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**THE MED POL BIOMONITORING PROGRAMME CONCERNING THE
EFFECTS OF POLLUTANTS ON MARINE ORGANISMS ALONG
THE MEDITERRANEAN COASTS**

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1. INTRODUCTION

It is now well established that chemical monitoring represents a suitable approach to determine and quantify the amounts of pollutants present in the different compartments of the marine ecosystem (i.e. water column, sediments and organisms).

However, due to the extremely high number of toxic compounds present in the environment (more than 4000 toxic substances have been identified in coastal waters) it is very difficult to define an area "unpolluted" from a chemical point of view. In this regard, it is important to note that organic and inorganic pollutants have been found not only in the coastal waters of industrialised areas but also, although in minimal amounts, in remote regions such as the Arctic and the Antarctic environments.

The difficulties in the evaluation of the chemical pollution level of a particular sea coast area are related to the fact that, different physical factors, such as the movement of sea water masses, the variable distribution of pollutants in the water column, the wave movement, as well as biological parameters such as algal blooms, particulate suspended matter and movement of organisms, can considerably affect the concentration of chemical contaminants. Therefore, complete chemical analysis of a marine coastal ecosystem needs a repetitive collection of numerous samples which usually render the assessment of pollution extremely expensive. In addition, this kind of data are frequently difficult to interpret, particularly when it comes to the comparison of pollution levels between different coastal sites.

Despite the difficulties that may be encountered for a correct interpretation of chemical data in the assessment of marine ecosystem alteration, and the fact that today it is not possible to extrapolate from the pollutant levels the possible effects on marine life, it must be stressed that the chemical approach remains a fundamental step in the monitoring of the marine environment.

However, in the last two decades the study of the effects of pollutants on marine organisms has allowed the identification of a certain number of biological parameters, usually referred to as "stress indices" or "biomarkers", suitable to evaluate the physiological status of the aquatic organisms. The concept of a biomarker is strictly linked to that of a stress syndrome, which is often defined as a measurable alteration of a physiological steady-state that renders the organisms more vulnerable to further environmental changes (Bayne, 1975). Biomarkers have been in fact defined as biological parameters whose variations may be utilised to point out and quantify the stress syndrome.

The use of biomarkers will therefore allow the evaluation of a stress syndrome induced on the organisms by the accumulation of pollutants in their tissues.

It is now generally accepted that in a polluted environment, the toxic chemicals taken up by the organisms will initially produce alterations at the molecular level (structure and function of proteins, enzyme-substrate interactions, metabolites, nucleic acids, etc.). Such events will cause dysfunction in cells and tissues and will produce alteration of the organism's physiology with a consequent reduction in growth,

and reproduction or survival capability (Jenkins and Sanders, 1986). These detrimental effects may also propagate from organisms to population and community level, thereby greatly affecting life in a polluted ecosystem.

Although the target of environmental research should be to evaluate pollutant effects on population and community in a particular ecosystem, this kind of study has been found, to present a number of drawbacks. In particular, the period of time needed for the toxic effects of pollutants to become evident at the population and community level could be very different (depending on sublethal/lethal pollutant concentration, effects of the chemical compound on the reproduction of relevant species in the ecosystem, etc.). In addition, the natural variability at the population and community level is high. Such a complex of facts can often make difficult (or even impossible) a clear distinction between biological changes which are due to pollutant effects from those which depend on naturally occurring variations.

The use of biomarkers can therefore represent a suitable way to correlate data relevant to the concentration of chemical pollutants in marine organisms with the alteration of their physiology. Such an approach can be a highly informative, low cost, complement to the chemical monitoring programme developed in the past years. As clearly reported by Shughart *et al.* (1992) the utilisation of biomarkers allows to integrate an organism's exposure to pollutants over a period of time. In addition, biomarkers can provide an integration of the additive (or synergistic) effects of a complex mixture of pollutants (and also variations of environmental factors such as temperature, salinity, etc.). Due to the rapid action of toxic chemicals at the molecular and cellular levels, biomarkers also provide an early warning for long-term pollutant effects. Finally, the use of biomarkers is a complement to ecological surveys correlating environmental alterations to a direct effect of contaminants.

