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The present document was prepared in the framework of the MED POL Programme and specifically, within the activities related to the biological effects monitoring for which the Dipartimento di Scienze dell' Ambiente e della Vita (DISAV) of the University of Alessandria, Italy (Professor Aldo Viarengo) acts as the scientific adviser.

The overall responsibility for the editing and compilation of the proceedings was entrusted to G. P. Gabrielides, MEDPOL consultant.

Le présent document a été préparé dans le cadre du Programme MED POL et plus particulièrement dans celui des activités relatives à la surveillance continue des effets biologiques pour laquelle le Dipatimento di Scienze dell'Ambiente e della Vita (DISAV) de l'Université d'Alessandria, Italie, (Professeur Aldo Viarengo) agit en tant que conseiller scientifique.

M. G. P. Gabrielides, Consultant auprès du Programme MED POL est responsable pour l'éditage, la mise en page et la conceptualisation de cet ouvrage.

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The thematic structure of the MAP Technical Series is as follows:

- Curbing Pollution
- Safeguarding Natural and Cultural Resources
- Managing Coastal Areas
- Integrating the Environment and Development

La Série des rapports techniques du PAM est présentée avec la structure suivante:

- Maîtriser la Pollution
- Sauvegarder le Patrimoine Naturel et Culturel
- Gérer les Zones Côtières de Manière Durable
- Intégrer l'Environnement et le Développement

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EXECUTIVE SUMMARY

A UNEP/MAP/MED POL workshop entitled “Workshop on the MED POL Biological Effects Programme: Achievements and Future Orientations” was organized on 20 and 21 December 2006, at the Department of Environmental and Life Sciences (Dipartimento di Scienze dell’ Ambiente e della Vita, DISAV) of the University of Alessandria, Italy. The workshop had as its aims:

(a) to review the work undertaken during Phase III. Under this item the participants had the opportunity to present the work accomplished during the last decade within national monitoring programmes and other comparable programmes;

(b) to discuss a proposal for the utilization of a 2-tier approach to rank the level of pollutant-induced stress syndrome in sentinel organisms sampled along the Mediterranean coast; and

(c) to make recommendations for MED POL Phase IV and other pertinent issues.

The workshop was attended by 22 Mediterranean scientists, participants of the MED POL biological effects programme, who also had the opportunity to present their work. Two outside experts also attended. Discussions concentrated on the evaluation of the results, the assessment criteria and the proposal for the use of a 2-tier approach.

The conclusions and recommendations of the workshop were the following:

The workshop:

1. Acknowledges with satisfaction the excellent work accomplished during MED POL Phase III and the data presented, in particular, from countries of the southern and eastern Mediterranean. The data presented during the workshop ranged from core biomarkers to new developed *omics* (genomics and proteomics) approaches in natural and caged sentinel organisms. Special achievements were obtained in data management and biomarker interpretation.

2. Recognizes the need for harmonization of the assessment criteria with those of the northern European organizations and Conventions. Harmonisation should include biomarker selection, standard operating protocols and data management as well as common inter-calibration exercises, training courses and databases.

3. Recognising that biological tools are useful for the evaluation of the impact of chemicals on marine life, considers that biomarkers and bioassays could be utilised as indicators in the European Marine Strategy and the ecosystem approach for the management of human activities impacting on the marine environment.

4. Recommends that MED POL includes in Phase IV the application of a 2-tier approach with caged molluscs: the first tier would include a single biomarker, namely, lysosomal membrane stability, and mortality. The second tier would include a whole set of biomarkers including lipofuscin accumulation, neutral lipid accumulation, micronuclei frequencies, oxidative stress, metallothionein content, acetyl cholinesterase activity, peroxisome proliferation, lysosome to cytoplasm ratio, and stress on stress.

5. Recommends that MED POL promotes biomonitoring in all Mediterranean countries and that it provides the necessary equipment, reagents, and training for the first tier to the countries that are in need. MED POL should also promote inter-calibration exercises on a Mediterranean scale.

6. Recommends to countries to include the 2-tier approach in their national monitoring programmes.

7. Recommends that MED POL provide environmental managers with simple biomarker integration indices (using the expert system or the multi-marker pollution index) to score adverse biological reactions.

RESUME EXECUTIF

Un atelier PNUE/PAM/MED POL, intitulé “Atelier sur le programme MED POL de surveillance des effets biologiques: réalisations et orientations futures”, a été organisé les 20 et 21 décembre 2006 au Département de l’environnement et des sciences du vivant (Dipartimento di Scienze dell’ Ambiente e della Vita, DISAV) de l’Université d’ Alessandria (Italie). Cet atelier avait pour objet :

a) d’examiner les travaux entrepris au cours de la Phase III. Au titre de ce point de l’ordre du jour, les participants ont eu l’occasion de présenter les travaux réalisés au cours des dix dernières années dans le cadre des programmes de surveillance continue et d’autres programmes comparables;

b) de débattre d’une proposition visant à utiliser une approche à deux paliers pour classer le niveau du syndrome de stress induit par les polluants dans des organismes sentinelles prélevés le long du littoral méditerranéen; et

c) de formuler des recommandations pour MED POL-Phase IV et d’autres questions pertinentes.

L’atelier a réuni 22 scientifiques méditerranéens, participant au programme MED POL sur les effets biologiques, qui ont eu aussi l’occasion de présenter leurs travaux. Deux experts extérieurs y ont également pris part. Les débats ont été centrés sur l’évaluation des résultats, les critères d’évaluation et la proposition d’utilisation de l’approche à deux paliers.

Les conclusions et recommandations de l’atelier ont été les suivantes:

L’atelier:

1. Prend acte avec satisfaction de l’excellent travail accompli au cours de MED POL-Phase III et des données présentées, en particulier par les pays du Sud et de l’Est de la Méditerranée. Les données présentées lors de l’atelier couvraient un vaste champ, depuis les principaux biomarqueurs jusqu’aux nouvelles approches "omiques" mises au point (génomique et protéomique) chez les organismes sentinelles à l’état naturel et en cage. Des progrès ont, en particulier, été obtenus dans la gestion des données et l’interprétation des biomarqueurs.

2. Reconnaît la nécessité d’harmonisation des critères d’évaluation avec ceux des organisations et conventions d’Europe du Nord. L’harmonisation devrait porter notamment sur la sélection des biomarqueurs, les protocoles opérationnels types et la gestion des données, et comporter des exercices d’interétalonnage, des sessions de formation et des bases de données réalisés en commun.

3. Reconnaissant que les outils biologiques sont utiles pour l’évaluation des impacts des produits chimiques sur la flore et la faune marines, considère que les biomarqueurs et bio-essais pourraient être utilisés comme indicateurs dans la Stratégie marine européenne et l’approche écosystémique de la gestion des activités humaines affectant le milieu marin.

4. Recommande que le MED POL intègre dans sa Phase IV l’application d’une approche à deux paliers avec des mollusques en cage; le premier palier comporterait un seul biomarqueur, à savoir la stabilité de la membrane lysosomiale, et la mortalité. Le second palier comporterait une batterie complète de biomarqueurs, dont l’accumulation de la lipofuscine, l’accumulation de lipides neutres, les fréquences de micronoyaux, le stress oxydant, la teneur en métallothionéines, l’activité acétylcholinestérasique, la prolifération des peroxisomes , le rapport lysosome/cytoplasme, et le "stress sur stress".

5. Recommande que le MED POL encourage la biosurveillance dans tous les pays méditerranéens et qu"il fournisse le matériel, les réactifs et la formation nécessaires pour le premier palier à tous les pays qui en ont besoin. Le MED POL devrait aussi promouvoir des exercices d'interétalonnage à une échelle méditerranéenne.

