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INTERCOMPARISON EXERCISE CONCERNING LYSOSOMAL MEMBRANE STABILITY AND METALLOTHIONEIN ANALYSIS

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The intercomparison exercise concerning lysosomal membrane stability and metallothionein analysis was conducted by the Centro per lo Studio della Biologia e Chimica dei Metalli in Traccia, Università di Genova, Corso Europa (Palazzo delle Scienze), Genova 16132, Italy

1. INTRODUCTION

Biological monitoring consists of evaluating the physiological status of animals (bioindicators) living in the ecosystem under study, by estimating the values of selected biological parameters that are known to vary in response to the toxic effects of pollutants. These biological parameters are often referred to as stress indices or biomarkers.

The activities for launching a MED POL biomonitoring programme for studying the pollution effects in the Mediterranean sea were initiated in 1991 by FAO/UNEP in the framework of MED POL Phase II; two meetings were organised in 1991 and 1992 to decide the strategies for the development of the programme.

The conclusions of the meetings pointed out that the chemical monitoring of the marine environment developed in the last 20 years represents the more appropriate scientific background to start biological monitoring with the aim of quantifying the deleterious effects of pollutants on marine life in the Mediterranean sea.

Moreover, it has been established that in the evaluation of biological effects of environmental pollution it is necessary to utilize, for a correct understanding of the results, a battery of tests (biomarkers) some of which are able to reveal the physiological status of the animal (general stress indices) and others to reflect specific responses to a particular class of contaminants (specific stress indices).

For the first application of the biomonitoring programme of the Mediterranean sea a battery of four simple, sensitive and low cost tests was selected: lysosomal membrane stability, DNA damage, EROD activity, metallothionein, and a fifth biomarker, stress on stress, was recommended in the countries where mussels are available.

During the above meetings it was also decided that before initiating the programme in the field, a set of methodologies had to be developed for distribution to all potential participants; thus, the same standardized protocols concerning the selected techniques were distributed to the different laboratories participating in the programme. In addition, training was provided to certain scientists from different countries in order to improve the correct utilization of the selected biomarkers; the training was either through teaching/training courses (University of Nice - University of Sunderland - University of Genoa) or at an individual level at the Universities of Nice and Genoa (Prof. Lafaurie and Prof. Viarengo) which acted as reference centres for this biomonitoring activity. This training was highly useful to upgrade the technical capabilities of the different Mediterranean laboratories to enable their participation in the biomonitoring programme.

The selected biomarkers must have some specific characteristics to be utilized by all the laboratories: the biological analysis must be simple, repeatable, low in cost and possibly inter-comparable. In fact, after the distribution of the methodologies , three assays, fundamental for this programme, were inter-compared: lysosomal membrane stability, EROD activity and metallothionein content. Intercomparison is an essential element in the utilization of biomarkers in a large scale biomonitoring

programme along the Mediterranean coast where many laboratories from different countries participate, many of which are beginners in this field.

The results concerning the intercomparison of the lysosomal membrane stability and metallothionein determinations are presented in this report. The list of participating Institutions appears in the Annex. The results of the intercomparison on EROD activity which was undertaken by the University of Nice are presented in another report.

As it is known, lysosomal membrane stability represents a general stress index able to give general information on the physiological status of the animals. Lysosomal membranes are destabilized by most of the contaminants present in the marine environment (heavy metals, organic xenobiotic compounds such as aromatic hydrocarbons, pesticides, etc.).

Metallothionein is a specific stress index utilized for monitoring the effects of heavy metals. Metallothioneins are metal inducible proteins and therefore high levels of metallothioneins are present in the cells of metal exposed organisms that accumulate in their tissues high levels of contaminant metals.

The interest for the simple spectrophotometric technique proposed for the metallothionein assay and the importance of such a parameter in the evaluation of heavy metal biological impact, have led six laboratories to participate in the first inter-comparison for this biomarker. Four research centres were involved in the more elaborate lysosomal membrane stability analysis.

The aim of this report is to present the results of this inter-comparison exercise, an approach that has been utilized for years to standardize the results of chemical environmental analysis but applied for the first time in the standardization of biological data in the Mediterranean sea biomonitoring programme.