2. THE MED POL BIOMONITORING PROGRAMME

In the early 1990's, MED POL started to develop the idea of a programme for monitoring the biological effects of pollutants along the coasts of the Mediterranean sea. In this context two meetings were organized in 1991 and 1992 to decide the strategies for the development of the programme. The conclusive points of these meetings pointed out that the chemical monitoring of the marine environment developed in the past 20 years represent the more appropriate scientific background to start a biological monitoring aimed at quantifying the deleterious effects of pollutants on marine life in the Mediterranean sea.

The basic concepts in the organisation of the first Mediterranean Biomonitoring Programme were essentially the following:

- a) The only way to obtain reliable results for assessing the effects of pollutants on marine organisms is to use a battery of tests on at least two different organisms occupying key positions in the coastal ecosystem. Research work carried out showed that the utilisation of a single biomarker, no matter how powerful (eg. MFO, MT etc.), was always found not suitable to describe the

effects of chemicals on aquatic life.

- b) As far as possible the sentinel organisms used must be the same in the different Mediterranean regions. When this is not possible similar species should be used for which sufficient biochemical, cyto/hystological and physiological information is available (in case of lack of basic biological information concerning the selected biomarker, background research should be developed in order to make easier the interpretation of the data collected from an ecological point of view).
- c) As the monitoring programme will extend to all Mediterranean countries the proposed biomarkers must not only be sensitive and reproducible, but also low-cost and possibly simple to use in order to allow a rapid but complete training of personnel from laboratories where no biomarker experience is available.
- d) The biomarkers selected should be intercomparable, in order to allow easy and routine comparison of data from laboratories from different countries. This will allow a uniform and correct interpretation of the effects of the chemical pollutants on the organisms in the different Mediterranean regions.

3. SELECTION OF THE BATTERY OF BIOMARKERS

In the initial phase of the biomonitoring programme a set of four simple, sensitive, reproducible, and low-cost biomarkers was selected. Among these, two biomarkers, lysosomal membrane stability and DNA damage, were used as general stress indices. These biomarkers reveal a syndrome characteristic of the animal response to a wide variety of environmental stressors i.e. they integrate the effects of the pollutants accumulated into the cells, taking also into account possible negative effects caused by variations of environmental parameters such as temperature, oxygen, salinity, etc.

Two of the biological tests selected are specific stress indices able to reflect the response of the organisms to a particular class of contaminants: (i) Mixed Function Oxygenase (MFO), evaluated as EROD (Ethoxyresorufin-O-deethylase) activity, a biomarker showing the biological responses to xenobiotic aromatic compounds such as PAHs, PCBs etc. (ii) Metallothionein concentration, usually considered a good indicator of the biological response to heavy metal pollution.

A fifth biomarker, the stress on stress response, was recommended to be used in countries where mussels are available.

Initially, another general stress index, that of scope for growth, was proposed. This biomarker is based on such parameters as feeding, digestion, respiration and excretion rates to provide an insight into the growth process. Scope for growth provides a quantitative evaluation of changes in the energy status of the animal, which reflects an integration of a wide variety of responses to environmental pollutants. This parameter

has been recently demonstrated to be of great sensitivity for the assessment of environmental pollution levels, being sensitive to low concentrations (in the range of ppb) of hydrocarbons and heavy metals.

Notwithstanding the importance of this biomarker, different MED POL laboratories have found its use not easy for routine applications. Moreover, the fact that scope for growth requires the use of living organisms, usually mussels, makes it not suitable for intercomparison quality control, thus reducing the possibility of comparing the data collected in different laboratories. For these reasons, this important biomarker was not included in the battery of tests selected to be utilised in the Mediterranean Biomonitoring Programme.