6. Recommande que les pays intègrent l'approche à deux paliers dans leurs programmes nationaux de surveillance continue.

7. Recommande que le MED POL fournisse aux gestionnaires de l'environnement des indices simples d'intégration de biomarqueurs (en ayant recours au système expert ou à l'indice de pollution multimarqueurs) pour noter les réactions biologiques défavorables.

PAPERS PRESENTED

Predicting health of the environment – lysosomal biomarker responses in mussels

by

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ABSTRACT

Cellular changes, such as reduced lysosomal membrane stability, lipofuscin (age or stress pigment) accumulation and other lysosomally related assays, are good indicators of cell injury and animal health status. In a situation where exposure to environmental stressors is likely to be sustained, lysosomal biomarkers can be used to predict that further pathological changes will occur. Lysosomal and autophagic functional perturbations also appear to have potential as measures of damage to ecological health. The potential prognostic use of lysosomal reactions to environmental pollutants has been explored in relation to predicting animal health in marine mussels and flatfish (dab and flounder), based on diagnostic biomarker data. Integration of multiple biomarker data has been achieved using multivariate statistics and then mapped onto "health status space" by using lysosomal membrane stability as a measure of cellular well-being. This is viewed as a crucial step towards the derivation of explanatory frameworks for prediction of pollutant impact on animal health; and has facilitated the development of a conceptual mechanistic model linking lysosomal damage and autophagic dysfunction with injury to cells, tissues and the whole animal. This model has also complemented the creation and use of cell-based bioenergetic computational models of molluscan hepatopancreatic cells that simulate lysosomal and cellular reactions to pollutants.

The use of coupled empirical measurements of biomarker reactions and modelling is proposed as a practical approach to the development of an operational toolbox for predicting the health of the environment. Current assessment methods are largely indicative of exposure to chemical contamination but do not necessarily indicate harmful effects on the health of sentinel animals. In contrast, we have clearly demonstrated a mechanistic link between responses of lysosomal and autophagic biomarkers, oxidative stress and early onset pathology. The lysosomal stability biomarker has also been shown to be significantly correlated with an indicator of ecological status.

INTRODUCTION

The current evolving ecosystem approach to marine environmental management requires that the cumulative effect of all conceivably relevant impacting human activities are considered. Although *performance indicators* play an important and essential role in environmental protection, these indicators do not allow us to say with confidence that the coastal seas are in a healthy state (Defra, 2005). A further category of indicators are required that can demonstrate that ecosystems are healthy. These are known as *indicators of state* (Defra, 2005).

Biomarkers include a variety of measures of specific molecular, cellular and physiological responses of key species to contaminant exposure (Depledge, 1994, 1999; Depledge et al., 1993; Moore et al., 2004). A response is generally indicative of either contaminant exposure or compromised physiological fitness. The challenge is to integrate individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation in health of a particular type of sentinel organism.

However, what we are currently lacking are integrated explanatory frameworks for evaluating complex environmental information and predicting harmful biological effects and their subsequent consequences for environmental health. And while it is clearly recognised that stress-induced changes at the population / assemblage / ecosystem / human health levels of biological

organisation are the ultimate concern; they are generally too complex and far removed from the causative events to be of much use in developing tools for the early detection and prediction of the consequences of environmental stress (Depledge et al., 1993; Moore et al., 2004).

Consequently, it is only at the lower levels of biological organisation that we will have the reasonable expectation of developing a basis of mechanistic understanding of how different environmental conditions can modulate organismal function, which in turn will ultimately help in linking causality with predictability of response (Livingstone et al., 2000; Marigomez & Baybay-Villacorta, 2003). This is in part due to our ability to make certain generalisations about biological organisation and function at the molecular and cellular level, which rapidly disappears as we ascend the hierarchical ladder. Hence, distress signals at the molecular, cellular and physiological levels of organisation should be capable of providing "early warning biomarkers" (molecular, cellular, physiological and behavioural) indicating reduced performance; some of which may be prognostic for impending pathology and severe damage to health of the animal (Depledge, 1994; Depledge et al., 1993; Galloway et al., 2002, 2004; Moore, 2002).

Understandably, however, the concerns of environmental managers and regulators are largely focused on the ecosystem level and not individual animals (Rice, 2003; Xu et al., 2003). Unfortunately, the necessary epidemiological data for pollutant impact on sentinel animals that should permit a more comprehensive understanding of possible causal links between animal and ecosystem health is often limited or fragmentary, both spatially and temporally (Rice, 2003; Xu et al., 2003). Consequently, alternative interdisciplinary approaches will be required to identify such links and we are proposing that the health status of representative sentinel species (e.g., blue and green mussels, oysters, clams, periwinkles, crabs, flatfish) be used in evaluating health of the environment (Allen & Moore, 2004; Depledge, 1994, 1999; Depledge et al., 1993).

Coastal marine ecosystems are sensitive to exposure to toxic contaminants. Pollutants either individually or in combination may have sub-lethal effects at the cellular, organ and individual level, (e.g. causing changes in genetic, behavioural and reproductive activity). Key species have been identified as indicators of this sensitivity including the edible mussel, periwinkles, crabs and several species of fish (Bayne *et al*., 1988; Stebbing *et al*., 1992). Biomarkers include a variety of measures of specific molecular, cellular and physiological responses of key species to contaminant exposure. A response is generally indicative of either contaminant exposure or poor health. In order to address the above mentioned challenge we propose Environmental Prognostics as a branch of systems biology that is specific to the reactions of organisms to both natural and anthropogenic stress (Allen & Moore, 2004; Moore et al., 2006a).
The key enabling concepts of environmental prognostics are:

1) Acknowledgement that reductionist science acts to disassemble ecotoxicological impacts into constituent processes;
2) Acceptance that biology is a cross-disciplinary science involving mathematics, physics, chemistry, engineering and information technology;
3) Moving towards the notion that biology is an information-based rather than a qualitative science;
4) The process requires the assembly of systems by modelling followed by disassembly and focused experimentation as an ongoing procedure.

The crux of the procedure is the definition and evaluation of models of the system in question. This requires the use of the following heavily interdependent tools: conceptual, statistical and numerical models, empirical experimental work and bioinformatics (Fig. 1). Many biomarkers only exhibit a response in a part of the health status space (Allen & Moore, 2004; Moore et al., 2004, 2006a). They indicate that the response has taken place and may even indicate health status within a narrow range, or what has induced the response, but they do not indicate the health status of the whole range from healthy to terminally ill. In terms of environmental prognostics, the first stage is to relate biomarker responses to health status of individual organisms and then to derive integrated explanatory frameworks (Allen & Moore, 2004; Moore et al., 2006a).

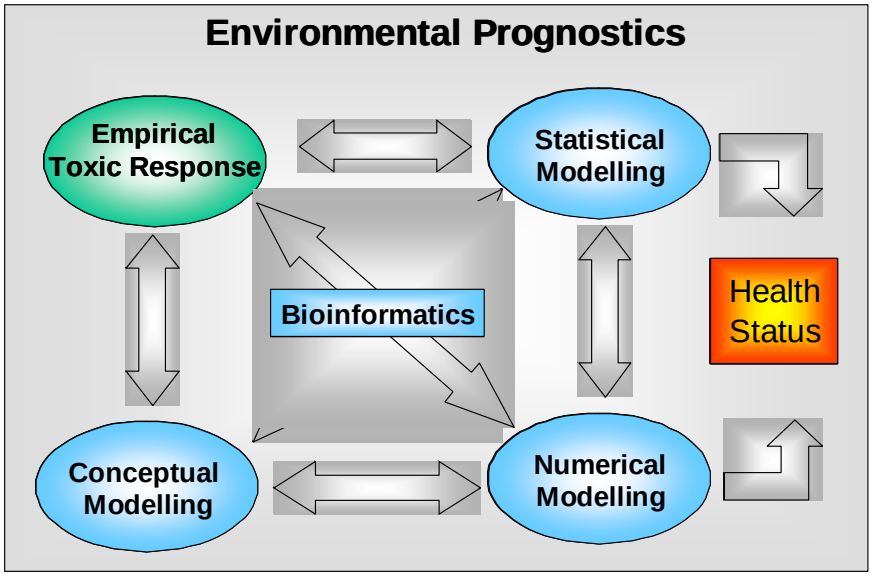

Fig. 1. Diagram showing a schematic of how the disciplines of environmental prognostics relate to each other for the development of explanatory frameworks (adapted from Allen & Moore, 2004).