2. MATERIALS AND METHODS

Chemicals

Leupeptin, PMSF (phenylmethylsulfonyl fluoride), EDTA (Ethylenediamine-tetraacetic acid), GSH (Glutathione reduced form), DTNB (5,5-dithiobis-2-nitrobenzoic acid), Naphthol As-BI N-acetyl-D-glucosaminide, polipep and fast violet b salt were purchased from Sigma Chemical Co. (Saint Louis U.S.A.); β-mercaptoethanol was obtained from Merck (Germany). All other reagents were of analytical grade.

Animals and incubations

Specimens of mussels (*Mytilus galloprovincialis*), 4-6 cm long, were obtained from a local farm (Palmaria - La Spezia) and rapidly transferred to aquarium with aerated re-circulating artificial sea water (1 litre/animal) at 15°C. After one day of stabulation under these conditions, treatment with heavy metal were started. In order

to destabilize lysosomal membranes, mussels were treated with $CuCl_2$ (40 µg/animal) for 3 days. In a different experiment, in order to stimulate metallothionein neosynthesis, mussels were treated with CdCl2 (200 µg/animal) for 7 days. In each experiment, control animals were maintained in pure artificial sea water for a period corresponding to the duration of the treatment. During all experiments, water and metal were changed daily.

Sample distribution

Animal treatment and tissue preparation were carried out in the laboratory of the Interuniversitary Research Centre for the study of Chemistry and Biology of trace metals (University of Genoa) and then blind samples, including treated and controls, were sent on dry ice by air mail to all the other laboratories involved in the intercomparison exercise.

Metallothionein assay

After mussel incubation, digestive glands were rapidly removed and homogenized in three volumes of sucrose 0.5 M - Tris 20 mM pH 8.6, containing 0.006 mM leupeptine, 0.5 M PMSF, 0.01% β-mercaptoethanol. Aliquots of the homogenate (3 ml) were stored at -80°C and thereafter distributed to all the laboratories involved.

Besides samples, each participant received GSH powder for standard curve evaluation.

All metallothionein evaluations were performed according to the following procedure.

The homogenate was centrifuged at 30000 x g for 20 min., and the resulting supernatant was mixed with 49% cold ethanol and 3.5% chloroform, vortexed and centrifuged at 6000 x g for 10 min. Aliquots of the 6000 x g supernatant were added to 3 volumes of cold ethanol, stored at -20°C for 1 hr and centrifuged at 6000 x g for 10 min. The pellet was washed with ethanol/chloroform/homogenization buffer (87:1:12), centrifuged, dried under gaseous nitrogen and resuspended in 300 µl of 5 mM tris - 1 mM EDTA, pH 7.

The resuspended metallothionein fraction was mixed with 4.2 ml 43 mM DTNB in 0.2 M phosphate buffer, pH 8 and after 30 min. the concentration of reduced sulphydryls was evaluated by reading the absorbance at 412 nm, utilizing GSH as reference standard (GSH 1 mg/ml in 5 mM tris - 1 mM EDTA, pH 7).

Lysosomal membrane stability assay

Small pieces (3-4mm^3) of digestive gland, each deriving from a different mussel, were rapidly dissected in order to obtain transverse sections of tubules and placed on an aluminum cryostat chuck. The chuck was placed for 40 sec. in a small plastic box containing N-hexane which had been precooled to -70°C with liquid nitrogen. Chucks were then sealed with aluminium foil and parafilm, immediately stored to -80°C

and distributed to participants.

10 µm sections were cut using a cryostat with a knife angle of 15 degrees. Sections were then flash-dried by transferring them to "warm" slides.

Slides were then placed in a Hellendal jar containing the lysosomal membrane labilising buffer (0.1 M Na-citrate Buffer - 2.5% NaCl, pH 4.5) for different times (0,3,5,10,15,20,30,40 min.) at 37°C in order to find out the range of pretreatment time needed to completely labilise the lysosomal membrane.