The section below describes some fundamental characteristics of the selected biomarkers, that render them most suitable for evaluating the physiological status of the animals (general stress indices) and for identifying the class of pollutants (heavy metals/organic xenobiotics) that determine the stress syndrome.

3.1 General stress indices

3.1.1 Stress on stress response

According to Bayne (1986) "stress" is defined as a measurable alteration of biochemical and physiological parameters induced by an environmental change which results in a reduced capacity of the individual to adapt to further environmental variations. This concept was applied practically to mussels, superimposing exposure to air, a natural stressor, over the effects of pollutants. Mussels are evolutionarily adapted to survive to short periods of aerial exposure, but they are also able to sustain prolonged emersions (days). It was clearly demonstrated that mussel mortality in air occurs more rapidly in animals pre-stressed by pollutant than in control mussels. In particular it was found that exposure to sublethal concentrations (nM) of pollutants, such as Cu^{2+} , DMBA, and Aroclor 1254, significantly reduced mussel capacity to survive in air. This effect was markedly dose-dependent, and was also strongly increased by pollutant mixtures.

Stress on stress response shows a sensitivity which is in the same range of other commonly used general stress indices such as lysosomal membrane stability. Moreover, the methodology is simple, does not require sophisticated equipment and is inexpensive. Therefore, the stress on stress response should be utilized in monitoring programmes as a general stress index for the assessment of contaminated coastal areas in all those countries where marine mussels are available.

3.1.2 Lysosomal membrane stability

The evaluation of lysosomal membrane stability represents a biomarker able to give a clear indication of the toxic effects of the chemicals accumulated in the tissues of marine organisms (Viarengo and Canesi, 1991).

It is well known that the cells are able to accumulate and compartmentalize in the lysosomal vacuolar system a wide variety of toxic compounds such as organic

xenobiotics and metals which in turn may alter the functions of these organelles. It was clearly demonstrated that heavy metals (Cu, Cd, Hg, etc.), PAHs and PCHs are able to destabilise the membranes of lysosomes also when present in the aquatic environment at minimal concentration (ppb). This causes an increase of the lysosomal activity which, at least initially, can induce an enhancement of the catabolic rate of cell macromolecules (protein, RNA, DNA etc.) leading to autophagy and cell damage (possibly also due to the release of hydrolytic enzymes in the cytosol) and ultimately, to cell death (Moore, 1976).

Most of the studies on this subject seem to indicate that the deleterious effects of the different stressors (i.e. the variation of physical, chemical or biological parameters as well as the presence of chemical pollutants in sea water) give additive effects on the destabilisation of the lysosomal membranes. Therefore, this stress index seems to be able to integrate the toxic effects of most of the different chemical compounds accumulated in the tissues of marine organisms, taking into account, at the same time, the basal level of stress that can be induced by natural variations of the environmental parameters.

3.1.3 DNA damage

The term genotoxicity refers to the ability of a chemical pollutant or other agent to alter the structure of DNA. Carcinogen and/or mutagenic chemical pollutants are able to interact with DNA either directly or indirectly through the production of active intermediates. DNA can be repaired by different mechanisms or can produce a stable hereditary genetic alteration.

Carcinogenic and mutagenic aquatic pollutants may exert an effect on a single individual and may be active through several generations, by producing irreversible modifications.

A high incidence of neoplasms have been described in marine vertebrates and invertebrates (Mix, 1986), in association with carcinogenic and mutagenic pollutants, in different geographic areas around the world (Malins *et al.*, 1985).

Different biomarkers are available to detect the level of DNA alteration, in terms of DNA damage, or chromosomal mutation. Studies on marine animals have allowed the selection of two tests: alkaline elution and micronuclei frequency. Alkaline elution quantifies DNA alteration in terms of single strand breaks in alkaline medium (alkali labile sites) (Kohn *et al.*, 1976; Bolognesi *et al.*, 1996). This test, together with DNA alkaline unwinding, represents a well recognised genotoxicity biomarker able to "photograph" the DNA alteration status in the cells of the sentinel organisms at the time of sampling.

