lysosomal membrane stability as general stress indices together with metallothionein determination and MFO induction as specific biomarkers.

4. Formulation of a biomonitoring programme

Test species

The discussion concentrated on the selection of test species for the four biomonitoring techniques chosen. Mussels, clams and periwinkles (Littorina littorea) have been used in lysosomal membrane stability studies and they could also be considered as the likely candidate species for the remaining techniques. For the Mediterranean, Mytilus galloprovincialis, Venus verrucosa, Littorina neritoides, Patella sp. etc. could be considered. Fish can also be used; however, to avoid inter-variation, the same kind of fish should be used. Dr. Lafaurie said that Mullus barbatus tends to prove difficult for MFOs due to certain peculiar features such as variation between sexes. He suggested the use of Serranus spp that are hermaphroditic, benthic and found abundantly near posidonia beds. The use of sea bass (Dicentrarchus labrax) in cage experiments cannot be excluded. All these species can be used to obtain simply a comparative and a relative monitoring assessment depending on the type of technique being used.

Methodology

As regards MFOs there's already a draft UNEP Reference Method (no. 60) available but since it does not go into details for some points Dr. Lafaurie undertook to distribute a detailed protocol for MFO assay using Serranus spp. As regards Metallothionein determination, Dr. Viarengo undertook to distribute a protocol in the near future. He said that this metallothionein method of quantification can be applied routinely and is very sensitive, although not specific to the type of metallothionein under investigation. A polarographic method can be used as an alternative, although it is less sensitive. For lysosomal membrane stability and DNA studies, he suggested the use of the protocol applied during the GEEP workshops.

Intercalibration

Intercalibration is possible for all the techniques by distributing samples on dry ice; however, for the DNA elution method this is not possible but high molecular weight DNA standards are available and can provide a solution. Both liquid nitrogen or dry ice can be used for transporting proteins and it is best to buy reagents from the same supplier. Metallothionein standards can be distributed to the participating laboratories by post at low temperature. As regards the intercalibration exercises, the Universities of Genova and Nice would be willing to act as focal points for lysosomal membrane stability and MFO studies respectively. Both control and exposed tissue samples should be distributed to the various participating laboratories and the data obtained should then be correlated. Collaboration will also take place for the interpretation of the data.

At this point Dr. Lafaurie said that he was authorised to inform the Group that IFREMER was planning a 40- 50 day cruise in the Mediterranean region during 1995 especially for the pilot programme and that the Group should examine the possibility of incorporating an intercalibration exercise within the 1995 cruise. He said that a relevant proposal should be submitted to IFREMER.

Sampling

Sampling should follow a specific protocol. It was envisaged that samples should be collected from 4 to 5 stations along a concentration/pollution gradient.

Pools of 5 fish or 10-12 mussels of the same size should be used for all techniques except lysosomal stability; 5-8 replicates are necessary. It was agreed that sampling should take place twice a year at each station before and after the reproductive phase.

Time schedule

Before deciding on a time schedule it was necessary to examine the capabilities of the potential participating laboratories. After a lengthy discussion and based on information provided by the Group members, it was apparent that it was not possible to initiate a pilot biomonitoring exercise in the beginning of 1993 using all four recommended techniques. Consequently it was agreed that the techniques should be introduced in groups of two starting with MFOs and lysosomal stability and that 1993 should be used as a preparatory phase for these two techniques. Year 1994 would be the first year of the exercise using MFOs and lysosomal stability but also the preparatory phase for metallothionein and DNA studies. More specifically the time schedule would be as follows:

1993: preparatory phase for the development of lysosomal membrane stability and MFO studies by those laboratories still incapable of performing these tests during this year.

autumn of 1993: intercalibration exercise for above two techniques

1994: collection of data using the above two techniques;
preparatory phase for the development of metallothionein and DNA alteration studies

autumn 1994: intercalibration exercise

end of 1994: meeting of participants to discuss progress of work

1995: biomonitoring exercise using all four techniques.

beginning of 1996: workshop to summarise results and make conclusions

5. Identification of potential participating laboratories

Based on information provided by the Group members, a list of Mediterranean Institutions was drawn up where the above recommended biomonitoring techniques are actually being used or are at the stage of development. Institutes which intend to develop them shortly were also included. These are:

Spain

- Centre for Research and Development, Barcelona
- Spanish Institute of Oceanography, Murcia

France

- University of Nice
- IFREMER, Toulon

Italy

- University of Genova
- University of Siena

Croatia

- Centre for Marine Research, "Rudjer Bošković" Institute, Rovinj and Zagreb

Greece

- National Centre for Marine Research, Athens

Turkey

- Middle East Technical University, Ankara

Malta

- University of Malta

Israel

- University of Tel-Aviv

Tunisia

- University of Sousse and Sfax

Algeria

- University of Annaba

Romania

- Romanian Marine Research Institute, Constanta

Going further into the activities and technical capabilities of the above Institutes, a short list was drawn up composed of the following:

- University of Nice
- University of Genova
- Centre for Marine Research, "Rudjer Bošković" Institute, Rovinj and Zagreb
- University of Malta
- University of Sousse and Sfax

and possibly

- Centre for Research and Development, Barcelona

- University of Tel-Aviv

- Romanian Marine Research Institute, Constanta

The participants' list will be finalised after each laboratory is contacted officially.