The slides were then incubated for 20 min. at 37°C in the substrate incubation medium containing 20 mg of naphthol As-BI N-acetyl-β-D-glucosaminidase previously dissolved in 2.5 ml of 2-methoxyethanol which was made up to 50 ml with 0.1 M Na-citrate Buffer - 2.5% NaCl, pH 4.5, containing 3.5 g of polipep. Subsequently the slides were washed in filtered sea water at room temperature and transferred to 0.1 M phosphate buffer, pH 7.4, containing 1 mg/ml of diazonium dye Fast Violet B salt for 10 min. Afterwards the sections was rinsed in running tap water for 5 min. and fixed with KAISER's glycerol gelatin.

Maximal staining intensity peak can be evaluated viewing the slides under microscopy or with an image analyzer system.

3. RESULTS AND DISCUSSION

The results presented in Table 1 show the data obtained by the 6 laboratories involved in the intercomparison of the analysis for the evaluation of the metallothionein content utilizing blind samples obtained from the digestive gland of control and Cd exposed mussels.

The results indicate that all the laboratories involved in the intercomparison exercise were able to clearly identify all the 4 digestive gland control samples as well as the 4 samples of Cd treated animals.

The means of the control data of the different laboratories vary from 139.8 ± 54.5 µg/g tissue w.w. to 427.9 ± 26.3 µg/g tissue w.w.; for the Cd treated samples the mean values range from 332.2 ± 122.1 µg/g tissue w.w. to 958.3 ± 130.4 µg/g tissue w.w.. Therefore, with the exception of laboratory 7, there are no overlapping data of the metallothionein values for control and cd treated samples (Fig. 1). The low standard deviation of the metallothionein content values clearly indicate that the spectrophotometric method employed for metallothionein evaluation is simple and easy to standardise for routing analysis in biomonitoring programmes.

As shown in Figure 2 the per cent increase of metallothionein content in Cd treated animals with respect to control is very similar in all the different laboratories. This is due to the fact that the variations among control values always correspond to similar variations of metallothionein values of Cd treated animals.

Table 1

Metallothionein concentration in the digestive gland of control and Cd exposed mussels. Metallothionein values are expressed as µg/g w.w. and represent the mean of at least 4 different samples

Laboratory no.	Control Mean ± S.D.	Cd exposed Mean ± S.D.
1	297.6 ± 9.5	658.4 ± 19.6
2	219.8 ± 15.4	575.8 ± 19.4
3	404.5 ± 25.9	932.0 ± 99.5
4	344.5 ± 54.8	930.2 ± 16.5
5	281.9 ± 41.5	754.5 ± 74.4
6	242.7 ± 34.2	506.8 ± 29.8
7	139.8 ± 54.5	332.2 ± 122.1
8	427.9 ± 26.3	958.3 ± 130.4

The values concerning the absorbance of metallothionein preparation reacted with DTNB are able *per se* to describe the control and Cd treated groups: the utilization of a GSH standard curve to quantify the results in terms of µg metallothionein/g tissue does not provide an improvement of the results (the data show similar standard deviation). However, the utilization of standard solution of GSH, the absorbance of which in the Elmann analysis for sulphydryl groups quantification is well known, will represent a self control test for the laboratories ensuring that the procedures and the analytical reagents employed during the analysis are working correctly (data not shown).

A slightly different approach in the intercomparison of the methodology utilized for the analysis was employed in the case of histopathological and cytochemical biomarkers such as lysosomal membrane stability. In this case the samples sent to the research centres were all preliminarily analyzed in Genoa to ensure that every laboratory involved in the intercomparison has received a sample (five replicates) of "controls" (digestive glands in which lysosomal membrane stability is over 30 min.) and a sample (five replicates) of "stressed" animals (digestive glands in which lysosomal membrane stability is less than 10 min.).

The data of the lysosomal membrane stability in the mussel digestive gland obtained by cytochemical analysis are shown in Table 2 and in Figure 3. Only 4 laboratories were involved in this kind of activity, indicating that the training for the utilization of this stress index was insufficient or that the equipment (although simple: a cryostat, a microscope and a shaking bath) were not always available in the different