Micronuclei's frequency

Micronuclei are an index of chromosomal damage. Micronuclei are small DNA-containing bodies which can be present near the nucleus (Heddle *et al.*, 1983). Micronuclei may include fragments of a chromosome and could therefore represent an

index of DNA damage or incorrect chromosome separation during the cellular division.

A very important point is that micronuclei provide an index of accumulated genetic damage during the lifespan of the cells and therefore they can be used to integrate over a defined period of time the genotoxic effects of pollutants.

3.1.4 Mixed Function Oxygenase (MFO)

Mixed Function Oxygenase (MFO) is a multienzyme system found to be present, although at very different levels of activity, in the smooth endoplasmic reticulum of vertebrate as well as invertebrate cells (Payne, 1977).

It consists of a brief electron transport chain containing NADPH cytochrome P450 reductase and cytochrome P450 and its main role is related to the conversion of endogenous or xenobiotic compounds (PAHs - PCBs etc.) to more hydrophilic products (Phase I reaction).

MFO shows two fundamental properties. The first one is the low substrate specificity, which is also related to the fact that cytochrome P450 was found to represent a "family" of cytochromes able to metabolize most classes of aromatic xenobiotic compounds. The second characteristic is that MFOs are inducible by various substances (aromatic lipophilic compounds) accumulated into the cells. These characteristics render MFO potentially suitable as a specific stress index for organic xenobiotic pollution, as these enzymatic activities increase as a response to a rise in the concentration of different organic xenobiotics in the cells (Livingstone, 1985).

MFO are highly active (and easily inducible) in the cells of vertebrates, but the basal activity can be very low in the cells of invertebrates where also the induction often results. Therefore, this very important biomarker provides usually the best results when utilised on vertebrate (fish) tissue (usually liver).

3.1.5 Metallothionein concentration

As reported by many authors, metallothionein evaluation represents a specific stress index able to give information about heavy metal pollution. As known, metallothioneins are cellular proteins inducible by the metals accumulated into the cells (Viarengo and Canesi, 1991). They are soluble, low molecular weight (usually about 7000 d) proteins having high affinity for heavy metals.

Metallothioneins show a typical aminoacid composition characterised by the virtual lack of aromatic aminoacids, histidine and methionine and by a high cysteine content (12-30 %). The high content of cysteine sulphhydrylic groups present in these proteins is essential to guarantee a high affinity of heavy metals binding through the formation of tetrathiolate bonds.

Metallothioneins can bind both essential metals such as Cu and Zn and non essential ones, such as Cd, Hg, Ag etc. that are generally considered among the main toxic inorganic cations present in the marine environment. Due to their inducibility and

biochemical characteristics, metallothioneins play an important role in the cells: they protect the cell structures from non-specific binding of heavy metals with biological molecules; at the same time, they can detoxify the excess of metal cations that penetrates into the cells (Viarengo *et al.*, 1987).

Metallothioneins have been identified in almost all living organisms and therefore this biomarker can be used on different organisms representing a number of ecosystems.

Data from experimental research demonstrated that this parameter may represent a sensitive specific stress index, the synthesis of metallothioneins being stimulated by ppb of heavy metals present in sea water.

In conclusion, all the biomarkers proposed are sensitive and provide reproducible data. Also the procedures for their determination are simple, low cost and intercomparable. The required equipment (i.e. cryostat and microscope for lysosomal membrane stability; centrifuge and spectrophotometer for metallothionein; centrifuge and spectrophotofluorimeter for EROD activity; light microscope for micronuclei) are moderately expensive but are common basic instruments found in laboratories for biochemistry, cellular physiology, cytochemistry and therefore are presumably present in most of the research centres participating in MED POL.

