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MANUAL ON THE RECOMMENDED BIOLOGICAL EFFECTS TECHNIQUES

(DRAFT)

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PREFACE

The scope of this manual is to serve as an initial source of technical reference for laboratories interested to start routine biological-effects pollution monitoring. A number of biomarkers have been considered in this manual, starting from those capable of giving a general indication of biological stress due to pollution. These *general stress indices* include the assessment of damage to genetic and subcellular components. Both the elevation of enzymatic activity of the mixed function oxygenase system and the induction of metallothionein proteins synthesis also termed as *specific stress indices*, are considered.

The potential use of the last two biomarkers is considerable, since the specific groups of contaminants are considered as being major pollutants in coastal areas. They seem to offer the best information on adverse changes to normal genetic, biochemical and cellular systems in marine organisms as *early warning signals* that environmental damage is in progress. They have been carefully characterized in a number of marine organisms and proved to be stable in reference areas unless during certain physiological stresses for which intervals have to be fully identified depending on the type of indicator species to be used. However, due to the complex nature of these biological responses, extreme caution should be exercised when coming to interpret monitoring results from field stations. These responses have to be assessed in view of the physiological state of the test organisms at time of sampling which can be ascertained by the measurement of the two general stress indices mentioned above. This manual also attempts to address a number of technical pitfalls and whenever possible, suggests ways how to enhance the certainty of biomonitoring results.

Important note on health safety

Safety deserves special attention. Most of the chemicals and equipment listed in the following sections are relatively harmless, provided they are not abused. Disposable items should be used wherever possible, as should safety items such as gloves, lab coats and special waste disposal containers for carcinogenic substances.

Acknowledgements

The first draft of this manual was prepared by Mr. Charles Galdies, Euro-Mediterranean Centre on Insular Coastal Dynamics (ICoD), Foundation for International Studies, University of Malta. A number of Mediterranean scientists, including Professor A. Viarengo of the University of Genova, provided comments all of which were incorporated into the present draft by Mr. Galdies. UNEP is grateful to everybody for their cooperation.

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1. INTRODUCTION

Upon exposure to harmful contaminants, marine organisms start manifesting a number of symptoms that are indicative of biological damage, the first ones appearing after a short while at the subcellular level. These *'sublethal'* effects, when integrated, often converge to visible harm to the whole population at a later stage, when it is too late to limit the extent of biological damage resulting from environmental deterioration.

Most of these symptoms have been reproducibly obtained in the laboratory and the various biological mechanisms of response to major xenobiotics are now fairly well understood. The use of *biomarkers*[1] has, since then, came into common practice by ecotoxicologists who adapted these responses into 'early warning' pollution monitoring tools to signal the onset of harmful effects at the cellular and sub-cellular levels. The following discussion briefly describes a number of common sublethal effects, exhibited by marine organisms as well as their correct application as biomarkers of biological harm resulting from marine pollution.

1.1 Mutagenic processes

Abnormal DNA replication, transcription as well translational processes of RNA (Eichorn, 1973) often represent the first kind of symptoms following the interaction between xenobiotics and the nuclear material. *In vitro* studies show that heavy metals, for example, can easily alter the complementing hydrogen bonding capability of nucleic acids and thus facilitate the onset of the above processes.

Metabolic by-products of bioaccumulated xenobiotics can also generate harmful effects on the integrity of genetic material. Within the eukaryotic cell, organic xenobiotics are biotransformed into several mutagenic and carcinogenic metabolites by cytochrome P-450 enzymes and the epoxide hydrase function (see 1.3 below). These metabolites are highly reactive by-products with an affinity for nucleophilic sites on cellular macromolecules, like DNA. The interaction of these reactive substances with DNA can lead to the formation of DNA lesions. Unless inhibited by certain types of contaminants, such as heavy metals, specialised nuclear enzymes are able to correct these lesions thus minimising misexpression of the genome. Inefficient repair of the genome also characterises one of the first steps which lead to chemical carcinogenesis and malignancy. This could have a serious implication if such processes are detected within the reproductive tissues of marine organisms, for this would negatively affect its reproductive success.

So far, the study of genotoxicity has mainly been confined to human and other mammalian systems. Direct correlation of those genotoxic effects onto other, different organisms, is not advisable since these may be influenced by quite different mechanisms, such as differential accumulation capabilities, differences in metabolic pathways or complements of enzymes which are characteristic of different phyla. Such

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[1] Two other categories fall under biological effects monitoring: (1) bioassay testing and (2) ecosystem responses

differences may not only be significant at the organismal level, but may also alter the way in which genotoxic damage is manifested at higher levels of integration (e.g. at the ecosystem level).

It was only recently that the successful use of standard methods to determine genotoxic damage has rendered it possible to substantiate mutagenic effects in marine invertebrates (Shugart, 1988) and reveal a direct correlation between chromosomal abnormalities, nuclear enzyme inhibition and other related events with the bioaccumulation of heavy metals (Dixon, 1983) and polyaromatic hydrocarbons (PAHs) (Bolognesi *et al.*, 1991).

The benefits of using these responses to monitor genotoxic risks is self-evident, and are creating the need for both the systematic screening of major marine pollutants for their DNA-altering potentials as well as unmasking suspected pollution sources. In fact they have been instrumental in detecting such pollution hazards along specified coastal stretches in the Mediterranean (Scarapto *et al.*, 1990; Bolognesi *et al.*, 1991).

1.2 Subcellular injuries

At a slightly higher level of biological organisation, eukaryotic cells respond to common pollutants by exhibiting a series of irreversible cellular changes leading to cellular death. Such changes are normally translated into general cellular disorganisation of plasma membranes and intracellular compartments.

One subcellular component which has proved to be very sensitive to the presence of extracellular contaminants is the lysosome. The importance of this organelle lies in its normal degradative role of cellular membrane constituents and extracellular macromolecules by means of hydrolytic enzymes segregated within (such as nucleases, phosphatases and peroxidases). Damage to lysosomes is translated into loss of integrity of their membranes, resulting in "leaky" vesicles thereby releasing their hydrolytic enzymes into the cytosol. Many environmental contaminants, including aromatic hydrocarbons, carbon tetrachloride, asbestos, silica, aminoazobenzene derivatives, beryllium, metal powders, viruses and metal ions are known to be sequestered in lysosomes under certain conditions (Moore, 1985). Overloading of the lysosomes leads to membrane destabilisation, with subsequent cellular necrosis due to the release of degradative lysosomal enzymes. Undoubtedly, a negative consequence of cellular atrophy would be a perturbed intracellular digestion, rapidly affecting the nutritional status of the organism.

The evaluation of lysosomal injury has now become widely accepted as a sensitive index of cellular health, where the destabilisation of the lysosomal membrane bears a to the quantitative relationship magnitude of the stress response. *In vitro* (Moore, 1990) and *in vivo* (Lowe *et al.*, in press) investigations on lysosomal damage have been successfully carried out along pollution gradients using both teleosts and invertebrates, and have been correlated with total tissue burdens and benthic sediments for a range of contaminants.

1.3 Functional alteration of normal biochemical pathways

1.3.1 Induction of cytochrome P450-dependent mono-oxygenase system

This multi-enzyme complex consists of a group of hemoproteins associated with the smooth endoplasmic reticulum. Among the wide range of different types of reactions, they are able to catalyse metabolically-important reactions such as oxidative hydroxylation of aromatic and aliphatic hydrocarbons, O-, N- and S-dealkylation reactions, N-oxidation, sulphoxidation and deamination reaction. In a nutshell, the role of cytochrome P450 is to convert relatively insoluble, redundant endogenous organic substances into water-soluble compounds (biotransformation) to facilitate their elimination from the body.

Of particular interest to pollution monitoring is that in vertebrates the P450 gene expression (and consequently their activity) can be markedly induced by a number of exogenous chemical classes which happen to be common environmental pollutants, including PAHs, polychlorinated dibenzo-p-dioxines and dibenzofurans, polyhalogenated biphenyls and other halogenated organic compounds such as pesticides and herbicides. The induced synthesis of the P450 gene family has therefore attracted the interest of ecotoxicologists for its application as a field sublethal bioassay that is diagnostic for PAH exposure. Their interest also focused on the biotransformation of these xenobiotics which, unlike normal endogenous compounds, tends to be deleterious since their transformation lead to a more chemically reactive species (Heidelberger, 1973), such as epoxides. Studies show that these intermediate metabolites are able to bind to DNA and promote mutagenesis.

Cytochrome P450 activity in marine vertebrates, and to some extent also in invertebrates, has proven to be one of the most sensitive indicators of environmental contamination. This assessment has been based on a sound knowledge of the properties and regulation of this enzyme complex (Stegeman, 1989). Toxicity studies demonstrate that this biomarker is a more sensitive indicator of pollution stress than other physiological variables such as osmoregulation and energy metabolism (Nikunen, 1985). This system responds relatively rapidly to a variety of organic environmental pollutants as well as to complex mixtures including municipal and industrial effluents. Induction can be detected fairly rapidly and elevated activities of P450 can persist for several weeks after a contaminant exposure has ceased (Kloepper-Sams and Stegeman, 1989).