6. Other aspects relevant to the implementation of the programme

The Working Group discussed the practical aspects for implementing the programme. Firstly the Group identified the expensive pieces of equipment which are necessary to carry out the four selected techniques. These are:

Lysosomal membrane stability: Cryostat, microscope, image analyser (optional)

DNA alkaline unwinding technique: Peristaltic pump, fraction collector, spectrofluorimeter

Mixed function oxidase induction: spectrophotometer, spectrofluorimeter, centrifuge (S-9), ultracentrifuge (optional)

Metallothioneins: spectrofluorimeter with plate reader (Perkin Elmer LS-50) or densitometer, electrophoresis equipment.

It was apparent, that unless large amounts of non-MTF funds are made available, there is no possibility of providing institutions with such equipment. An effort will be made to include a special budget line in the MTF for the biennium 1994/95 to assist laboratories with the purchase of reagents and small equipment.

The training needs of potential participating institutions were also discussed. The Universities of Nice and Genova indicated their willingness to accept staff from other laboratories for training but would prefer people who are involved with the technique to shorten the time of training and to be able to evaluate the data obtained. On-job training was also discussed and some participants considered it preferable. From the discussion it was apparent that many laboratories have training needs and the necessary funds should be sought. The MED POL budget could cover part of the needs.

7. Conclusions

The main conclusions which can be drawn are the following:

a) There is an urgent need to initiate biological effects monitoring in parallel with the chemical monitoring in the Mediterranean and the Black sea.

b) Some laboratories of the region have initiated or are in the process of initiating biomonitoring studies but their number is not sufficient to enable the implementation of a fully-operational Mediterranean-wide biomonitoring programme.

c) It is necessary to strengthen and upgrade the technical capabilities of more laboratories, especially those from the South, to enable a full participation of as many countries as possible.

d) A pilot biomonitoring exercise can be initiated towards the end of 1993 if intensive preparatory work takes place during the year.

e) A pilot biomonitoring exercise is imperative before a full programme can be implemented.

8. Closure of the meeting

The FAO/UNEP representative, in his closing remarks, expressed satisfaction for the results of the meeting and thanked the Group members for their invaluable contribution to its success. He also expressed his appreciation to the Euro-Mediterranean Centre on Marine Contamination Hazards (Council of Europe) for organising and hosting the meeting.

The Director of the Centre expressed his gratitude to all those present and his staff who helped make the meeting a success.

ANNEX I

LIST OF PARTICIPANTS

Victor AXIAK
Marine Ecotoxicology Laboratory
Department of Biology
University of Malta
Tal-Qroqq
Msida
MALTA

Tel. 356-342488
Fax. 356-336450
Tlx. 407 HIEDUC

Amor EL ABED
Laboratoire de l'Environnement et de Physiologie des poissons
Ecole Nationale d'Ingénieurs de Sfax
Route de Soukra
B.P. "W", 3038 Sfax
TUNISIE

Tel. 216-4-74088/74409/74418
Fax. 216-4-74437
Tlx. 40982 ENIS

Marc LAFAURIE
Maître de Conférences
Laboratoire de Biotransformation et Cancérogénèse
Faculté de Médécine
Université de Nice
Avenue de Valombrose
06107 Nice Cedex 2
FRANCE

Tel. 33-93377721
Fax. 33-93536121
Tlx. 970281

Mirjana KRAJNOVIĆ-OZRETIĆ
Centre for Marine Research-Rovinj
"Rudjer Bošković" Institute
Giordano Paliaga 5
P.O. Box 150
52210 Rovinj
CROATIA

Tel. 38-52-811544/811567/816633
Fax. 38-52-813496

Aldo VIARENGO
Istituto di Fisiologia Generale
Università di Genova
Corso Europa (Palazzo delle Scienze)
16132 Genova
ITALY

Tel. 39-10-509101
Fax. 39-10-3538267

FOOD AND AGRICULTURE ORGANIZATION OF THE
UNITED NATIONS

Gabriel P. GABRIELIDES
Senior Fishery Officer (Marine Pollution)
Co-ordinating Unit for the Mediterranean
Action Plan
P.O. Box 18019
Leoforos Vassileos Konstantinou 48
116 10 Athens
GREECE

Tel. 30-1-7244536-9
Fax. 30-1-7291160
Tlx. 22564 MEDU GR

EURO-MEDITERRANEAN CENTRE ON MARINE
CONTAMINATION HAZARDS

Anton MICALLEF
Director
Euro-Mediterranean Centre on Marine
Contamination Hazards
Foundation for International Studies
St. Paul Street
Valletta 07
MALTA

Tel. 356-240746
Fax. 356-230551

Charles GALDIES
Project Officer
Euro-Mediterranean Centre on Marine
Contamination Hazards
Foundation for International Studies
St. Paul Street
Valletta 07
MALTA

Tel. 356-234121/224067
Fax. 356-230551

ANNEX II

AGENDA OF THE MEETING

1. Opening of the meeting
2. Background and scope
3. Objectives of the biomonitoring pilot programme
4. Identification of suitable biomarkers of environmental contamination
5. Formulation of a biomonitoring programme
6. Identification of potential participating laboratories
7. Other aspects relevant to the implementation of the programme
8. Conclusions
9. Closure of the meeting