Stress on stress response in mussels is so sensitive, simple and inexpensive test that its utilisation is suggested in all the countries where mussels are available.

A group of experts selected the simpler, more sensitive and reproducible methodologies to evaluate the proposed biomarkers and a manual was prepared to guarantee that all the laboratories involved in the programme can utilize for the analyses, standard operating conditions.

4. ORGANISMS PROPOSED FOR THE PROGRAMME

Mussels (*Mytilus galloprovincialis*, Lam.), where available, represent a suitable sentinel organism. In fact, these lamellibranch molluscs are sessile, intertidal, filter feeding organisms, able to accumulate in their tissues most of the pollutants present in the surrounding water.

However, mussels are not the best organisms for MFO determination and therefore a fish should represent a correct complement to provide a better information on the biological effects of organic aromatic pollutants. Fish from resident populations, possibly with feeding habits related to benthic environments, should represent the best choice. As known, pollutants tend to accumulate in the sediment, therefore, the feeding habit of the selected fish species could be important in terms of chemical compound accumulation.

In field experiments carried out by the University of Nice (Prof. M. Lafaurie), *Scilla cabrilla* was used as the sentinel organism for studies on wild population. However, it must be pointed out that studies utilising caged animals seems to be more appropriate to reduce data variability and to ensure that fish responses are strictly related with the pollution of a well defined coastal area. For this approach, molluscs and fish are usually obtained from commercial farms and the utilisation of mussels and fish of the species *Dicentrarchus labrax* was suggested on the basis of previous experience of the University of Genoa and Nice (Prof. Viarengo and Prof. Lafaurie).

Where the suggested sentinel species were not available, different molluscs and fish should be utilised, taking into account previous experience in the use of the selected organisms for biomarker application in monitoring programmes.

Initially, due to the relatively high cost of the cage system, analysis of biomarkers on wild populations was suggested. However, a pilot project in countries where the approach with caged animals can be undertaken is considered of great importance.

5. QUALITY ASSURANCE (QA)

Quality assurance represents the pool of activities devoted to guarantee controlled data of high quality. Therefore, quality assurance must be considered an essential element in a large, international, monitoring programme.

To reach such a target, different activities were promoted before the realization of the biomonitoring programme:

- a) A group of experts prepared a manual containing the standard operating procedures concerning all the biomarkers proposed for the implementation of the programme.
- b) Three training courses were organized (Nice - Sunderland - Genoa) ensuring the participation of scientists involved in the development and utilization of the biomarkers proposed for the Mediterranean programme. Individual training was also offered to the staff of the different Mediterranean laboratories in selected, internationally acknowledged research institutes (Centro Interuniversitario Chimica e Biologia dei metalli in traccia, Genoa, Istituto tumori, Genoa - Lab. Toxicologie marine, Nice).
- c) A number of research centres were visited to ensure the adequacy of the laboratories to participate in the programme (staff, equipment and financial support).
- d) Finally, an intercalibration (and intercomparison) exercise was organized for 3 of the techniques (EROD, Lysosomal membrane stability and Metallothioneins). Reports on these exercises are submitted separately.

6. INTERCOMPARISON OF BIOMARKER TESTS

The previous experience developed during MED POL concerning the chemical monitoring of the Mediterranean sea clearly showed that an intercalibration programme is essential to ensure a good level of the quality of the data obtained in the different laboratories involved in the programme, and to provide a correct comparison of the results collected in the different countries, in different seasons or years.

For the same fundamental reasons, before starting the pilot phase of the MED POL biomonitoring programme, an intercomparison exercise was organised. It is important to point out that this is essentially the first time that quantitative/semiquantitative biological analyses were intercompared with the aim of ensuring that in all the Mediterranean laboratories involved, an alteration of the physiological status of the sentinel organisms could be interpreted in the same way, and that the quantitative data could be compared with the results of the other laboratories.