In field situations P450 activities in fish (EROD) were shown to give sensitive responses to a PAH pollution gradient over a broad area in the north-western Mediterranean region, validated by a concomitant PAH concentration in the sediments (Garrigues *et al.*, 1990). Preliminary investigations in the Mediterranean to measure benzo(a)pyrene-mono-oxygenase (BaPMO) activity in *Mytilus galloprovincialis* seems to indicate the possibility of using these molluscs for the detection of PAH pollution gradients (Selli *et al.*, 1994). However, further research is required to fully understand this response in molluscs.

1.3.2 Increased cytosolic levels of metal-binding proteins

Another biological response occurring at the subcellular level is the induced rate of synthesis of a class of metal-binding proteins, known as metallothioneins, following exposure to heavy metals. This is considered to be a sublethal detoxification response, but the induced synthesis of these proteins can result in biological costs which reduce the fitness of the individual. Energy which may be otherwise diverted for growth, reproduction and maintenance for example, may be used to further drive detoxifying and excretory mechanisms.

Extensive studies using many different organisms (Hamer, 1986) indicate that these proteins have three major physiological roles: (1) *Detoxification of elevated internal metals* (Goering and Klaassen, 1984), which is strongly supported by the presence of a eukaryotic gene sequence coding for Cu-MT of which expression is induced under conditions of high metal exposure (Hamer *et al.*, 1985); (2) *Internal homeostasis of Cu and Zn* by (a) keeping high toxic intracellular concentrations of free Zn and Cu at a low level; (b) binding excess metals in a non-toxic form as well as (c) acting as a storage of Cu and Zn for later use and reactivation of *apo*proteins that require these metals for their activity (Brouwer *et al.*, 1986) and (3) *Participation in nutritional and metabolic functions,* including scavenging of free radicals (Thornalley and Vasak, 1985) and protection against damage due to ionising radiation (Karin, 1985).

The use of this biomarker for biomonitoring of environmental metal impact has now been quite well validated and a number of biomonitoring exercises using this index have been conducted in various areas within the Mediterranean (Hamer *et al.*, 1985; Pavicic *et al.*, 1991; Galdies, 1995).

1.4 Utilisation of sub-lethal responses as diagnostic tools

1.4.1 Monitoring criteria and sample acquisition

Sampling of biological material to monitor sublethal responses has its special requirements. Allowance should be given to a number of biotic and abiotic factors which can potentially influence, and thereby disturb, the analysis. The investigator has to make sure that changes in the magnitude of the responses are only due to temporal pollution fluxes rather than to any other source of variation; to do this, the stress indices have to be measured in the same test species at the same time and place from year to year (UNEP/FAO/IAEA, 1993). Inconsistent sampling tends to add noise to the data and makes it harder to identify meaningful trends. Temporal monitoring should also take into account fluctuations in the population density in a way not to interfere with statistical sampling. Allowance should also be given to geographical variation which might affect the physiology of certain bioindicator species, particularly marine gastropods (Cossa *et al.*, 1979).

Once collected, samples are to be handled in the same way as in previous sampling occasions and are to be adequately stored to prevent degradation of their biochemical entities or activities. Studies show that most biochemical parameters can only survive short periods of time at ambient temperatures and for this reason, either dry

ice or liquid nitrogen is required for temporal storage of tissues prior to storage in a deep freeze at -80°C.

1.4.2 Choice of species

The choice of the test organism must be guided by several criteria including its abundance and geographical range in the Mediterranean, longevity (to allow sampling of more than one-year class if desired), ease of sampling all year round, whether it is amenable to laboratory investigations, etc. Having done this, one soon realises that there is available a very limited choice of marine vertebrates and invertebrates[2].

Teleosts such as combers (*Serranus* species) tend to be highly sensitive to PAH pollution (Narbonne *et al.*, 1991) and are therefore chosen as test organisms to monitor the activity of their cytochrome P-450. The selection of this hermaphroditic species provides the investigator with some advantages, both in terms of eliminating sex-linked variations in the magnitude of the response as well as due to its sedentary mode of life. Seabass (*Dicentrarchus labrax*) also responds well towards PAH contamination in terms of P450 activity. Sexually immature representatives (weighing up to 75±17g) are preferred to eliminate sex-type differences in enzyme activity levels.

Striped mullet (*Mullus barbatus*) has also been successfully used and proved to be an excellent bioindicator to monitor P-450 activities (Mathieu *et al.*, 1991). It has been categorised as belonging to the high hepatic xenobiotic-metabolising activity fish group, showing a higher enzymatic activity than do combers, seabass and other fish. Mullets exhibit an increased enzyme activity from October to February which decreases just before spawning. Sex-type differences in P450 activity is also exhibited by this species, with males showing a higher P-450 activity than females during the reproductive period.

Sessile marine invertebrates for biological monitoring are generally preferred because they exhibit the necessary criteria that qualifies them as good bioindicators (Viarengo and Canesi, 1991). They can bioaccumulate a number of pollutants and exhibit the existence of a number of *specific* biomarkers, including PAH-metabolising enzymes (Livingstone and Farrar, 1972) without exhibiting any significant seasonal changes in their enzymatic activity (found to be responsive to PAH contamination of more than 1000 µg/g sediment and 4ppb in water, (Ribera *et al.*, 1989)) and metallothioneins (Roesijadi *et al.*, 1988). However, mussels are not widely distributed in the Mediterranean and therefore other species have to be selected where absent or when spatial biomonitoring data is to be compared. Other drawbacks for those wanting to use these molluscs include their low level of both their MFO activity as well as its responsiveness to organic xenobiotics. As a comparison, the level of benzo(a)pyrene hydroxylase activity is increased by only about 50% in mussels from polluted areas (Suteau *et al.*, 1988) but by at least three times in fish collected from the same area

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[2] Apart from animals, investigations are being carried out to use similar responses in marine plants, such as *Posidonia oceanica*

(Addison and Edwards, 1988). With regard to MT measurements, mussel tissues (such as gills) prove to be a good indicator of Cd, Cu and Hg, but not of Zn pollution (George and Olsson, 1994).

One other thing regarding invertebrates is that although many aspects of metabolising systems are highly similar, there nevertheless exist significant differences between various invertebrates even within the same phyla (Goksoyr *et al.*, ...). For example, the ability to synthesise MT proteins in response to Cd is clearly not uniform among molluscs and is due to interspecific differences in bioaccumulation (Langston *et al.*, 1989). Oysters, for example, tend to naturally accumulate very high concentrations of Cu and Zn in their tissues and in doing so, they can mediate a different biomarker response from other species with a much lower affinity for these metals. Other molluscs, such as whelks and scallops, are able to synthesise a different kind of metal-binding proteins as a response to intracellular metal contaminants, thereby competing with that mediated directly by MT protein. Consequently, these molluscs are usually ruled out from biomonitoring activities. Under such circumstances, it might be more opportune to shift your choice to a single teleost species which could cater for all biomarkers without posing of such problems.

General stress indices (such as genetic and subcellular injuries) seem to be ubiquitous in all eukaryotic cells, thus precluding problems when coming to choose the test species. Obviously, care should always be taken to try to establish a good signal-to-noise ratio as much as possible, but when compared to the specific stress indices, they tend not to be problematic.

1.4.3 Interpretation of results: Some practical considerations

The prerequisites for using these indices do not only include their specificity to certain contaminants, but also knowledge of their normal physiological functioning. Consideration has to be given to any background variability in order to obtain a good signal-to-noise ratio; after all, these are normal homeostatic mechanisms which are responsive towards changes in both their physiological status as well as to their immediate surroundings.

For example, it has been observed that the integrity of the lysosomal membrane can be destabilised by non-chemical stressors such as hypoxia, hyperthermia, osmotic shocks, changes in light, pressure and other stress factors like handling, noise, ionising radiation, etc. (Moore, 1985). These factors were found to modulate the response of the P-450 enzymes to environmental pollutants. Changes in salinity, temperature, and oxygen availability are among the most significant factors influencing mono-oxygenase activity (Goksoyr and Forlin, 1992) and metallothionein levels (Engel, 1988) in fish.

A good example of a major physiological factor influencing the activity of some of these biomarkers in vertebrates, particularly mono-oxygenase activity, is a change in the levels of reproductive hormones. In many fish species, mono-oxygenase activity usually decreases shortly before or during spawning season. Differences in liver mono-oxygenase activities between fish sexes has also been recorded (Stegeman *et*

al., 1986). Reproductive stress may also pose urgent requirements of some essential metabolic precursors, such as essential metals like Cu and Zn which in turn may stimulate the appropriate storage, transport, and release of these metals to metabolically-important sites. This process exactly fits one of the functions of metallothionein proteins (see section 1.3.2), and justifies its transient elevated synthesis during such kind of physiological states.

One may therefore conclude that confident application of these biochemical systems in environmental monitoring very much depends upon the understanding of the regulatory processes involved and characterisation of the magnitude and timing of these changes.