LYSOSOMAL BIOMARKERS

Lysosomal Reactions to Pollutants

Responses of the lysosomal-vacuolar system may provide a solution to the question of prognostic biomarkers, since injurious lysosomal reactions frequently precede cell and tissue pathology. Lysosomal perturbations have been widely used as early indicators of adverse effect to various factors, including pollutant exposure (Galloway et al., 2004; Moore, 2002; Moore et al., 2004). Consequently, lysosomal function can be used across a range of animals, including annelids, molluscs, crustaceans and fish to detect responses to environmental stress (Cajaraville et al., 2000; Galloway et al., 2004; Hankard et al., 2004; Hwang et al., 2003; Köhler, 1990; Köhler et al., 1992, 2002; Lekube et al., 2000; Lowe et al., 1981, 1992, 1995; Svendseb & Weeks, 1995; Wedderburn et al., 1998).

Lysosomes are highly conserved multi-functional cellular organelles present in almost all cells of eukaryotic organisms from yeast to humans. Their function in the cellular economy includes the degradation of redundant or damaged organelles (e.g., mitochondria and endoplasmic reticulum) and longer-lived proteins as part of autophagic cellular turnover (Klionsky & Emr, 2000). Lysosomes are also involved in the digestion of materials ingested by endocytosis and phagocytosis (i.e., intracellular digestion) (Moore et al., 2006a).

Lysosomal reactions are involved in normal physiological responses as well as many cell injury and disease processes: these include augmented sequestration and autophagy of organelles and proteins (Cuervo, 2004; Klionsky & Emr, 2000; Moore, 1990, 2002). Stress-induced macroautophagy, such as that triggered by nutrient deprivation, is regulated by the mTOR kinase (mammalian target of rapomycin) in eukaryotic cells from yeast to mammals (Klionsky & Emr, 2000). Such reactions have been widely documented for many adaptive and developmental physiological and disease processes; and lysosomal responses have been shown to be involved in generalised reactions to environmental stress (Cajaraville et al., 1995; Köhler et al., 2002; Moore, 1985, 1990, 2002). The functional stability of the lysosomal membrane is a good indicator of lysosomal integrity and has been used widely to measure responses to environmental perturbation in fish and molluscs (Allen & Moore, 2004; Hwang et al., 2002; Köhler et al., 2002; Moore, 2002; Moore et al., 2004).

Lysosomes are also remarkable for the vast and diverse array of chemicals and pharmaceuticals that they can sequester and accumulate (De Duve et al., 1974; Moore, 1990, 2002; Moore et al.,

2004; Rashid et al., 1991). These range from metal ions such as iron, copper and mercury, transuranics, asbestos, polycyclic aromatic hydrocarbons (PAHs), heterocyclics, anti-psychotic drugs to nanoparticles, to name but a few (De Duve et al., 1974; Gould, 2004; Howard, 2004; Moore, 1985, 2006; Moore et al., 1985, 1997; Nott & Moore, 1987; Panyam & Labhasetwar, 2003; Rashid et al., 1991).

Lysosomal functional integrity is a generic common target for environmental stressors in all eukaryotic organisms from yeast and protozoans to humans (Cuervo, 2004), that is evolutionarily highly conserved, and lysosomal membrane stability is a good diagnostic biomarker of individual health status (Allen & Moore, 2004; Bayne & Moore, 1998; Broeg et al., in preparation; Burlando et al., 2002; Cajaraville et al., 1995, 2000; Dondero et al., 2006a, b; Galloway et al., 2002, 2004; Hankard et al., 2004; Klionsky & Emr, 2000; Köhler, 1991; Köhler & Pluta, 1995; Köhler et al., 1992, 2002, 2004; Lehtonen et al., 2006; Lekube et al., 2000; Lowe, 1988; Lowe et al., 1982, 1992, 1995, 2006; Marigomez & Baybay-Villacorta, 2003; Moore, 1976, 1985, 1988, 1990, 2002; Moore et al., 2004a; Moore et al., 2006a,b,c; Nicholson & Lam, 2005; Svendsen & Weeks, 1995; Svendsen et al., 2004; Winston et al., 2002). Dysfunction of lysosomal processes has been mechanistically linked with many aspects of pathology associated with toxicity and degenerative diseases (Cuervo, 2004; Köhler, 1990; Köhler et al., 2002, 2004; Moore et al., 2006a, b). Recent studies have shown that lysosomal autophagy provides a second line of defence against oxidative stress (Cuervo, 2004; Moore et al., 2006c), and the capability to effectively up-regulate this process is probably a significant factor contributing to the ability of some organisms to tolerate stressful and polluted environments.

Lysosomal stability can also be used prognostically to predict liver damage and tumour progression in the liver of various fish species (Broeg et al., 1999 a, b; Köhler et al., 2002; Köhler, 2004), and hepatopancreatic degeneration in molluscs (e.g., blue and green mussels, freshwater bivalves and snails, periwinkles, oysters), coelomocyte damage in earthworms, as well as enhanced protein turnover (i.e., lysosomal autophagy) as a result of radical attack on proteins; and energetic status (i.e., scope for growth) as a predictive indicator of fitness of individuals within a population (Allen & Moore, 2004; Kirchin et al., 1992; Köhler et al., 2002; Moore et al., 2004a, 2006a; Nicholson & Lam, 2005; Svendsen & Weeks, 1995; Svendsen et al., 2004).

Lysosomes are known to accumulate many metals and organic chemical xenobiotics as indicated above. Adverse lysosomal reactions to xenobiotic pollutants include swelling, lipidosis (pathological accumulation of lipid), lipofuscinosis (pathological accumulation of age/stress pigment) in molluscs but not fish, and loss of membrane integrity (Köhler et al. 2002; Moore, 1988; Moore et al., 2006a, b, 2007; Viarengo et al., 1985a). Metals such as copper, cadmium and mercury will also induce lysosomal destabilisation in mussels (Viarengo et al., 1981, 1985a, b), and if oxyradicals are generated then lipofuscinosis can also occur (Viarengo et al., 1985b).

Lysosomal stability and other lysosomal biomarkers such as lipofuscin are strongly correlated with mussel tissue concentration of PAHs (Fig. 2), which are ubiquitous contaminants (Cajaraville et al., 2000; Krishnakumar et al., 1994; Moore, 1990; Moore et al., 2006a, b, c; Viarengo et al., 1992), as well as organochlorines and PCB congeners in liver of fish (Köhler et al., 2002).

Lysosomal stability of various species of mussel and fish from different climate zones clearly reflects gradients of complex mixtures of chemicals in water and sediments (Da Ros et al., 2002; Pisoni et al, 2004; Schiedek et al., 2006, Barsiene et al., 2006; Sturve et al., 2005), single pollution events and accidents (Einsporn et al., 2005; Broeg et al., 2002, Nicholson & Lam, 2005) and also serves for the discovery of new “Hot Spots” of pollution (Bressling, 2006; Moore et al., 1997, 1998a,b, 2004).