Three biomarkers, lysosomal membrane stability, metallothioneins concentration and EROD activity (MFO), were intercompared among laboratories participating in the programme.

Genetic stress indices such as DNA damage (alkaline elution and micronuclei frequency) were not included in the intercomparison exercise because most of the laboratories were not yet ready to start the application of these biomarkers, and more work was necessary before this test could be applied widely.

For what concerns the organization of this intercomparison exercise, it must be noted that all the laboratories received a detailed set of protocols concerning the utilisation of the different biomarkers. This way, most of the researchers involved in the programme were able to use the same methodologies in order to evaluate the proposed stress indices.

The Centro Interuniversitario di Biologia e Chimica dei Metalli in Traccia of the Genoa University organized the intercomparison on lysosomal membrane stability and metallothionein concentration, while the Laboratoire de Toxicologie Marine of the Nice University managed the intercalibration on EROD activity.

6.1 Lysosomal membrane stability

Only 5 laboratories provided results for this cytochemical intercomparison (Croatia, France, Greece, Italy and Monaco). This fact may probably indicate that the training for the utilization of this stress index was insufficient or that the equipment (although simple: a cryostat, a microscope and a thermostated shaking bath) was not always available in the different Mediterranean laboratories involved in the biomonitoring programme. However, it must be stressed that this is a powerful general stress index and its application need to be extended to a larger number of laboratories in the near future.

The results obtained in all the laboratories involved in the cytochemical lysosomal membrane stability intercomparison exercise demonstrated that, each research group was able to find out control and stressed animals (exposed to 0.6 μM Cu for three days); controls were correctly estimated showing a latency time of about 21-30 min (with the exception of a laboratory that found a lower value of 15 min). All the participating laboratories estimated that Cu-exposed mussels had a lysosomal membrane stability in the range of 4.5-7.4 min. By comparing pairs of data collected in each laboratory, it was possible to assess that the percent variations of latency times between control and treated animals were quite similar in different laboratories.

6.2 Evaluation of Metallothioneins concentration.

Eight laboratories from five countries participated in this intercalibration exercise (Italy, Croatia, Malta, Monaco, Spain). All the laboratories utilized the spectrophotometric method developed at the University of Genoa and proposed in the Manual. The researchers received 8 blind samples of digestive gland homogenates. Four were from control mussels and 4 were obtained from Cd-exposed mussels. A preliminary analysis in the Genoa laboratory establishes an increase in metallothionein concentration by about 200% in the Cd-treated samples with respect to control samples. A certain amount of a GSH standard (SIGMA Chemical Company) was also sent to the laboratories. The material was despatched in solid CO_2 and in the Genoa laboratory it was also verified that no decrement in metallothionein concentration was detectable in samples maintained frozen for up to 3 months.

The result was that all the laboratories involved in the intercomparison activity were able to identify the samples obtained from either control or Cd-exposed animals. Although the mean absolute values varied from 139.8-427.9 $\mu\text{g/g}$ ww tissue in the controls and from 332.2 to 958.3 in Cd exposed mussel digestive glands, the percent differences between controls and Cd exposed were quite similar (about 200%) for all the laboratories.

6.3 EROD activity

Eleven laboratories from eight different states (Croatia, France, Israel, Italy, Malta, Monaco, Spain, Turkey) participated in the intercalibration of EROD activity. In this case not all the laboratories used the same method to evaluate the enzymatic activity in the microsomal pellet obtained from the liver of control and β -naphthoflavone treated fish. All the laboratories were able to identify the biological samples obtained from control and treated animals, the variation in specific activity was found to vary from 6.8 to 15.9 (with the exception of one laboratory) in the control samples, and from 69.0 to 165.1 (with the exception of one laboratory) in the treated fish. The percentage increase in the xenobiotic treated animals with respect to the control was very similar for all the laboratories (\approx 11 times).