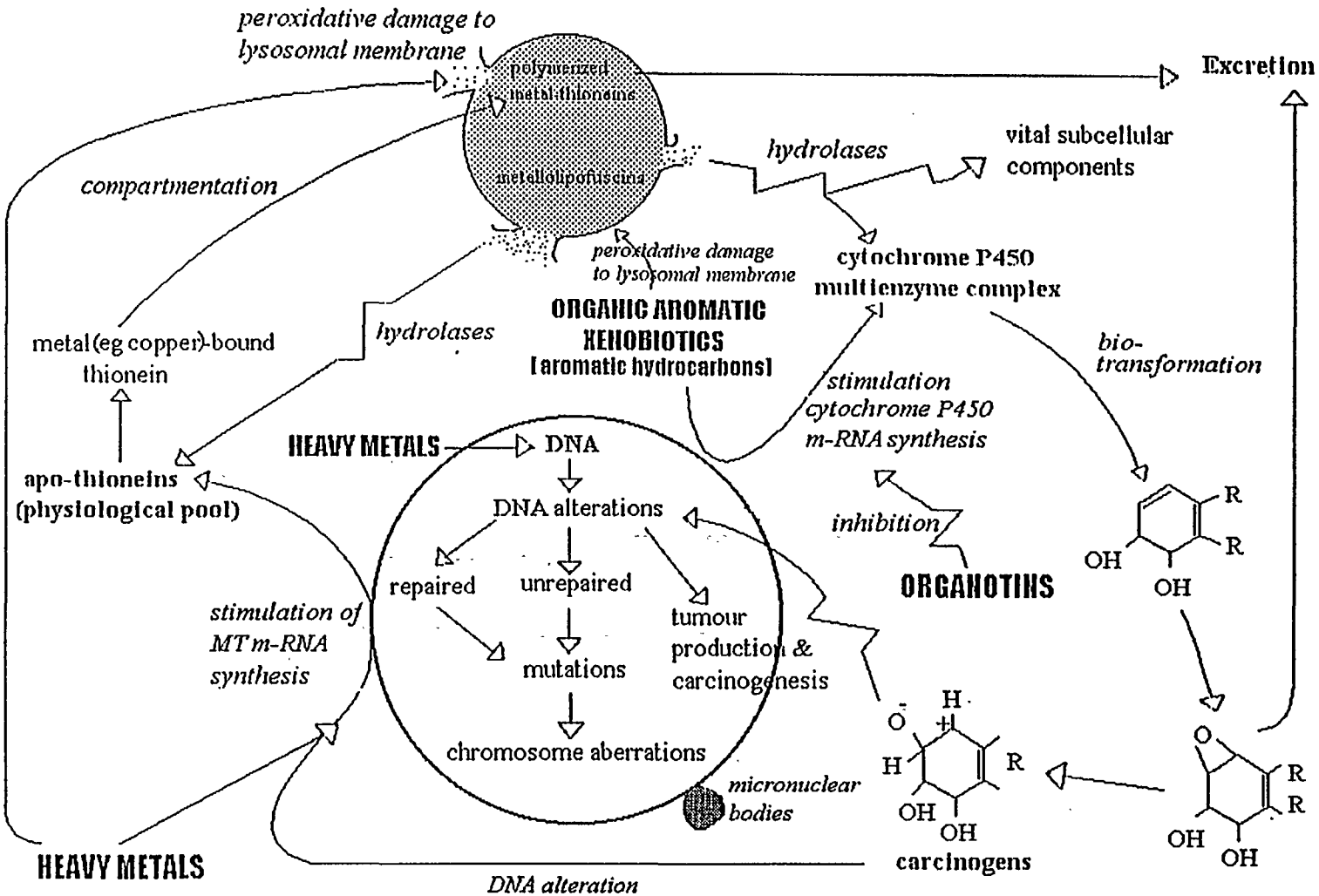
1.4.4 Effects due to complex mixtures of pollutants

Apart from the above a/biotic factors, these biochemical indices can also be variably affected by the many thousands of individual chemicals present in real contaminated situations where the result can be a synergistic and/or antagonistic effects on the activities of these biomarkers depending on the type of contaminant. It is very difficult to reproduce realistically these complex mixtures in the laboratory; but readers can at least glimpse at the interactive effects between the biomarkers and major type of contaminants by referring to the diagram below.

Studies suggest that the presence of organic xenobiotics in the sampling environment can reduce the synthesis of metallothionein proteins and their compartmentation within the lysosomes (Viarengo *et al.*, 1988). We have already seen that these organic xenobiotics tend to bioaccumulate in lysosomes and destabilise the integrity of the lysosomal membrane thus promoting catabolism of cellular proteins, including the integrity of the biomarkers, by liberating hydrolytic enzymes (Viarengo *et al.*, 1987).

Moreover, concentrations down to nanomolar levels of Cu^{2+}, Hg^{2+} and CH_3Hg^+ in the reaction mixture are able to significantly inhibit P450 activity as detected by a catalytic assay whereas at micromolar concentrations, the enzyme is totally inhibited (Viarengo *et al.*, 1994a). This has important implications in the utilisation of MFO activity as a tool for the evaluation of marine pollution due to organic xenobiotics and can explain the low correlation sometimes observed between P450 activity levels and the amount of organic xenobiotic compounds accumulated in fish liver cells.

Another significant interference is caused by the presence of bioaccumulated tributyltin (TBT) compounds. The leaching of this biocide from antifouling paints is considered to be a major pollution problem in enclosed areas where there are significant yachting and shipping activities. Studies show that mono-oxygenase activities can be reduced in the presence of even minimal levels of TBT, indicating considerable effects of TBT on this enzyme complex (Cassar and Axiak, 1994). This strongly suggests that exposure of fish to this widespread marine contaminant may alter the induction response to other environmental contaminants, particularly PAHs, thus conflicting with the use of this biomarker in pollution monitoring.



Synergistic effects of a mixture of pollutants on various metabolic pathways. Sequence of events is initiated by contact of cells to exposure of different types of pollutants (see text for discussion).

1.5 Conclusion

Some may say that the implication of these interactive effects vis-à-vis the interpretation of those stress indices under field situations which are not specific to any particular class of contaminants, such as the changes in the lysosomal hydrolase latency and mutagenic events, is not critical. Rightly so. However, specific stress indices, like the MFO system and MT which are susceptible to interactive effects, are to be interpreted with caution when applied to field situations having mixtures of both organic and inorganic xenobiotics.

It is hoped that this brief introduction highlighted both the intricacies of utilising these sublethal responses as biomarkers of biological harm, as well as pitfalls often encountered if results are not interpreted correctly. Analysis of isolated homeostatic mechanisms is never encouraged, and a general picture should always be considered and translated into the overall state of health of the organism, and eventually of the population.

2. EVALUATION OF LYSOSOMAL MEMBRANE STABILITY

2.1 Background

It is very difficult to evaluate structural changes caused by an altered permeability of the lysosomal membrane. These analyses require extensive purified lysosomal membrane preparations and their examination at molecular level. An easier way to assess this parameter is to examine whether its normal physiological function has been altered or disrupted following exposure to pollutants.

One tool which links both descriptive morphology and biochemistry to observe such pathological alterations is cytochemistry. Apart from permitting the use of very small samples of tissue, this technique is ideal to detect changes in particular target cells and tissues.

Cytochemistry has been successfully applied to assess lysosomal integrity by visualising the hydrolytic enzymes within the lysosome, and has proved to be a rapid and sensitive investigative tool both for organic xenobiotics and other injurious agents at very low intracellular concentrations. This generalised response occurs in all cell types ranging from fungi to vertebrates, so that such cytochemical testing can be applied on a fairly widespread basis.

2.2 *In vitro* demonstration of latent activity of lysosomal hydrolases for assessment of lysosomal stability

2.2.1 Principle

The following protocol is a cytochemical procedure for the determination of lysosomal membrane stability, based on the evaluation of the activity of N-acetyl-β-hexosaminidase, a lysosomal enzyme. Lysosomal destabilisation is measured as the

increased permeability of the substrate (naphthol AS-BI N-acetyl-β-glucosaminidine) visualized by the reaction with the enzyme into the lysosomes in presence of diazonium salt. The preparation of tissues for the examination of cell structures requires the use of specialised methodology to produce high-quality stained sections. In this section all observations are related to frozen material, and it is these preparative techniques that will be described.

2.2.2 Solutions and chemicals

Lysosomal membrane labilising buffer (Solution A)

- 0.1 M Na-citrate Buffer - 2.5% NaCl w:v, pH 4.5

Substrate incubation medium (to be prepared just 5 minutes before use) (Solution B)

- 20 mg of napthol AS-BI N-acetyl-β-D-glucosaminidine (Sigma, N4006) are dissolved in 2.5 ml of 2-methoxyethanol (Merck, 859) and made up to 50 ml with solution A, containing also 3.5g POLYPEP (Sigma, P5115; low viscosity polypeptide to act as a section stabiliser).