Pathology-Related Biomarkers and “Health Status” in Molluscs

Lysosomal membrane integrity or stability in blue mussels is correlated with oxygen and nitrogen radical scavenging capacity (TOSC), protein synthesis, scope for growth and larval viability (oysters – *Crassostrea gigas*); and inversely correlated with DNA damage (incidence of micronuclei), lysosomal swelling, lipidosis and lipofuscinosis, which are characteristic of failed or

incomplete autophagy (Dailianis et al., 2003; Domouhtsidou & Dimitriadis, 2001; Kalpaxis et al., 2004; Krishnakumar et al., 1994; Moore et al., 2004a, b, 2006a; Regoli, 2000; Ringwood et al., 2004). In fish liver, lysosomal membrane stability is strongly correlated with a suppression of the activity of macrophage aggregates (Broeg, 2003; Broeg et al., 2005), lipidosis and lipofuscinosis (Broeg et al., 1999 a,b; Broeg et al., in preparation; Köhler , 2004).

Furthermore, lysosomal stability is also directly correlated with diversity of macrobenthic animals as demonstrated in an investigation in Langesundfjord in Norway (Fig. 3; Moore et al., 2006c).

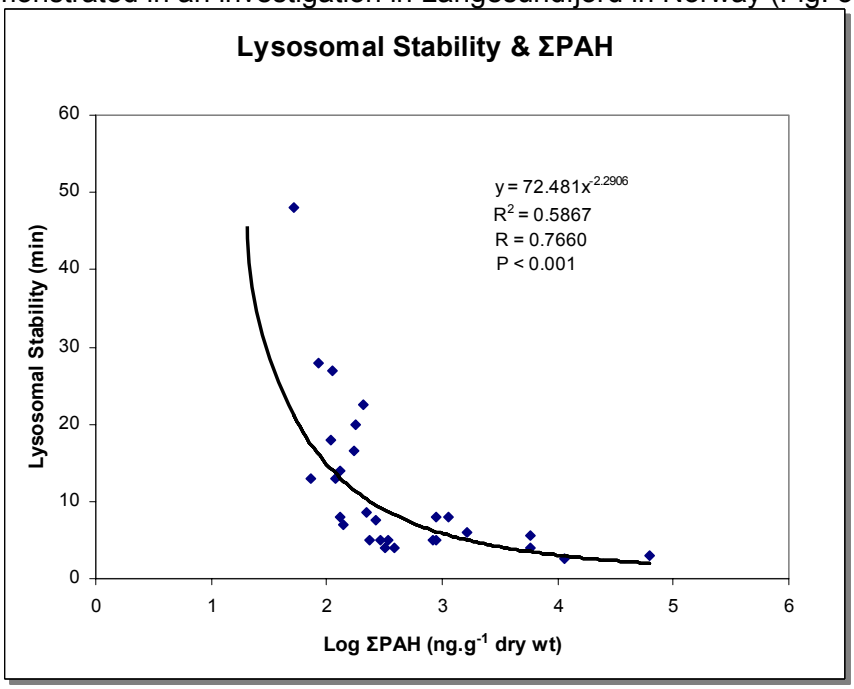

Fig. 2. Statistical model of lysosomal stability in mussel tissue and ΣPAH concentration. The data is a compilation of results from Puget Sound (USA), SW England and the Lagoon of Venice (Krishnakumar et al., 1994; Lowe et al., 1995; Moore et al., 2006a; and unpublished data) .

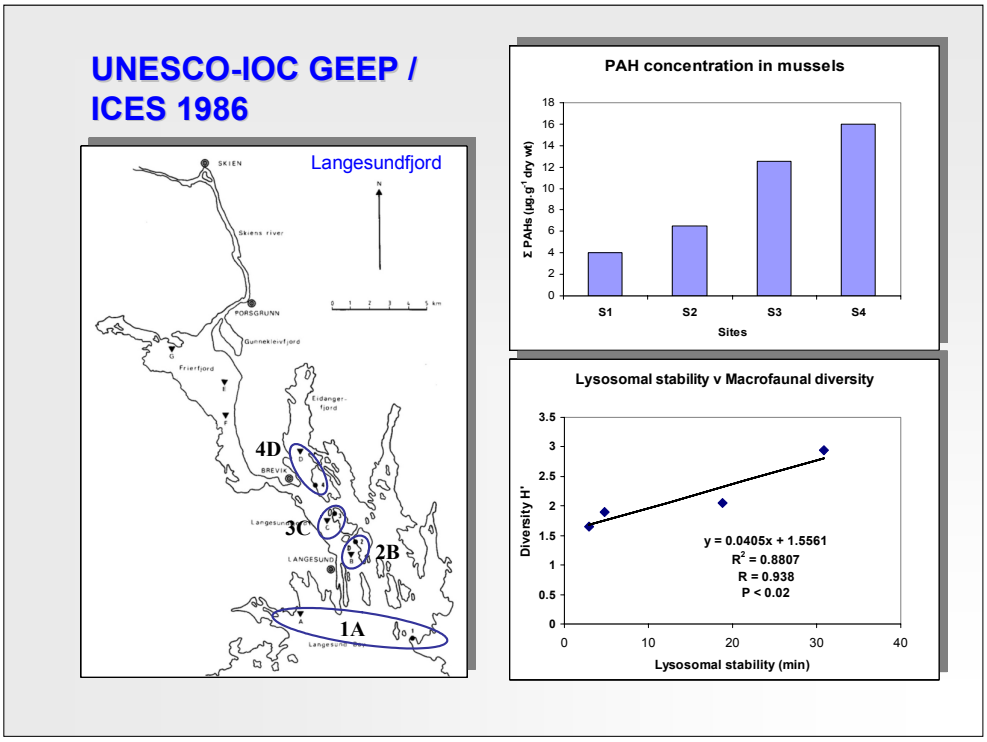

Fig. 3. The summed concentrations of selected polycyclic aromatic hydrocarbons (ΣPAH) in mussel tissues from four sites in Langesundfjord Norway showing a gradient of contamination. The relationship between macrofaunal diversity and lysosomal stability in mussel hepatopancreatic digestive cells from the 4 sites (1-4), shows that they are directly correlated (Moore et al., 2006c).

Integration of multiple biomarker data is achieved using multivariate statistics and then mapped onto “health status space” by using lysosomal membrane stability as a measure of cellular well being (Allen & Moore, 2004; Moore et al., 2006a). This is viewed as a crucial step towards the derivation of explanatory frameworks for prediction of pollutant impact on animal health; and has facilitated the development of a conceptual mechanistic model linking lysosomal damage and autophagic dysfunction with injury to cells, tissues and the whole animal (Moore et al., 2006a, b).

The subcellular pathological reactions induced by many contaminant treatments is known to be linked to augmented autophagic sequestration of cellular components (e.g., bulk cytoplasmic segregation and breakdown of organelles and proteins). Previous studies have shown that all of the stressors used are capable of inducing cellular autophagy in the cells of the mussel hepatopancreas (Moore et al., 2006a,b,c; 2007).

Based on these findings and previous investigations (see Moore et al., 2006a, b), a conceptual mechanistic model has been developed, as stated above, linking lysosomal damage and autophagic dysfunction with injury to cells, tissues and the whole animal. This conceptual model has in turn facilitated complementary development and use of a cell-based bioenergetic computational model of molluscan hepatopancreatic cells that simulates lysosomal and cellular reactions to stress including pollutants (Fig. 4; Allen & McVeigh, 2004; McVeigh et al., 2006; Lowe, 1988; Moore et al., 2006a, b, c).