It is important to point out that the percent increase in the treated fish was also similar when utilizing the S9 supernatant instead of the microsomal fraction. As known, S9 supernatant can be prepared with centrifuges usually present in all the laboratories (able to reach 12-20000g); microsomal fraction can be prepared only

utilizing ultracentrifuges (able to reach at least 150000g), which are highly sophisticated and expensive instruments present only in biochemistry laboratories of well organized research centres.

On the basis of these results it is suggested that in future, activity S9 supernatant be utilized to evaluate EROD activity in biomonitoring programmes. This choice was also made by IFREMER.

7. A PILOT BIOMONITORING PROGRAMME

During 1996, the laboratories of 8 Mediterranean states participated in the pilot biomonitoring programme but data were received only from Croatia, France, Israel, Italy, Monaco and Spain.

Looking at the results presented in their reports it may be concluded that the first year of biomonitoring of the pollution effects along the Mediterranean coasts has been a good success; this is essentially the first attempt to quantify the deleterious effects on living organisms of the toxic compounds discharged in these coastal waters.

This kind of studies are very important to correctly preserve aquatic life in the Mediterranean sea; however, it must be pointed out that in a biomonitoring programme organised on such a large scale, the comparison of the results collected in the different states could be a problem. Therefore more work is needed to achieve a better coherence among the results.

As a positive result of the first year of activity we can say that most of the laboratories involved in the programme were able to correctly identify the deleterious effects of pollution on molluscs and fish sampled from different sites distributed along a well known pollution gradient, which had been previously characterized from a chemical point of view.

Another important point was that most of the research teams involved in the programme utilized as a common base the set of biomarkers suggested for the programme and used in the training courses. Moreover, the laboratories improved the programme by using additional stress indices typically related to their specific field of research.

This approach will make each laboratory free to improve and develop new biomarkers and utilize them within the programme, as well as to eliminate those biomarkers whose application in these studies has proven unsuitable on the tested organisms. Therefore, the programme will allow each group to fully develop its potential in this research field giving its own contribution to the continuous development of the common monitoring programme.

In this context it is important to note that the data collected by different laboratories confirmed some general indications; it has in fact been verified that the biomarker based on MFO activity in molluscs (and mussels in particular) is not as

powerful as in fish and therefore in the future biomonitoring programmes, MFO estimated as EROD activity will be evaluated only in fish liver, unless new technical developments occur to render this biomarker suitable also on invertebrate tissues.

In particular, it must be mentioned that four laboratories were able to use lysosomal membrane stability obtaining excellent results. Unfortunately, this powerful stress index is not yet widely used in the Mediterranean laboratories. Concerning this biomarker, it must be mentioned that the utilization of the "in vivo" lysosome membrane stability test gave results that must be considered in depth before proposing a general utilization of this methodology on marine species not yet sufficiently studied. The use of standard analytical conditions (also related to the particular organism utilized) is also a prerequisite.

Only 2 laboratories utilized the test for DNA damage; this general stress index is usually considered of great importance to define the physiological status of the organisms, and therefore larger emphasis is suggested to be given in the future training courses.

EROD activity is one of the best known biomarkers, and although its utilization was in general successful, it must be said that high pollution conditions could alter the liver physiology of fish so that minimal MFO activity and organic xenobiotic metabolism may occur. This fact can, at least in part, explain some low level of EROD activity obtained in the liver of fish living in high polluted sites and therefore the results confirm that the specific stress indices must be utilized always in association with biomarkers able to define the health status of the animals (general stress indices).

However, it is possible to assume that from the results presented in the report the inducibility of MFO in *Scilla cabrilla* (collected in the Adriatic Sea) is lower than in the *red mullet* (collected along the Spanish coast). Additional research is therefore needed to ascertain the best species to be utilized by testing the effects of xenobiotic pollutants on wild fish population.

Stress on stress response utilized in 3 laboratories (Genoa, Nice, Monaco), gave successful results.