Diazonium dye (Solution C)

- 0.1M Na-phosphate buffer, pH 7.4, containing 1 mg/ml of diazonium dye Fast Violet B salts (Sigma, F1631) (Note: saturated solution)

  Other dyes can be utilised such as:
  Fast Garnet GBC (Sigma)
  Fast Red Violet LB (Difco)
  Fast Blue BB (Sigma)
  Fast Blue RR (Sigma)

Fixative (Solution D)

- calcium formol: 2% Ca-acetate w:v + 10% Formaldehyde v:v

Mounting Medium: aqueous Mounting Medium (Vector Laboratories H1000) or Kaiser glycerine gelatin

Liquid Nitrogen

2.2.3 Preparation of tissue

Rapidly excise 5 small pieces (3-4mm^3) of the target organ/tissue obtained from five different samples and rapidly place them on an aluminium cryostat chuck (i.e. aligned in a straight row across the centre). Tissues from field samples must be rapidly dissected out to impose no additional stress.

While dissecting, leave the chuck on ice and then place it for 40 seconds in

a small plastic box containing pre-cooled N- hexane[3] at -70°C using liquid nitrogen. Seal the chuck with 4-5 pieces of Parafilm and immediately store at -80°C. (At this temperature the tissues maintain their integrity for months).

Using a Bright's Cryostat (cabinet temperature below -25°C), cut 10 µm thick sections using a 15° knife angle. Transfer the sections to "warm" slides (at room temperature) to flash-dry them. The slides can be stored in the cryostat (for at least 4 hours).

2.2.4 Enzymatic determination of membrane stability

Place the sections in a Hellendal jar containing solution A for different times (0, 3, 5, 10, 15, 20, 30, 40 minutes) at 37°C in order to find out the range of pre-treatment time needed to completely labilise the lysosomal membrane. In the last five minutes add bath shaking.

Transfer the set of slides to solution B and incubate the slides for 20 minutes at 37°C in a Hellendal jar preferably in a shaking water-bath.

Wash the slides in filtered sea-water at room temperature or with a saline solution (3% NaCl) at 37°C for 2 to 3 minutes. Transfer the slides to solution C containing the diazonium coupler for 10 min at room temperature. Rapidly rinse the slides in running tap water for 5 minutes. Fix the sections for 10 minutes in solution D at 4°C (or mount directly with glycerol gelatin), rinse in distilled water and mount in aqueous mounting medium.

The pre-treatment procedure can be further refined by first incubating the sections as indicated above to determine the approximate pre-treatment time required to labilise the lysosomal membrane, and then carrying out a second series of pretreatments at 1 minute intervals in the appropriate time period to obtain greater precision.

2.2.5 Interpretation of results

View the slides under a microscope and divide each section into four areas for statistical interpretation (see diagram below). Lysosomes will stain reddish-purple due to the reactivity of the substrate with N-acetyl-β-hexosaminidase. The average labilisation period (LP) for each organ tissue corresponds to the average incubation time(s) in this acid buffer that produces maximal staining reactivity. LPs for the other samples (in this case n=5) are similarly obtained.

Now analyse the respective quarters of each set of tissue sections pertaining to the same organ tissue and compare the staining intensity with the respective area of the section showing maximal staining (i.e. from which the LP has been derived). A mean

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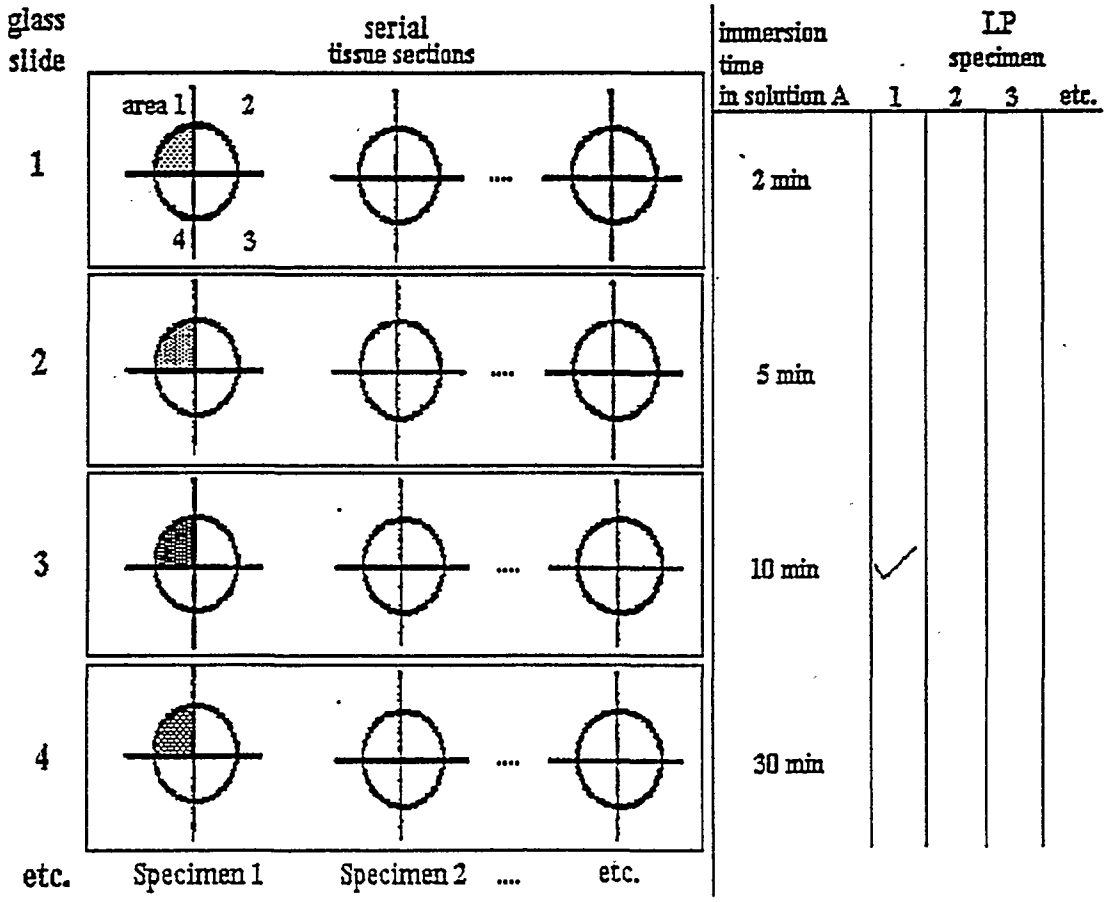
[3] Hexane cooling prevents the formation of ice in the tissue and hence it reduces any structural damage to the subcellular components

value is then derived for each section, corresponding to an individual specimen and successively for the other sections present on the slide derived from the four different samples. Compare test samples with those taken from reference area and determine gradient of cytotoxicity. Reduction in the LP along the expected pollution gradient would indicate cellular stress due to pollution.

Any decrease in staining intensity in successive sections following that with maximal staining may be due to loss of enzyme by diffusion from fully labilised lysosomes. If there are two peaks of staining intensity, then consider only the main staining peak as the LP. This may be due to differential latent properties of the lysosomal hydrolase concerned.

"0" time will be utilised only to verify the correct lysosomal enzyme activity and it will not be considered in the evaluation of the maximal staining intensity peak.



Staining Intensity:

Area 1 (10 min) > Area 1 (30 min) > Area 1 (15 min) ...etc.
∴ average labilisation period for specimen 1 is 10 minutes

This technique does not usually require the degree of precision obtained from a pre-treatment sequence at 1-min intervals. Intervals of 3 or 5 min are generally satisfactory for most test situations, and in this case, the data can then be statistically tested using the Mann-Whitney U-test (Speigel, 1961) and compared with reference or base-line data. For mussel digestive gland, timing intervals of 3, 5, 10, 15, 20, 30 and 40 minutes are normally utilized.

2.3 *In vivo* determination of lysosomal membrane stability: neutral red retention assay

Neutral red is lipophilic and as such will freely permeate the cell membrane. However, within the lysosomes, the compound becomes trapped by protonisation and can be visualised microscopically. The degree of trapping of this lysosomotropic marker depends on the pH of the lysosome as well as the efficiency of its membrane associated proton pump (Segien, 1983).

The acid environment of lysosomes is maintained by a membrane Mg^{2+}-ATPase dependent H^+ ion proton pump (Ohkuma *et al.*, 1982), the neutral red retention assay reflects on the efflux of the lysosomal contents into the cytosol following damage to the membrane and, possibly, impairment of the H^+ ion pump (Lowe *et al.*, 1992), so any impairment of this latter system will result in a reduction of the dye retention assay. Studies indicate that the neutral red retention assay follows the trend of published contaminant levels (Lohse, 1990). The following protocol has been specifically adapted to be used on mussels.

2.3.1 Chemicals and solutions

- Physiological saline

  4.77g Hepes
  25.48g NaCl
  13.06g $MgSO_4$
  0.75g KCl
  1.47g $CaCl_2$

  Dissolve these in 1 litre of distilled water. Gas for 10 minutes (95%O_2:5% CO_2) and adjust to pH 7.3 with 1M NaOH. Store solution in refrigerator, but use it at room temperature.

- Neutral Red dye

  Prepare stock solution by dissolving 20 mg of neutral red powder in 1 ml DMSO. Transfer 5 µl of stock solution into 995 µl of physiological solution (working solution). Keep neutral red in the dark and in fridge when not ulilized. The working solution must prepared freshly before analysis.