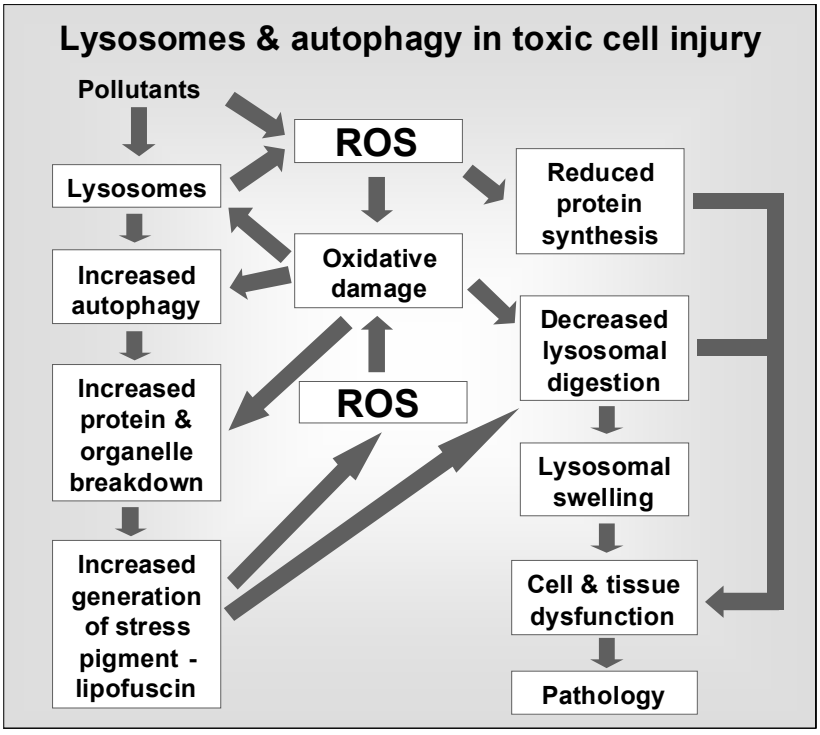

Fig. 4. Simplified conceptual model for interactions of pollutant induced oxidative stress with lysosomal and autophagic processes leading to cell injury and pathology, based on empirical data where there is a reasonable mechanistic basis for making assumptions about the linkages. Reactive oxygen species (ROS) are produced by normal metabolism and enhanced by the action of many chemical toxins. ROS attack proteins and essential cellular organelles, such as mitochondria, contributing to lipid peroxidation and the formation of protein carbonyls, protein aggregates and stress or age pigment (lipofuscin), which is currently thought to produce further free-radicals and may also inhibit autophagic breakdown of cell constituents. It is hypothesized that repeated stimulation of augmented autophagy by various environmental factors that induce mild stress, will result in a more effective recycling of cellular proteins and organelles, before major oxidative damage occurs, consequently reducing lipofuscin formation and thus protecting the cell from further injury. Adapted from Moore et al. (2006a, b).

The integration of biomarker data can be achieved using multivariate statistics and then mapped onto a two dimensional representation of "health status space" by using lysosomal membrane stability as a measure of cellular well-being (Fig. 5; Allen & Moore, 2004; Clarke, 1999; Dagnino et al., 2007; Dondero et al., 2006a; Lowe, 1988; Moore, 1988; Moore et al., 2006a). This is viewed as a crucial step towards the derivation of explanatory frameworks for prediction of pollutant impact on animal health.

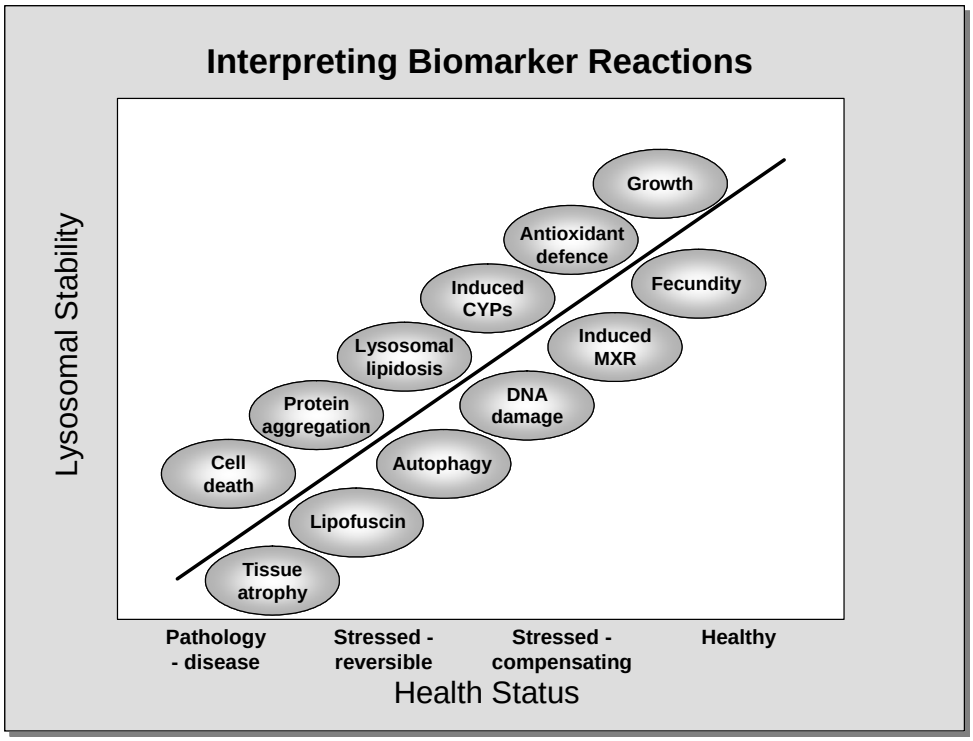

Fig. 5. Idealised diagram of a putative tool for interpreting the probable adaptive or dysfunctional significance of physiological and biomarker responses and pathological reactions in relation to health status. Lysosomal stability is used as an indicator of physiological fitness (Allen & Moore, 2004; Moore et al., 2006a). CYPs are cytochromes P-450 and MXR is multixenobiotic resistance protein (based on Minier & Moore, 1996). Adapted from Moore et al. (2006a).

Autophagy as a Protective System

Changes in lysosomes have been used as biomarkers of ageing in a wide range of organisms including nematodes, fruit flies, molluscs and mammals (Cuervo, 2004). In general there is a trend for decreasing proteolytic capability with increased age that has been linked with a gradual decline in the efficiency of the autophagic process (Bergamini et al., 2003; Moore et al., 2006b). However, it has been proposed that repeated triggering of the autophagic system by diet or caloric restriction will prevent the decline in proteolytic capacity and, hence, contribute to increased lifespan probably through the maintenance of more efficient "cellular housekeeping" (Bergamini et al., 2003; Moore, 2004; Moore et al., 2006a, b, c, 2007) . This may parallel the situation of animals like mussels that live in an environment where autophagy is repeatedly switched on and off, thus maintaining an effective capacity for the removal of proteins, membranes and organelles that are damaged by free-radicals (i.e., reactive nitrogen and oxygen species, RNOS). Further investigation of the role of lysosomal autophagy in conferring resistance to stress is required but the possibility raises provocative questions about the possible role of low levels of stress in the evolution of tolerance (Moore et al., 2006b, c, 2007).