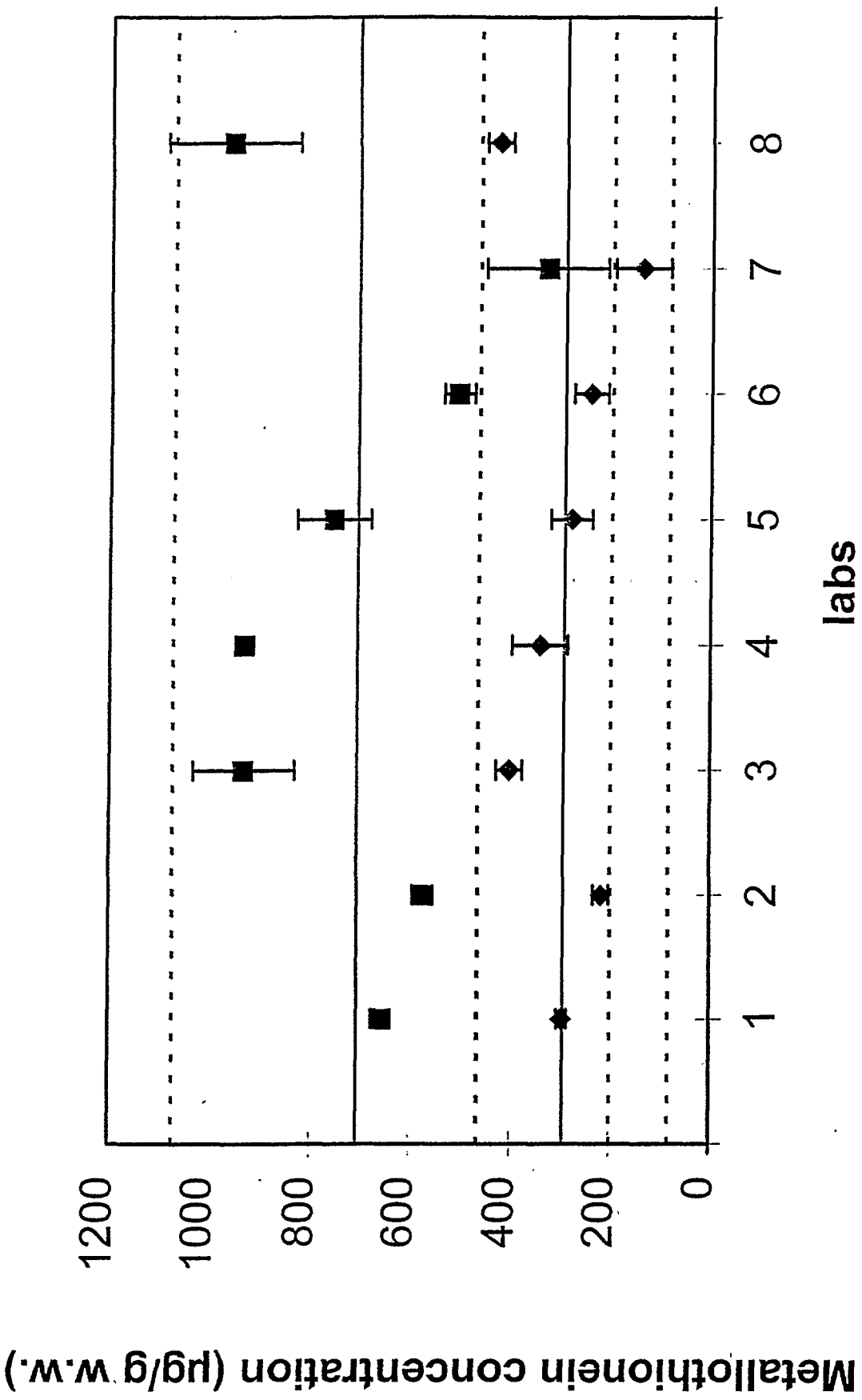

Fig. 1 Graphic representation of the values of the metallothionein content in the digestive gland of control (◆) and Cd exposed (■) mussels. The values reported ± standard deviation represent the mean of at least 4 different samples