2:3.2 Practical evaluation

The following procedure is according to Lowe and Pipe (1994), Moore (1985) and Moore and Simpson (1992).

Fill the eppendorf tubes with sigmacote (SIGMA) for 10-30 minutes, then return sigmacote to container (it is reusable). Put 2 µl of Poly-L-Lysine (SIGMA), diluted 1 to 10 with distilled water, on a microscope slide and spread out with a cover slip. Leave to dry in a hymidity chamber.

Insert scissors half way along the ventral surface of the mussel and partially disclose the valves to allow the insertion of the hypodermic syringe. Drain the water from the shell. Fill an hypodermic syringe with 0.5 ml of physiological saline and then aspirate 0.5 ml of hemolymph from the posterior abductor muscle of the mussel. After obtaining the hemolymph sample, discard the needle and expel the content in a siliconised eppendorf tube.

Dispense 40 µl of hymolymph-saline mixture on the slide, in the same position where the poly-l-lysine was added and incubate in humidity chamber for 30 minutes to allow the cells to attach. Carefully drain the excess solution from the slide by placing the slide on its side and letting the liquid run off. Add 40 µl of the neutral red working solution and leave in a humidity chamber for 15 min. Apply a coverslip and inspect the preparation under a microscope.

Look at the slides every 15 minutes for the first hour then every 30 minutes for the next two hours thereafter. Determine the time at which 50% of the dye that had been taken up into the individual lysosomes (turning them red) leaches out in the cytosol. Derive a mean value for each specimen and successively for the other specimens from the same pool. Compare pool samples with those taken from reference area and determine gradient of cytotoxicity. An increase in leaching rates would indicate cellular stress due to pollution.

Results:

Samples	0	15	30	45	60	90
control	+	+	+	+	+	+
treated	+	+	±	-	-	-

Key:
+ more than 50% of the lysosomes in the cells retaining neutral red
- less than 50% of the lysosomes in the cells retaining neutral red

3. EVALUATION OF GENOTOXIC EFFECTS IN MARINE ORGANISMS

3.1 Alkaline filter elution method

The following protocol, commonly known as the alkaline filter elution method (AFE) is a widely used method to determine the extent of genetic damage in a wide range of marine organisms (Erickson *et al.*, 1980). Damaged breaks or weak points in the DNA are identified by measuring the rate at which single-stranded DNA passes through a membrane filter of known porosity under alkaline denaturing conditions.

The sensitivity of the method depends on the complexity of the DNA, which differs considerably among the different taxa. Thus, DNA from a lower taxon will eluate faster than one of a higher one, even if completely undamaged. One good advantage in using this method is that it allows the determination of genotoxic damage in live animals, for in many instances, small tisue biopsies may be sufficient. Additionally, microfluorimetric DNA determination (Cesarone *et al.*, 1979) increases the sensitivity and reproducibility of the alkaline elution method.

3.1.1 Equipment

- peristaltic pumps with multiple channels (flow rate 1-10 ml/h)
- spectrofluorimeter: excitation: 360 nm/emission: 450 nm
- Fraction collector
- pH meter able to measure pH>12
- Filter holders FP 025/1, PK/10, Schleicher & Schuell (Labotec)
- Filters (Millipore), Type GV 0.22 µm, GVWP 02500
- inverted microscope
- centrifuge
- micro-syringe filter holder[4]
- " stainless extension barrel (10 ml capacity)[4]
- " stainless support screen[4]
- O-ring teflon filter sealing[4]
- Flat gasket, Teflon[4]

3.1.2 Chemicals and Solutions

- Homogenization buffer

  0.14M NaCl
  1.47mM KH2PO4
  2.7mM KCl
  8.1 Na2HPO4
  0.1M EDTA
  bring the solution to pH 7.4 using NaOH

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[4] Millipore Corp. USA

- HANKS' balanced salt solution 2X

  0.2738M NaCl
  0.0107M KCl
  0.8 mM $MgSo_4.7H_2O$
  2.5 mM $CaCl_2.2H_2O$
  0.67 mM $Na_2HPO_4.2H_2O$
  0.88 mM KH_2PO_4
  8.3 mM $NaHCO_3$
  10.09 mM D-Glucose.H_2O

- Lysing solution

  2M NaCl
  0.02M EDTA
  0.2% N-laurylsarcosinate, sodium salt (Sigma L5125)
  bring solution to pH 10 using NaOH

- Washing solution

  0.02M EDTA pH 10.2 (using NaOH)
  bring solution to pH 10.2 using 1N NaOH

- Eluting solution

  0.04M EDTA. Bring to pH 12.3 using tetraethylammounium hydroxide (Merck 822149.0250)

- Working Bis solution

  Prepare $2.5x10^{-4}$M of BIS solution by dissolving 8 mg of bisbenzimide (33258 Hoechst: 2-[2-4-hydroxy-phenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)-benzimidazole trihydrochloride (Farbwerke Hoechst, Frankfurt, Germany), MW: 533.9) in 100 ml distilled water. Make 1 ml aliquots in Eppendorf tubes and store at -20°C. This solution remains stable for at least 1 week when stored at 4°C in dark glass bottles and wrapped in tinfoil. Prepare the working solution by add 100 mls of water containing 0.154M NaCl and 0.015M Na_3citrate to 1 ml of BIS stock solution. The final solution is the working BIS solution.

- DNA standard

  Calf thymus DNA was purchased from Sigma Chemical Company (St. Louis, Mo.), dissolved in sterile SSC, pH 7.0, sonicated for 10 seconds to increase the solubility, and diluted to a concentration of 1 mg/ml. This stock solution was diluted with SSC, pH 7.0.

3.1.3 Sample preparation from tissues of aquatic organisms:

Avoid damaging DNA during handling procedures by using high EDTA concentrations (0.03-0.1M). Always keep materials on ice and try to work fast.

3.1.3.1 For fish liver:

Excise liver and wash in homogenization buffer to remove blood residues. Proceed immediately to the next step or store at -80°C. Homogenize the liver in the buffer using 1:5 w/v.

3.1.3.2 For mussel gill cells:

Open mussels and remove gill cells. Isolate gill cells by enzymatic digestion with a solution of dispase (Boehringer Mannheim), 0.1 mg/mL in modified (2X) Hanks' balanced salt solution for 10 minutes at 37°C. The cellular suspension obtained by filtration is centrifuged at 1,000 rpm for 10 minutes.

3.1.3.3 For mussel haemolymph:

Introduce a hypodermic syringe in the large adductor muscle and draw out some haemolymph. Dilute the sample with equal volume of HANKS' solution 2X.

3.1.4 Sample application

Load sample onto filter at a flow rate of 0.2 ml/min (10-20 mg fish liver per filter; 1 to $2x10^6$ hemocytes or gill cells per filter). Count cell concentration using a counting chamber.

3.1.5 Lysing of cells:

Wash the filters with 5 ml of lysing solution using a flow rate of 0.2 ml/min. Repeat washing using another 5 mls of washing solution at the same flow rate.

3.1.6 Elution of single-stranded DNA:

Perform the elution under reduced light conditions.

Elute DNA through Millipore filters (25mm diameter, 0.2 µm pore size) placed on filter-holders (Millipore Corp. USA) with 10 ml of eluting solution at a flow rate of 0.05 ml/min (i.e. 2 mls per fraction). Collect this volume in 5 tubes each containing 2 mls.

Recover the remaining DNA by removing the filter and immersing it in 4 ml of eluting solution. Shake vigorously.

Rinse the filter holder and tubes with 4 ml of alkaline solution. This is denoted as 'dead' volume.

3.1.7 Microfluorimetric determination of DNA

Place 1 ml aliquots of each elution fraction, the DNA retained on the filter, and a wash of the filter holder in 13x100 mm disposable glass tubes. Neutralise each tube with 0.4 ml of 0.2M KH_2PO_4 and add 0.6 ml of water to bring the volume up to 2 mls. Finally, add 1.0 ml of working BIS solution and vortex. Determine the increased fluorescence, due to binding of the fluorochrome to DNA, using a spectrofluorometer with the excitation wavelength set at 360 nm and the emission at 450 nm.