Simulation Modelling of Pollutant Effects

The lysosomal system occupies a central and crucial role in cellular food degradation (intracellular digestion), toxic responses and internal turnover (autophagy) of the hepatopancreatic digestive cell of mussels and other molluscs (Moore et al., 2006a). Understanding the dynamic response of this system requires factors affecting performance (conceived as a function of the throughput, degradative efficiency and membrane stability) to be defined and quantified. A previous version of the carbon/nitrogen flux model has been augmented by separately identifying lysosomal 'target' material (e.g., autophagocytosed or endocytosed proteins, carbohydrates and lipids) and 'internal' material (e.g., digestive enzymes & lipid membrane components) (Allan & McVeigh, 2004; McVeigh et al., 2004, 2006). Additionally, the whole cell energetic costs for maintaining lysosomal pH and productions of these internal components have been incorporated; as has the potentially harmful effect of generation of lipofuscin on the transitory and semi-permanent lysosomal constituents. Inclusion of the three classes of nutrient organic compounds at the whole cell level allows for greater range in the simulated response, including deamination of amino acids to provide molecules as a source of energy, as well as controlling nitrogen and carbon concentrations in the cytosol. Coupled with a more functional framework of pollutant driven reactive oxygen and nitrogen species (RNOS) production and antioxidant defence, the separate and combined effects of three stressors (nutritional quality, nutrient quantity and a contaminant polycyclic aromatic hydrocarbon - phenanthrene) on the digestive cell have been simulated and compared with real data (McVeigh et al., 2004, 2006).

In tandem with this modelling effort, a new decision support system (expert system), able to integrate biomarker data, has been developed by Dagnino et al. (2007) that integrates a suite of biomarkers to interpret complex biological data by transforming it into a relatively simple, easy to understand and objective evaluation of the changes in the organism physiology induced by pollutants. This expert system is based on a classification scale that considers the various characteristics of the biological responses to environmental stressors and can be used to categorise harmful environmental impact. A feature of this system is that it incorporates an indicator or biomarker of cellular or tissue health such as lysosomal stability (Dagnino et al., 2007).

CONCLUSIONS

Lysosomal membrane stability and accumulation of age pigment (lipofuscin) within lysosomes are clearly good predictive indicators for cell injury and pathology; and supporting evidence indicates that these parameters are probably generic in the animal kingdom (Moore et al. 2006a, b, c, 2007). We have also addressed the complex problem of evaluating and predicting health of environmental sentinel animals, such as blue mussels and the flatfish flounder and dab, through a coupled biomarker testing and modelling approach in the wider context of forecasting risk (Fig. 6; Allen & Moore, 2004, Moore et al, 2006a). The approach described above will facilitate the validation, and further the essential new development of robust diagnostic and prognostic tools that can be used along with other chemical, biological and ecological tools as indices of sustainability (Fig. 6). Efforts must also focus on an integrated approach to the validation of biomarkers and relating these to indices of ecological functional integrity that are prognostic for population, community and possibly human endpoints (Moore et al., 2004, 2004a, c, 2007; Rice, 2003; Xu et al., 2003).

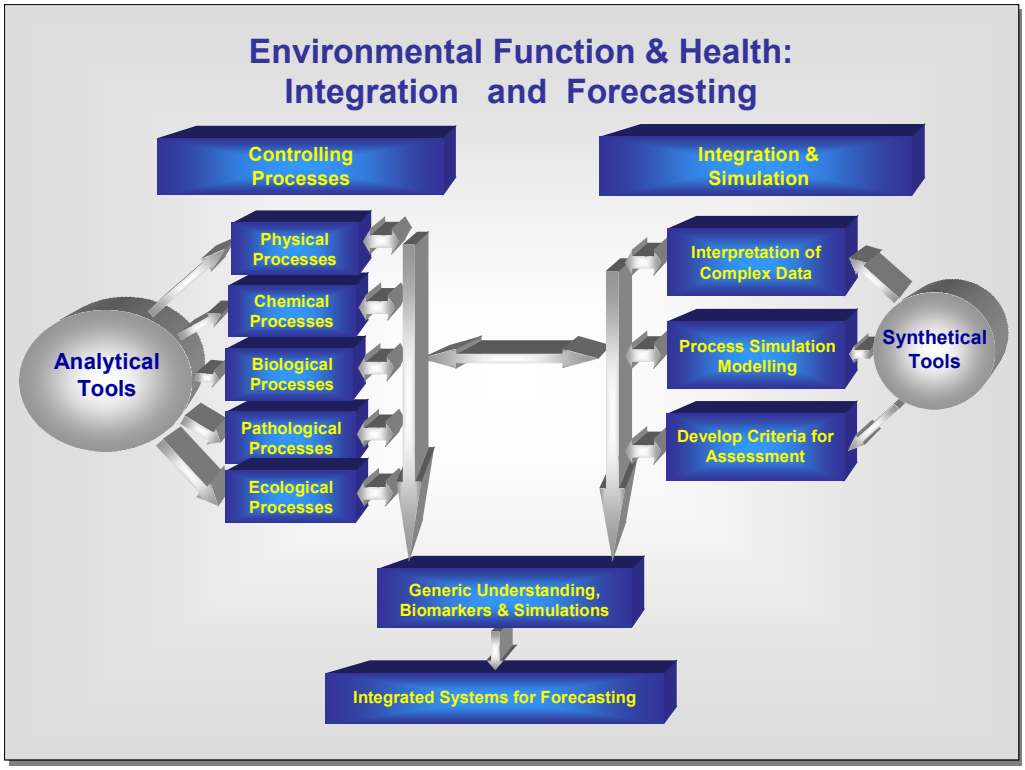

Fig. 6. Proposed process-based synthesis for the evaluation of "health" of the environment and predicting the consequences of future change (Moore et al., 2006a).

A key aim of environmental science is to derive robust, practical and relatively low cost procedures for assessing risk to the health of the biosphere and to use this capability to predict the likely consequences of exposure to potentially harmful toxic pollutants (Moore et al., 2004). Until relatively recently, risk assessment procedures have been oriented towards protecting human health. Now, it is widely acknowledged that such procedures must also ensure that complex biotic communities in natural ecosystems are protected if the quality of the environment in which we live is to be maintained. Environmental risk assessments are currently based on a suite of information derived from studies on the physical chemical characteristics of compounds (the QSAR-based approach), and from laboratory-based toxicity tests (see Moore et al., 2004). Although these procedures constitute a relatively low cost, pragmatic means of ranking the toxicity of potentially hazardous chemicals, they do not directly evaluate the sublethal toxicity, or other adverse effects (e.g., disturbance of ecological relationships) on organisms exposed to complex mixtures of pollutants in the highly fluctuating conditions that prevail in the environment (see Moore et al., 2004).

There is therefore, a priority requirement to implement the use of robust but simple, easy to learn, cost-effective test systems that can identify early diagnostic changes in biota, which can be linked to ecologically relevant endpoints. The selected endpoints must be capable of facilitating a predictive ranking of the condition of particular ecosystems, thus highlighting environmental situations where a more detailed analysis is justified, as indicated by the use of lysosomal stability (neutral red retention) in the Black Sea (Moore et al., 1997, 1998a, b), the Mediterranean Sea (Cajaraville et al., 2000; Lowe et al., 1995; Viarengo et al., 2000), Southeast Asia (Nicholson & Lam, 2005) and North America (Hwang et al., 2002).