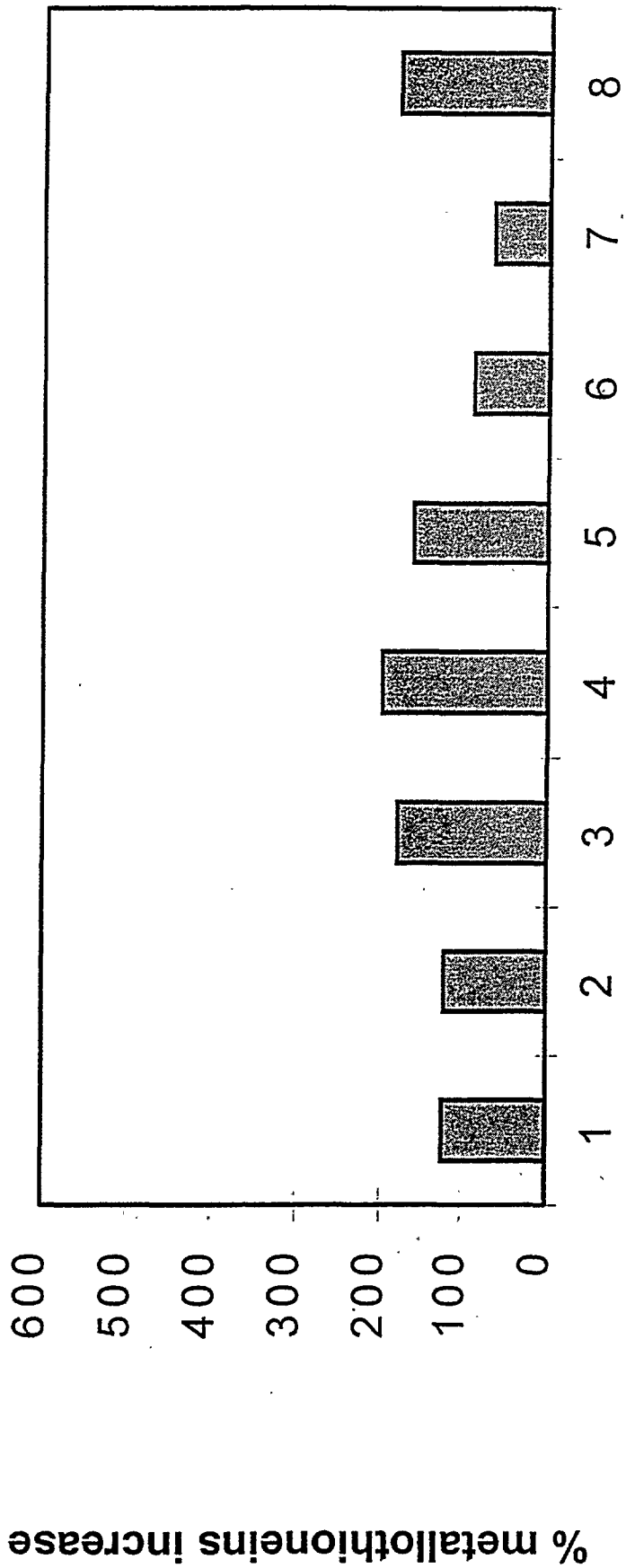

Fig. 2 Per cent increase of the metallothionein content in samples from control and Cd exposed animals analyzed in each different laboratory involved in the intercomparison exercise