3.1.8 Calculation:

3.1.8.1 Calculate the slope of the elution curves (elution rate)

For first order kinetics of alkaline elution:

$$y=ae^{-kt}$$

where
y = the fraction of DNA retained on the filter after the elution of volume v
a = the quantity of DNA present on the filter at the 0 solution volume
t = vol

$$\ln y = -kt + \ln a;$$

or

$$\ln y = -k\ vol + \ln a$$

and

$$k= -\ln y/vol$$

3.1.8.2 Calculate strand-scission factor

A value characterising the relative number of DNA-strand breaks, referred to as a "standard scission factor", can be calculated by taking the absolute value of the $\log_{10}$ of the percentage of DNA retained in the treated sample divided after a known elution volume by the percentage of DNA retained in the control sample eluted int he same amount of volume. Therefore, a strand scission factor of 0 indicates no DNA strand breaks. Values greater than 0 indicate a relative value for DNA breaks in the exposed cells (Meyn and Jenkins, 1983).

i) Percentage of DNA eluted in 6 mls of eluting solution

Example:

$\ln y = -0.018x + \ln 101.2$ ..... for 6 mls
$y = e^{-(0.018*6) + \ln 101.2}$
$y = 90.8$

ii) Strand scission factor (SSF)

$$SSF = \log \frac{(\%\ DNA\ eluted\ in\ 6\ mls\ from\ test\ sample)}{(\%\ DNA\ eluted\ in\ 6\ mls\ from\ control\ sample)}$$

Example:

For control sample:	For treated or polluted samples:
ln y = -0.018x + ln 101.2 for 6 ml elution volume: y = 90.8	ln y = -0.046x + ln 100.3 for 6 ml elution volume: y = 76.2

$\therefore$ SSF = log (76.2)/(90.8) = -0.076 at 6 ml elution volume

3.2 Determination of micronuclei frequency

3.2.1 Background

Micronuclei are small DNA-containing bodies which can be present near the cell nucleus during interphase resulting from both chromosome breakage and spindle disfunction. The type of mutations that could contribute to micronuceli production include:

a) mutations to kinetochore proteins, centromeres and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase;

b) unrepaired DNA strand-breaks induced by environmental and endogenous genotoxic agents which may result in acentric chomosome fragments.

Studies indicate that the relative occurrence of micronuclei can provide an indication of accumulated genetic damage throughout the life span of the cells even during short phases of contamination. These considerations suggest the suitability of this test to monitor the extent of gentoxic damage in marine organisms in an time-integrated manner. The following protocl has been devised to assess the frequency of micronuclei in mussel cells.

Table 1

Grid table showing presentation of arbitrary data

Control samples

DNA Standard: 1 µg/ml			fluorescence 160			
Fraction number	Volume (ml)	X value total volume (ml)		Total fluorescence	% retained	Y value ln%
1	1.8	1.8	45	81	98.4	4.58
2	1.9	3.7	45	85	96.7	4.57
3	1.8	5.5	42	76	95.2	4.55
4	1.8	7.3	39	70.2	93.8	4.54
5	2.1	9.4	40	82	92.3	4.52
Dead	4		38	152		
Filter	4		1138	4552		
		Total fluorescence		5098		
		Total DNA (µg)		31.8		

Treated or exposed samples

DNA Standard: 1 µg/ml			fluorescence 160			
Fraction number	Volume (ml)	X value total volume (ml)		Total fluorescence	% retained	Y value ln%
1	2.0	2.0	145	290	82	4.40
2	1.6	3.6	84	134.4	73.65	4.29
3	2.0	5.6	74	148	64.5	4.16
4	2.0	7.6	63	126	56.6	4.03
5	2.4	10.0	35	84	51.4	3.93
Dead	4		22	88		
Filter	4		185	740		
		Total fluorescence		1610		
		Total DNA (µg)		10.4		

3.2.2 Equipment

- centrifuge
- optical microscope

3.2.3 Chemicals and solutions

- 3% Giemsa
- methanol:acetic acid (3:1)
- saline buffer, pH 7.4

3.2.4 Practical evaluation and interpretation of results

Open mussels and remove gill tissues. Homogenize gills in saline buffer, pH7.4 at 4°C and spin at 1,000 rpm to get the cellular pellet. Fix aliquots of the cellular pellets obtained from mussel gills in methanol:acetic acid (3:1) for 20 minutes. Centrifuge at 1,000 rpm for 10 minutes. Resuspend the pellet and smear on slide. Dry in air and stain smear with 3% Giemsa.

Determine the frequency of micronuclei per mussel. The following criteria have to be met during scoring:

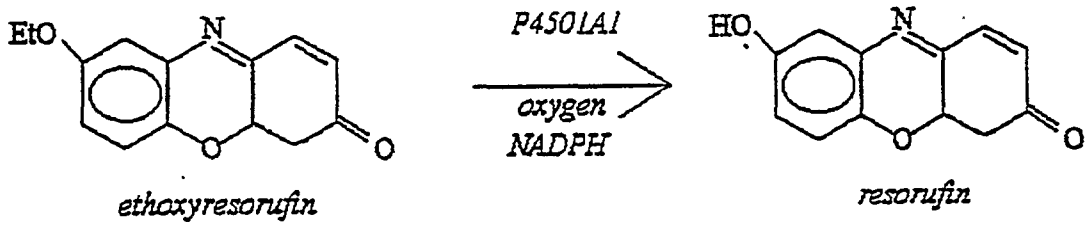
- consider only intact cells are to be scored
- chromatin structure and colour intensity have to be similar to those of the main nucleus
- cells have to be on the same optical plane as the main nucleus
- micronuclei should not be fragmented (so as to exclude small stain particles and apoptotic cells).

Note and compare the frequencies of micronuclei in the cells collected from various pools collected along a pollution gradient with reference and control samples. High micronuclei frequencies signify an increased damage to the nuclear material.

4. ELEVATION OF MIXED FUNCTION OXIDASE ACTIVITY

4.1 Background and principle

This section comprises the full procedure to determine the activity of the cytochrome P450 multi-enzyme complex in fish as a biomarker of biological stress imposed by organic xenobiotics in the marine environment. It describes methods for preparing MFO suspensions, determination of the catalytic activity of ethoxyresorufin O-deethylase (EROD) and estimation of microsomal protein content. The catalytic estimation is based on the incubation of a substrate (ethoxyresorufin) in a fluorimeter cuvette together with an enzyme preparation and cofactor (NADPH) in appropriate buffer, and the fluorescence increase due to resorufin production is recorded.



ethoxyresorufin *resorufin*

This procedure has been well tested both in the field and the laboratory as a monitor of contamination, mainly by PAH, PCB and chlorodibenzodioxins. It can also be applied to pentoxy- or benzyloxy-resorufin O de-alkylase (PROD and BROD) to indicate induction of other P450 isozymes (Burke and Mayer, 1983).

4.1.1 Sampling

A problem of great importance in field sampling of fish is the collection and storage of samples until they can be processed in the laboratory. The hemoprotein degrades rapidly in intact tissue or subcellular fractions; even the use of liquid nitrogen for storage may affect enzyme activity (Forlin and Andersson, 1985). Samples that have been thawed or stored at -20°C are of no use in catalytic measurements. One should also take care that all samples for inter-laboratory comparison are to be treated in the same way, and simple techniques for fixation of samples should be considered if liquid nitrogen is not available.

General guidelines for collection of fish[5]:

- fish should be sampled outside the species-dependent spawning season and the gonadosomatic index should be recorded;
- fish should be sampled within a species-dependent defined length-range;
- either male (usually higher EROD levels than females) or female (higher induction ratio in comparison of high and low polluted sites) individuals should be used. Data from males and females should not be mixed;
- the numbers of individuals sampled must be representative for each site to enable appropriate statistical treatments (at least 10 per site);
- only fish without external and internal visible diseases should be used for further processing, and
- bottom water temperature should be measured at the time of capture.

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5 Report of the Workshop on Biological Effects Monitoring Techniques, Aberdeen, Scotland, 2-6 October 1995

4.1.2 Equipment

- Top-pan balance weighing to 0.1 g
- Conventional dissection instruments
- Ice bucket
- Range of small beakers
- Electric drill capable of 2700 rpm
- Potter-Elvehjem teflon-glass homogenizer (5 or 15 ml)
- Measuring cylinder (10 or 25 ml capacity)
- Refrigerated ultracentrifuge
- Graduated tubes, 5-15 ml
- Pasteur pipettes
- Gilson P1000, 200, 20 pipetman
- Nalgene cryotubes
- Micropipettes with disposable tips to deliver 10, 25, 50, 100, 200 µl
- Glass pipette to deliver 2 ml
- Fluorimeter

4.1.3 Solutions and chemicals[6]

- Solution A
  150 mM KCl

- Solution B
  10 mM HEPES containing 250 mM sucrose; 1 mM Na_2EDTA, adjust to pH 7.4 with KOH (34-36%, Prolabo)

- Solution C
  80% solution B + 20% glycerol (V/V)

- Solution D
  18.2% KH_2PO_4 (Prolabo) 50mM + 22.2% Na_2HPO_4 (Prolabo)
  200 mM bring to 100% with water, pH 7.44

- Storage of solutions and chemicals

  + 4°C Solutions A, B, C, D, G-6PDH
  -20°C NADP, G-6-P, Resorufin
  -80°C Ethoxyresorufin (preferably)

4.1.4 Preparation of samples for analysis

This section describes the steps for preparing microsomal samples prior to the measurement of MFOs. It is always convenient to prepare in advance as many reagents and solutions as possible. Most, like those required for protein determinations,

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6 All chemicals are from Sigma

are stable and will withstand freezing and thawing if kept in plastic bottles. It is usually not possible to prepare nucleotide co-enzyme solutions in advance, however, and since (usually) small amounts of these are needed and as they are relatively expensive, it is desirable to preweigh appropriate amounts of these, and keep them (cooled and desiccated) in small vials.