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Integrated Biomarker Responses (IBR) Index as a useful tool for environmental assessment evaluated using transplanted mussels in the NW Mediterranean

by

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ABSTRACT

Mytilus galloprovincialis mussels from a clean area were transplanted to several stations in the Bay of Cannes and Nice (North-Western Mediterranean Sea) including a site considered as reference, for one month at the end of spring (May) or in autumn (September). Several biomarkers (AChE, GST and CAT activities, TBARS and MT concentrations) were measured in the transplanted organisms. The concentrations of metals (Cd, Cu and Zn) were determined in the transplanted mussels, PAH and PCB analyses were performed in the mussels caged in 2004. The integrated biomarker response (IBR) was calculated; pollutant concentrations in mussels were displayed as star plots and compared to IBR star plots. Visualization was thus possible between sites for comparison with exposure conditions. Results obtained in the bay of Cannes demonstrated that the mussels from the old harbour site (VP) are characterized by elevated copper and PCB concentrations, those from Canto harbour (PC) presented high PCB contents and those from the mouth of the Siagne River (ES) high PAH concentrations compared to the animals transplanted in the reference site (IL). The agreement between the copper gradient and the PCB gradient measured in the caged mussels and the IBR variation was good whereas the PAH gradient did not seem to contribute to the IBR demonstrating that the chosen biomarkers did not respond to PAHs. In 2005, IBR showed that other contaminants, not measured, might be present in VP, PC and ES compared to the reference station (IL). GST and catalase expression were evaluated using western blotting and were correlated with enzyme activity in most cases demonstrating the precision of the GST biomarker in particular.

Key words: Biomarkers; chemical pollutants; Mediterranean mussel; transplantation experiment

INTRODUCTION

Transplanting molluscs from a reference site to a polluted area can be a feasible strategy for bio-monitoring the effects of environmental changes in coastal or estuarine zones (Da Ros et al., 2000 and 2002, Roméo et al., 2003 a and b). In these papers, biomarkers were used to evaluate the effects of exposure to chemical contaminants and detect responses to environmental stress. The present study aims at measuring a set of biomarkers in mussels transplanted in different places in the North-Western Mediterranean Sea and at computing integrated biomarker response (IBR) as defined by Beliaeff and Burgeot (2002). These authors have established a simple method summarizing biomarker responses named "IBR" that simplifies the interpretation in biomonitoring programmes. In the present work, "active" biomonitoring experiments (De Kock and Kramer, 1994) were conducted with mussels since these animals are of great value in terms of biomonitoring due to their sedentary life and nutritional status (filter feeders). These organisms accumulate a wide range of contaminants and reflect changes in the contaminant status of the environment. Biomarkers associated with chemical analyses were measured in transplanted mussels. The glutathione *S*-transferase (GST EC 2.5.1.18) activity was used as biomarker of organochlorine compounds in bivalve molluscs (Fitzpatrick et al., 1997; Hoarau et al., 2001, 2004). Another biomarker, the catalase activity (EC 1.11.1.6), although not responding specifically to a group of contaminants but to oxidative stress, was measured, it is considered as the primary defence against oxidative damage and has been studied in bivalve molluscs (Pellerin-Massicotte, 1997). Moreover, a marker of oxidative stress, the thiobarbituric acid reactive substances, termed as

TBARS, was also determined as reflecting the state of lipid peroxidation of the membranes (Knight et al., 1988). Acetylcholinesterase (AChE E.C. 3.1.1.7.) is an enzyme involved in the synaptic transmission of nerve impulses and is inhibited by neurotoxic compounds like organophosphate and carbamate pesticides targeted to cause this mode of toxicity (Galgani and Bocquené, 1989).

The responsiveness of AChE to other chemicals such as heavy metals, detergents (Guilhermino et al., 1998) and algal toxins (Lehtonen et al., 2003) has been also acknowledged. Metallothionein (MT) measurements were also performed in transplanted mussels since they are useful indicators of Cd, Hg and possibly Ag and Cu exposure (Cosson and Amiard, 2000). When possible, metals (Cd, Cu and Zn), polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs) concentrations were determined in transplanted mussels. In some cases, immunoblotting techniques were performed in caged mussels to determine the expression of GST and catalase proteins using specific antisera. Cajaraville et al. (2000) underlined the interest in using immunochemical techniques for biomarker measurement since these methods provide a significant increase in the sensitivity and a reduction in the animals required for measurements. Moreover, increased protein levels can be detected even though the enzyme activity has been lost (for instance, in cases of complex mixture of other xenobiotics interfering with the biomarker-inducing xenobiotic). Lastly, stress on stress response was also measured in caged mussels according to Viarengo et al. (1995), since mortality in air would presumably occur more rapidly in pollutant stressed animals than in animals collected from clean areas. The stress on stress response can be integrated as a general stress index for the assessment of contaminated coastal areas.

The studied area, located in the North-Western Mediterranean Sea, has been surveyed; especially the Bay of Cannes, where both pleasure and fishermen's boats are present. In this area, the French RNO (Réseau National d'Observation) already monitored spatial and temporal trends of contaminant (metals, PAHs and PCBs) concentrations in sediments (RNO, 1998). Antifouling paints with tributyltin (TBT) are banned for small boats, in compliance with the French legislation, and copper is used in antifouling paints and is found in increasing concentrations in Mediterranean harbours (Roméo et al., 2003 a).

In this paper, IBR was computed with biomarker measurements obtained in transplanted mussels. The mussels originate from a relatively clean site of the coast (not enclosed bay) at Théoule-sur-mer, 10 kms from Cannes. The cages were located at different stations in the Bay of Cannes shown in Fig. 1.

One was immersed directly from a pier of the "old" harbour (Vieux Port : VP), one was put outside the pier of the "new" harbour (Port Canto : PC), one cage was placed at the mouth of the Siagne River (Embouchure de la Siagne : ES), known for its contamination (R.N.O., 1998) and the last cage was located 3 kms southwards from the old harbour, near the island Sainte-Marguerite (Lérins Archipelago), considered as a relative pristine area, this station is called Ile de Lérins (IL). Transplantation experiments were performed in 2004 and 2005 at the end of spring (May-June). An autumn caging was realized in September 2005 where GST and catalase activities were measured associated with their protein expression in the bay of Cannes (VP and IL) and in the Bay of Nice (Fig. 1), located 30 kms to the East of Cannes, mussels were transplanted in the harbour of Nice NH, at three stations near it called St. 4, St. 5 and St. 6 and at the mouth of the Paillon River EP, a less polluted river than La Siagne due to efficient waste water treatments.