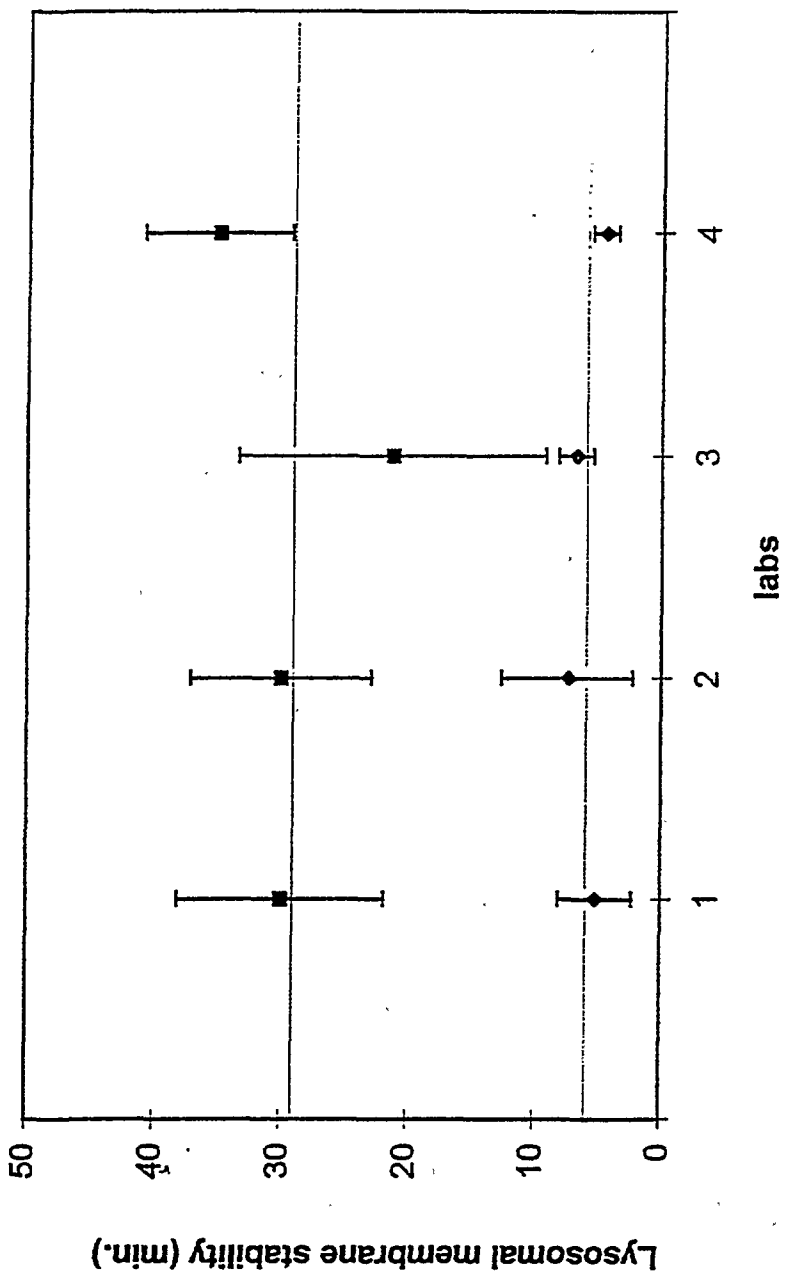

Fig. 3 Graphic representation of the values of the lysosomal membrane stability in the digestive gland of control (◆) and Cu exposed (■) mussels. The values reported represent the mean of at least 4 different samples

Mediterranean laboratories. However, it must be stressed that, this is a powerful general stress index and its applications should be extended to a greater number of laboratories in the next future.

The results obtained in all the laboratories involved in the cytochemical intercomparison demonstrate that, also in this case, each research group was able to find out control and stressed animals (exposed to 0.6 µM Cu for three days); the controls were correctly estimated showing a latence time of about 30 min. (with the exception of a lab that find a low value of 21 min.). All the participating laboratories estimated that Cu exposed mussels had a lysosomal membrane stability in the range of 4.5-7.5 min. It is interesting that the variation of lysosomal membrane stability values between control and treated animals, looking at the pair of data collected in the different labs (also in this case) was quite similar (Fig. 4).

Table 2

Lysosomal membrane stability data in the digestive gland of control and Cu exposed mussels. Lysosomal membrane stability is expressed as the time (min) occured to completely destabilize lysosomal membranes. The values represent the mean of at least 5 different samples ± S.D.

Laboratory no.	Control Mean ± S.D.	Cu exposed Mean ± S.D.
1	5.2 ± 2.9	30 ± 8.1
2	7.4 ± 5.2	30 ± 7.1
3	6.75 ± 1.4	21.25 ± 12.1
4	4.5 ± 1.0	35 ± 5.8

The results presented, clearly demonstrated that the intercomparison exercise is a suitable form of control of the quality of the data collected in the different laboratories involved in large biomonitoring programmes. This is the case of the biomonitoring of the Mediterranean sea where a routine activity of intercomparison of the selected methodologies will permit a survey that will improve the standard level of the activity of the labs of the different countries.

Finally, it is important to point out that the positive results obtained in this intercalibration exercise do not mean that all biological tests for which a correct intercalibration is not possible must be eliminated from the programme or underconsidered. In some cases, research is needed to prepare an intercalibration exercise for example for the analysis of DNA damage. This is the only biomarker selected for the Mediterranean sea biomonitoring programme that until now it is impossible to correctly intercalibrate due to the impossibility to freeze the tissue samples without increasing the level of DNA damage.