Microsomal fractions are generally prepared by ultra-centrifugation (e.g. 100,000xg for 90 mins) of homogenates from fresh or frozen tissues (such as liver or hepatopancreas). Cytochrome P-450 measurements and assessment of its activity can also be made in the 9,000xg supernatant (commonly known as the S-9 fraction); however, microsomes are the preferred subcellular fraction as the hemoprotein is concentrated in this fraction.

4.1.4.1 Microsomal preparation

Dissection and isolation of tissue:

Kill fish by severing spinal cord at the level of the pectoral fins and by insertion of a scissor's blade in the brain. Weigh fish with an accuracy of + 1%.

Dissect out the liver and avoid rupturing the gall bladder, since bile may contain MFO inhibitors. Weigh the liver (+ 1% accuracy) and place in a beaker on ice.

All subsequent operations should be performed at 4°C.

4.1.4.2 Homogenization of tissue:

Mince weighed liver (ideally ≥ 1 g, weighed to ± 0.1 g) with scissors, rinse it in solution A and blot dry on tissue paper.

Ajust solution B by adding 1% v/v 0.1 DDT (dissolved in distilled water) and 0.1M PMSF (dissolved in isopropanol).

Place the liver in a Potter glass homogeniser tube on ice and add solution B in ratio 5:1, v:w. Homogenize with 3 vertical strokes at 2700 rpm, keeping the tube cooled in ice. This produces the "crude homogenate".

4.1.4.3 Preparation of S-9 and 100,000xg homogenate:

Place the homogenate in centrifuge tubes and spin for 15 minutes at 9,000xg in a centrifuge. Transfer the resultant supernatant (S-9), without the lipid phase, to an ultracentrifuge tube and recentrifuge again at 100,000xg for 50 minutes. Discard the supernatant (or cytosol) and resuspend in 1 ml of solution C (adjusted to 1% DDT and PMSF as metnioned above for solution B) per fresh liver sample.

Transfer quantitatively this suspension into the Potter, re-homogenise with 5 hand strokes using a teflon tip and keeping the homogeniser cooled in ice. Transfer the homogenised suspension in a graduated tube and record its volume. Hold on ice.

This is the microsomal preparation and is now ready for quantification of protein concentration and enzymatic activities.

Freeze this suspension in small aliquots (100 and 200 µl) in Nalgene 1.5 ml cryotubes (dispensed with yellow tips fitted on a Gilson P 200 pipetman) and stored at -80°C or in dry ice if required for future reference.

4.1.5 Protein determination:

This is in accordance with Bradford (1976).

In 1.5 ml polystyrene spectrophotometer cuvettes, dispense 500 µl of Pierce Protein Assay Reagent using blue fitted Gilson P 1000 pipetman.

Since the optical density (OD) is not directly proportional to protein concentration, it is important to make a standard calibration curve using bovine serum albumin as a standard.

Dilute various BSA concentrations in solution C up to a final volume of 20 µl (using yellow tips fitted to Gilson P20 pipetman) using 1, 2, 5, 10, 20 µl of BSA from a 1 µg μl^{-1} solution C.

Dispense 10 µ1 of l0-fold dilution of solution C and 5 µ1 of non-diluted microsomes into the wall of the cuvette. Also add 480 µ1 of MilliQ water for the reference and the samples, and vortex.

Read the absorbance at 595 nm against a blank containing only the reagents without protein. Plot BSA calibration curve and extrapolate the protein concentration by regression.

4.1.6 Ethoxyresorufin O-deethylase (EROD) determination (Suteau *et al.*, 1988)

4.1.6.1 Preparation

Add sequentially in a tube:
1/100 volume of ethoxyresorufin (from a stock of 123 µM in DMSO)
1/10 volume of glucose-6-phosphate (25 mM in H_2O)
1/10 volume NADPH (25 mM in H_2O)

Bring the mixture to the desired final volume with solution D. Add glucose-6-phosphate dehydrogenase (G-6-PDH) to obtain a final concentration of 1 unit ml^{-1}. Warm the medium for 5 minutes at 30°C in a water bath.

4.1.6.2 Enzymatic reaction:

While the above medium is warming, dispense individual microsomal samples (10 to 100 µg in solution C) into Falcon 2018 polypropylene tubes.

For each sample, set a time "zero" in duplicate and a time "five" in duplicate.

To set a time 'zero' reaction, add 2 ml cold acetone onto the microsomes.

Now transfer all the tubes to the water bath and every 10 seconds add 1 ml of the warm medium to the samples and the time 'zero' reaction using an Eppendorf Multipette fitted with a 50 ml syringe. Vortex the tubes immediately.

Stop the reaction after 5 minutes by adding 2 ml cold acetone except the time 'zero' reaction tube. Vortex again the tubes.

4.1.6.3 Quantification of the resorufin produced

Having checked the extinction coefficient of the resorufin standard, add 100 pmoles of resorufin to a new tube (using a 2 mM standard resorufin solution in DMSO in 5 µl of a 1/100 dilution of solution C) and add 1 ml of reaction medium and 2 ml of cold acetone and vortex.

Measure the fluorescence using a spectrofluorimeter with an excitation wavelength of 537 nm and an emission wavelength of 583 nm. Transfer the samples carefully into cuvettes leaving behind any precipitated protein. Calibrate at 20 fluorescence units for the 100 pmoles of the standard (i.e. directly divide the values read by the 5 minutes of the reaction time) to get the fluorescence units in pmoles min^{-1}.

Autoblank on the time 'zero' reaction tube.

4.1.7 Calculation of the activity

Convert the fluorescence units in pmoles/min. Divide this value by the quantity of the protein in µg used in the assay thus obtaining the result in pmoles/min/µg of protein. Express specific activity in nmoles/min/mg of protein.

4.1.8 Interpretation of results

The EROD measurement is a convenient way of assessing P-450 1A1 catalytic activity and has gained widespread use in biomonitoring studies with fish. The catalytic assay can be viewed as a very useful primary test to identify biological responses due to PAH contamination. The occurrence of hepatic lesions should be recorded; it is a good idea to preserve representative sub-samples of hepatic tissue for future histological examination. Confirmation of increased EROD response can be obtained by determining PAH adducts[7] in fish as evidence that the EROD response is being mediated by PAH compounds.

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[7] PAH metabolites are not indicative of biological effects *per se* but can provide a sensitive marker of exposure to bioavailable levels in the environment. PAH bioavailability can vary markedly in different fish species living in environments similarly contaminated with PAHs

However, there are a number of issues which have yet not been resolved and standardized on an international basis. The choice of the catalytic method is one example. Two different ways of analysing the activity are in common use: (1) the fluorimetric assay (Burke and Mayer, 1974) and (2) the spectrophotometric assay (Klotz *et al.*, 1984), both measuring the formation of reaction product directly. Although reported to give similar results, differences in the detection principle may result in variations in sensitivity.

Standardization problems include the intercomparability of EROD activities in S-9 and microsomal (i.e. - ultracentrifuged) fractions. This often tends to produce somewhat ambiguous results. Another is the use of different protein estimation methods by different laboratories. A more pressing issue, is the use of different extinction coefficients for the reaction product - resorufin (phenoxazone) - which was found to vary between 20 to 73mM^{-1} cm^{-1} (Eichorn, 1973; Lowe *et al.*, in press).

Therefore, if the task is to intercompare and assess the EROD values amongst various regional laboratories, then it is important to fully standardized the catalytic assay before any actual biomonitoring takes place.

4.1.9 Future developments

Apart from the above problems, the catalytic method demands the availability of fresh samples, time and relatively high cost per assay and the use of carcinogenic and/or radioactive substrates.

Ongoing research is taking place to detect immunochemically the inductive response of P450 using antibody probes for both protein levels and mRNA levels (Goksoyr *et al.*, 1991a). In this way, the amount of a specific antibody probe cross-reacting with the P450 protein is measured chemically.

Immunochemical detection of m-RNA can prove highly advantageous since catalytic activity of induced P-450 (Gooch *et al.*, 1989) may be inhibited by certain inducers (such as organochlorines). Consequently, analysis of catalytic activity alone might show no response, but strong induction can still be seen by immunochemical analysis of the P-450 protein or its mRNA. Apparently different types of inducers can also modulate the catalytic activity, as can endogenous compounds. In other cases the catalytic activity may be lost due to bad storage (e.g. in field sampling situations), or the sample or tissue may be too small to give measurable catalytic activity (as with fish eggs and larvae) (Goksoyr *et al.*, 1991b). In all of these cases, immundetection of P450 has been able to detect inductive responses that would not have been possible with catalytic measurements alone (Goksoyr *et al.*, 1991c).

Another technique for the biological monitoring of pollutants in aquatic environments is also available which uses a complementary DNA (cDNA) probe. It has been estimated that such a technique is at least 20-500 times more sensitive than measurement of EROD activity (Haasch *et al.*, 1989).