Finally, it must be always considered that fish population, including the so-called resident species, shows large territorial movements at least within the range of distances (usually few km) relative to the dimensions of a highly polluted site. Moreover, water current movement can highly change the pollution conditions and the possibility that fish from unpolluted waters can cross for brief periods of time contaminated areas, must also be taken into account.

Metallothionein, the second specific stress index was also utilized by all the laboratories involved in the programme. This biomarker is related to pollution due to heavy metals such as Cd, Hg, Cu, etc.. The results concerning the use of this biomarker, although successful, seem to indicate that heavy metal pollution is not, to day, the major pollution problem along the Mediterranean coast.

Finally, it must be stressed that biomarkers such as the evaluation of mRNA of metallothionein or P450 must be considered as a very important tool for environmental research and, even though it is not yet possible to suggest a wide use of these methodologies in the Mediterranean biomonitoring programme it is clear that these techniques will represent a future development and integration of the biomarker battery suitable to quantify the stress syndrome of organisms living in polluted environments.

Moreover, the laboratories that evaluate stress proteins clearly confirm that such a stress index needs more research and technical development before being utilized on marine organisms in field programmes. Other biomarkers (i.e. Phase II biotransformation enzymes, acetylcholinesterase activity, etc.) were found of great interest to complement the basal battery of stress indices proposed for the programme.

All together, the first year results of the Mediterranean monitoring programme on the biological effects of pollution, clearly indicate that more work must be done to ensure that all the laboratories utilize routinely the general stress indices in order to improve the quality of data and their interpretation in terms of global evaluation of the physiological status of marine organisms. Finally, it is important to point out that, with the exception of the DNA damage tests, all the other biomarkers utilized in the Mediterranean programme have also been selected by OSPAR for biomonitoring the effects of pollutants on marine fish.

8. GENERAL CONCLUSIONS AND RECOMMENDATIONS

A list of very important points must be taken into account in the future development of the programme.

- (i) For future activities, basic environmental parameters such as sea water temperature at sampling time must be recorded; a clear indication of the position of the sampling sites is also needed.
- (ii) The size, the number and the sex of animals utilized must be accurately described.
- (iii) A standard procedure for organ extraction and/or the transport of the animals from the field to the laboratory must be accurately described and then utilized in all the laboratories involved.
- (iv) Sample storage procedures in the different laboratories should be similar and always mentioned in the report.
- (v) Biomarkers for which an intercomparison is possible, should be preferred. Biomarkers which make use of living cells or animals should be added, as an integration to other tests or utilized where not all laboratory facilities are available.

- (vi) Intercomparison exercises are essential for a correct interpretation of the results on a large scale in the Mediterranean area. In the future, the intercomparison exercise must be performed during the period of sample analyses to ascertain the accuracy and comparability of the results.
- (vii) During the biomonitoring programme both mussel and fish must be obtained from the same site and on the same days. Collection of the animals from different sites and in different periods of the year doesn't allow a correct interpretation of the battery of stress indices employed.
- (viii) The caging system is without doubt expensive and it may not always be utilized without an adequate financial support. However, when possible, the utilization of this kind of approach should be adopted as good results were obtained in the RAMOGE pilot experiment and in other studies in different countries.
- (ix) An accurate choice of the animals to be utilized in the programme must be defined. In fact, the comparison and the interpretation of results will be simplified if the programme utilizes a minimal number of widespread different animal species.
- (x) It must be stressed that only few countries were able to reach the target in the first year of activity. Therefore, in the future, activities must be developed to ensure the involvement of more laboratories from other countries in this programme.

This could be achieved by organizing training courses devoted only to researchers from these countries. UNEP support to these laboratories should ensure the basic requirements for the researcher involved.

Moreover, it would be useful to provide a video support explaining in detail the most important biomarkers.

Medium (4 months) and long term (1-2 years) fellowships could be provided by developed countries to support this activity which will greatly contribute to the good implementation of the programme.

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