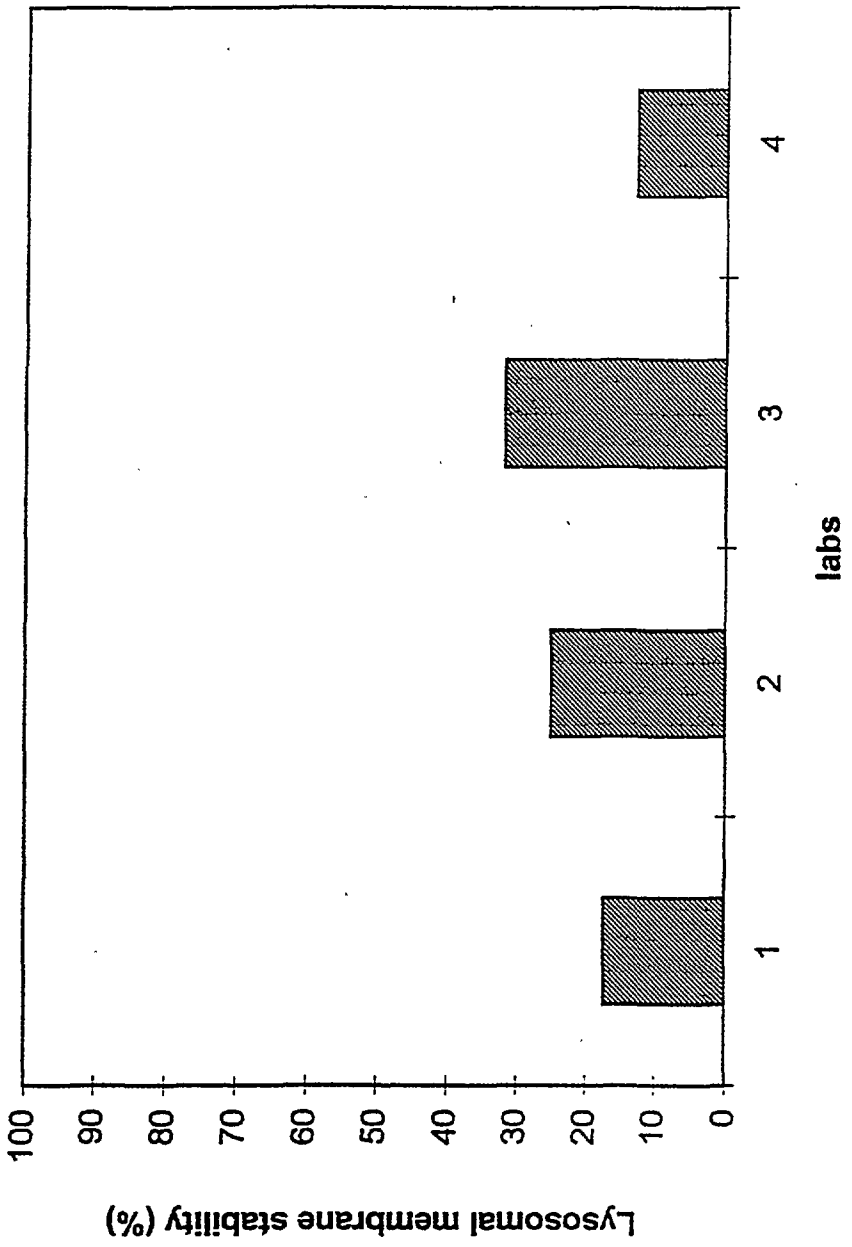

Fig. 4 Per cent variation of the lysosomal membrane stability in samples from control to Cu exposed animals analyzed in each different laboratory involved in the intercomparison exercise

However, in this context, it must be stressed that there are now simple biomarkers that in the future should be easily added to the battery of selected tests, although for some of them which utilize living cells or organisms any intercalibration is until now not possible.

ANNEX

List of participating institutions

Metallothioneins

Center for Marine Research-Rovinj Division, "Rudjer Bošković" Institute, Giordano Paliaga 5, P.O. Box 150, 52210 Rovinj, Croatia

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Lysosomal Membrane Stability

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