5. ELEVATION OF METALLOTHIONEIN (MT) PROTEINS LEVELS

5.1 Background

Routine quantitation of absolute MT levels often proves to be problematical due to the lack of a measurable biological activity of this metalloprotein. This has forced investigators to explore unique structural features to be exploited for quantitative purposes. Research efforts have relied for quite some time on estimates of (1) metal content bound to this protein (e.g. by competitive metal displacement or direct quantitation techniques) and (2) physical (e.g. absorption measurements), and chemical (e.g. measurement of sulphyhydryl groups and immunochemical affinities) characteristics.

Each of these approaches has its own strength and weakness. One major disadvantage common to most of these methods is the indirect estimation of this protein, which may lead to inconsistent results concerning the absolute value of the metalothionein concentration in the tissues. In addition, most of these procedures require expensive laboratory equipment (e.g. ultracentrifuges, AAS, chromatographic systems, etc.) and sample preparation and assay optimization require large commitments of time.

For this reason, investigators are adapting simpler but still accurate and sensitive techniques to quantify the levels of MT in biological tissues. Biotechnological assays, such as the measurement of MT m-RNA, are providing some hope in developing an easy and very sensitive technique (Swapan *et al.*, 1991), although further chemical analysis should be undertaken so as to determine the type of insulting metal which is stimulating metallothionein RNA transcription. Specific immunoassays for MT are available, but the limited inter-species compatibility provides a challenge for future development (Kay *et al.*, 1991).

The following methodology is based on the estimation of the sulphyhydryl content of MT proteins. This method has been reported to be a sensitive, time saving, and low-cost technique able to detect metallothionein content in the tissues of marine organisms (Viarengo *et al.*, 1994b) and is currently being intercalibrated and standardized by a number of laboratories within the Mediterranean.

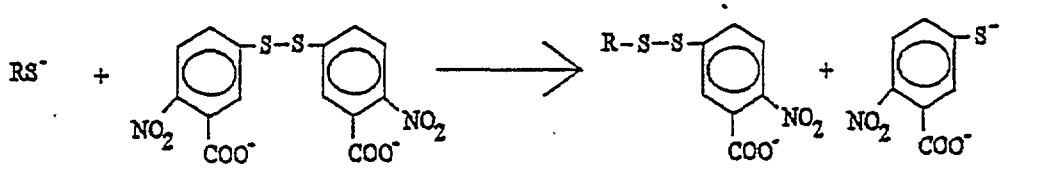
5.2 Colourimetric Determination of the -SH groups using Ellman's reagent

5.2.1 Principle

The method here described (Kay *et al.*, 1991) consists of the ethanol/chloroform fractionation of the cytosolic containing fraction, to obtain a partially purified metallothionein fraction. Metallothionein concentration in the samples is then quantified by evaluating the SH residue content by a spectrophotometric method, using Ellman's reagent (Ellman, 1959), (DTNB - 5,5 dithiobis 2 nitrobenzoic acid). As is known, metallothioneins are characterized by an extremely high cysteine content (about 20-30%) when compared to other proteins eventually present in the ethanolic extracts and therefore the metallothionein determination based on the SH detection allows a more

selective evaluation of these metalloproteins.

Illustrated below is the reaction between DTNB and protein SH groups. The reaction produces stoichiometric amounts of TNB (thionitrobenzoate), a yellowish compound with maximum absorbance at 412nm.



Thionitrophenylated protein *coloured thionitrobenzoate anion*

The analytical procedure has been adapted to be used on mussels, although other molluscs can be used.

5.3 Practical evaluation

To detect metallothionein content in biological tissues by the DTNB reaction, the samples have to be prepared under rigorous reducing conditions (0.01% beta-mercaptoethanol) as described in the protocol. The ethanol/chloroform fractionation allows both the elimination of low molecular weight soluble thiols, which reacting with DTNB, could interfere with metallothionein quantification, and the partial concentration of metallothioneins of which level in the tissues of uncontaminated animals is shown to be often low. During ethanolic fractionation the addition of RNA and acid is essential to allow a quantitative metallothionein recovery. A final "washing" of the metallothionein extracts eliminates the possibility of thiol contaminants, such as reducing agents present in the cells (GSH, cysteine, etc.) or those added during sample preparation (β-mercaptoethanol). The concentrated MT pellet is resuspended in 0.25 M NaCl with addition of HCl and EDTA (to remove heavy metal cations still bound to metallothioneins), followed by the addition of a known amount of DTNB reagent in a high ionic strength medium (to completely denature metallothioneins). A calibration curve of GSH or purified Cd, Zn thionein can be utilized to quantify the metallothionein content in mollusc tissues. Absorbance is evaluated at 412 nm.

5.3.1 Equipment

- Cooling centrifuge (having swing- and fixed-type angle rotors)
- Spectrophotometer
- Motor driven teflon/glass Potter homogenizer with teflon tip.
- Freezer
- Nitrogen gas cylinder

5.3.2 Solutions and Chemicals

- Homogenization buffer

Make up:

0.5M Sucrose-20mM TRIS pH 8.6.
Leupeptin stock solution of 1 mg/ml).
ethanolic stock solution of Phenylmethylsulphonyl fluoride 58 mg/ml).

To the desired volume of Sucrose-TRIS buffer add 3.0 µl/ml leupeptin, 1.5 µl/ml PMSF and 0.1 µl/ml β-mercaptoethanol (equivalent to 0.01%).

- GSH stock solution: freshly prepared before analysis
- 0.25M NaCl
- 0.2M phosphate buffer pH 8 containing 2M NaCl
- DTNB
- RNA (100 mg/ml) store in ice
- cold (-20°C) absolute ethanol
- chloroform
- 37% HCl
- 1N HCl/EDTA 4 mM

5.3.3 Sample preparation and enriched of protein fraction

5.3.3.1 Homogenization:

Rapidly dissect and blot the digestive gland using tissue paper. Weigh a pool of tissues of at least 10 animals and homogenize in 3 volumes of homogenizing buffer containing β-mercaptoethanol, PMSF and leupeptine, with 8 strokes in a motor driven teflon/glass Potter homogenizer.

5.3.3.2 Centrifugation:

Centrifuge the homogenate at 30,000 x g for 20 minutes to obtain a soluble fraction containing MTs.

Note on safety: Always equilibrate the tubes before centrifuging. Use specific tubes PYREX© or COREX© (circa 16 mls volume) for your centrifuge.

5.3.3.3 Ethanolic precipitation:

Precipitate the high molecular weight soluble thiols from the supernatant using absolute ethanol. To 1 ml of the 30,000xg supernatant add 1.05 ml of cold (-20°C) absolute ethanol and 80 µl of chloroform. Vortex for few seconds. Centrifuge in a fixed angle or oscillating rotor at 6,000xg for 10 minutes at 0-4°C. Collect the supernatant and measure the volume using a pipette.

Now, to the 6,000xg supernatant add 40 µl of 37% HCl and 10 µl of a solution of RNA (1 mg/10 µl) followed by 3 volumes of cold ethanol. Store at -20°C for 1 hour.

Re-centrifuge at 6,000xg for 10 minutes using an oscillating rotor. Discard the

supernatant and wash the pellet with an Ethanol/Chloroform/Homogenizing buffer (cold -20°C) solution (87:1:12 v/v) without the addition of β-mercaptoethanol, PMSF and leupeptin.

Centrifuge for 10 minutes at 6,000xg using an oscillating rotor. Remove supernatant and dry pellet under nitrogen gas stream.

Note: all tubes must be kept on ice.

5.3.3.4 Resuspend the pellet in 150 µl 0.25 M NaCl. Add 150 µl 1N HCl containing 4 mM EDTA

5.3.4 Colourimetric determination using Ellman's reagent:

- Dissolve 1 mg of the GSH in 1 ml of 0.25 M NaCl. Store in ice.
- Just before analysis dissolve 0.43 mM (7.14 mg/42 ml) DTNB in 0.2 M phosphate buffer pH 8 containing 2M NaCl. Store in darkness at room temperature.

Prepare the following matrix:

	GSH stock solution	0.25M NaCl	1N HCl 4 mM EDTA	DTNB stock soln
test samples	-	150 µl	150 µl	4.2 ml
standard: 20 µg of GSH	20 µl	130 µl	150 µl	4.2 ml
40 µg of GSH	40 µl	110 µl	150 µl	4.2 ml
80 µg of GSH	80 µl	70 µl	150 µl	4.2 ml

To all samples add 4.2 ml DTNB stock solution. Centrifuge at 3.000xg for 5 min. Measure the absorbance using a spectrophotometer set at 412 nm utilizing reduced glutathione (GSH) as a reference standard.

5.3.5 Calculation and interpretation of results

Plot standard curve and determine concentration of sulphyhydryl content in samples. A higher level of sulphyhydryl content (≡MT) relative to the basal pre-existing level in reference, clean samples would *generally* indicate the presence of a metal pollution stress in the sampling location. However, for the reasons already discussed in the introductory part of this manual, high MT levels can also be related to other

factors which can influence its synthesis.

Any true evidence of pollution has to be presented clearly and convincingly. Coastal areas or gradients containing organisms with high MT level relative to reference areas should be further investigated. In this case, chemical analysis of biota and sediments is advisable to identify the type of insulting metal in that particular area. It would therefore be sensible to reserve a subsample for metal analysis should the need arises.

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