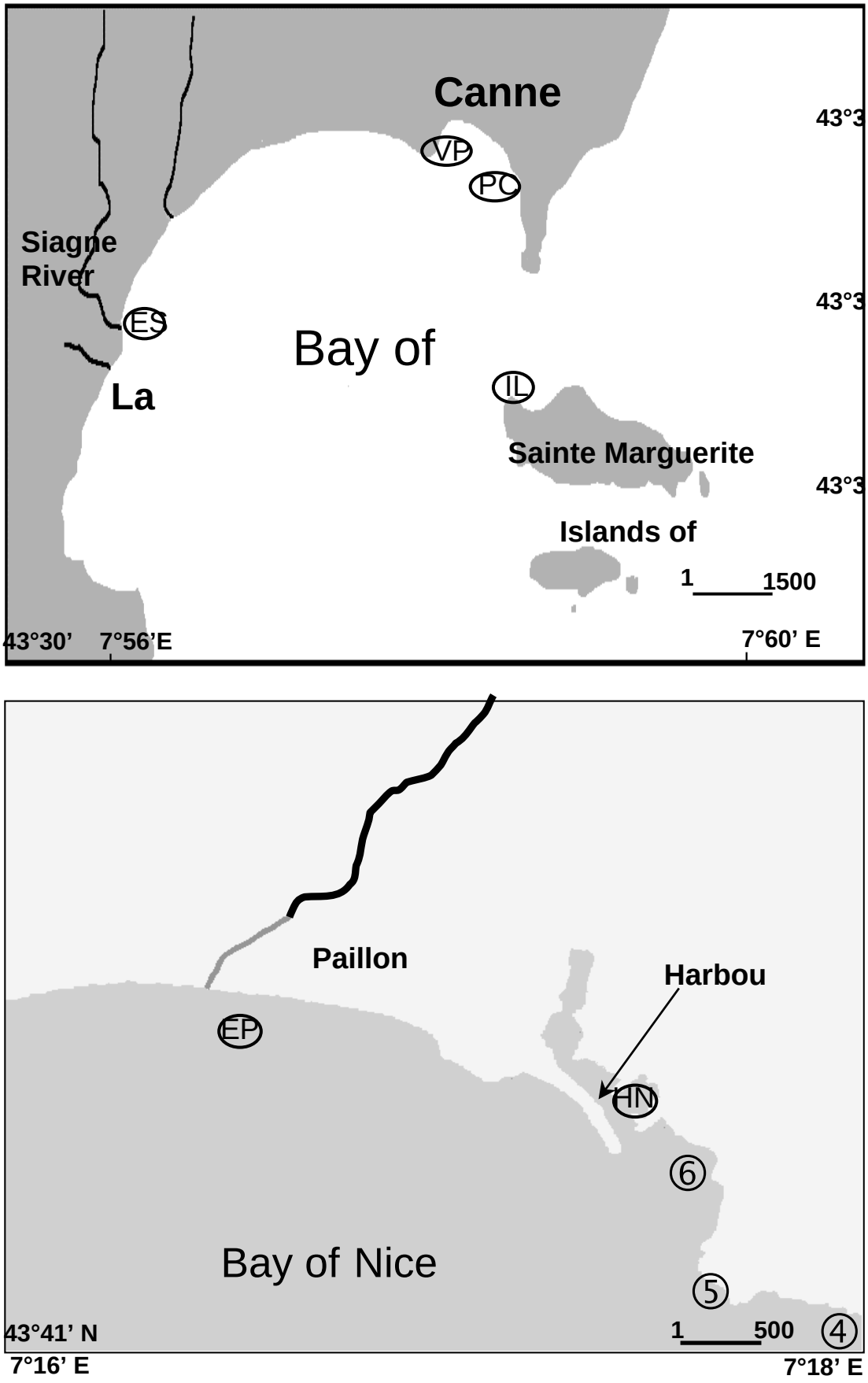


Fig. 1. Location of the cages containing the transplanted mussels in the Bays of Cannes and Nice (NW Mediterranean).

MATERIALS AND METHODS

Wild mussels were collected from the same place at Théoule-sur-mer. Mussels of the same mean shell length (45 ± 5 mm) were collected from the same population. Metal, PAH and PCB

concentrations were measured in the whole soft tissues of mussels after collection (methods and precision displayed below), giving the following results on a dry weight basis: 0.66 ± 0.07 µg Cd/g, 4.7 ± 0.3 µg Cu/g, 122 ± 15 µg Zn/g (*n* = 10 in each case), 0.089 mg ΣPAH/kg and 0.021 mg CB153/kg (*n* = 2 pools of two mussels in each case). These results reveal a good "chemical quality" of the mussels by comparison of the median values of chemicals determined in the mussels along the French coasts (IFREMER, 2003).

After collection, mussels were transported in a cold container from Théoule-sur-mer to the boat. Rectangular cages (20 x 30 x 50 cm) constituted by polyethylene netting and containing ca 120 mussels were used. Cages were immersed by scuba-diving at 5-10 m depth and maintained at anchor for one month.

After one month, cages were collected and transported in cold containers to the laboratory. Surviving animals were counted and compared to the initial number of mussels. Dead animals were thrown away. Upon return to laboratory, soft tissues were cut out of the shells, then weighed and frozen at – 80° C in polyethylene bags. For the stress on stress response, 10 x 4 animals from each station were subjected to anoxia by air exposure at 15 ± 1° C in humidified chambers. Survival was assessed daily. Death symptoms were considered to be open valves and absence of muscular activity. Dead animals were recorded until 100% mortality was reached.

Biochemical analyses
All procedures were carried out at 4 °C. Soft tissues were homogenized in a TRIS buffer (TRIS 50 mM, NaCl 150 mM, pH 7.4), 0.1% antiprotease cocktail, 1 mM DTT (dithiothreitol) in a 1/4 ratio (w/v) using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. Homogenates were then centrifuged at 4 °C for 25 min at 9000 g. Aliquots of the supernatant (called S9 fraction) were frozen at – 80 °C until use. All determinations were performed on S9 fractions.
Each biochemical measurement was performed in ten individual mussels (whole soft body tissues). Total proteins were determined according to Bradford (1976).

GST activities were measured spectrophotometrically at 340 nm by following conjugation of the acceptor substrate 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (Habig et al., 1974). Catalase activities were assayed as described in Clairbone (1985): the variations in absorbance at 240 nm caused by the dismutation of hydrogen peroxide were measured (ε= 40 $M^{-1}.cm^{-1}$). Lipid peroxidation was estimated by the formation of thiobarbituric reactive substances (TBARS). TBARS, considered as "malonedialdehyde (MDA)-like peroxide products", were quantified by reference to MDA absorbance (ε = 156.10^3 $M^{-1}.cm^{-1}$). Results are expressed as TBARS levels. Acetylcholinesterase activity was determined using the method of Ellman et al. (1961) adapted to microplate reader by Galgani and Bocquené (1991).

For MT, analyses according to Viarengo et al. (1997) could be carried out only in June 2005. Digestive gland tissues from 10 individuals were pooled and one pool per station was considered. Tissues were homogenized 1:3 (w/v) in reducing conditions (0.05 M sucrose TRIS buffer, pH 8.6, containing 0.01% β-mercaptoethanol). Homogenates were centrifuged at 30,000 *g* at 4 °C for 20 min. Resulting supernatants were collected and ethanol/chloroform fractionation was used to obtain a partially purified metalloprotein fraction. The concentration of MT was measured by spectrophotometric determination of -SH groups using Ellman's reagent (DTNB).

SDS - PAGE electrophoresis and Western blotting analysis for GST and CAT
SDS-PAGE was performed as described by Laemmli (1970) in S9 fractions of the gills of a pool of four mussels using a Bio-Rad Mini-Protean II electrophoresis unit, with a 12% resolving gel and a 4% staking gel. Samples were submitted to denaturing agents: β-mercapto-ethanol (5%), dithiothreitol D.T.T. (50 mM) and Laemmli buffer (5 fold concentrated). Samples were heated to 100°C for 8 min and immediately cooled in ice. A GST protein standard (GST from rat liver, SIGMA) is prepared in the same manner. Samples (25 µg proteins) were then underlayed into the wells. The electrodes were connected and electrophoresis was performed for 45 min at 150V and 25 mA. Proteins were then transferred on Immobilon-P membrane (Millipore) at 4 °C for 90 min at 100 mA. Non specific binding sites were saturated by incubation in 50 mM Tris-HCl, 150 mM NaCl,

pH 7.4, 0.1% (v/v) Tween 20 (TBS-T), containing 5% (w/v) of skimmed dry milk for one hour at room temperature. The blots were then incubated with a first antibody (dilution 1 : 500 in TBS-T + 1% skimmed milk) which was obtained from a rabbit immunized against the clam *Ruditapes decussatus'* GSTs by Hoarau et al. (2001).

The incubation lasted overnight under gentle agitation at 4°C. The membrane was then rinsed three times with the buffer TBS-T for 10 min and incubated with peroxidase-conjugated goat anti-rabbit IgG from Bio-Rad diluted 1 : 2500 with TBS-T + 1% skimmed milk for one hour at room temperature. The proteins that cross-reacted with the antibody were detected by chemiluminescence (ECL) according to the Amersham protocole. For catalase, the conditions were the same except that the resolving gel was at 10%. The protein standard was purchased from Sigma (catalase from bovine liver). The first antibody was a commercial rabbit anti-catalase polyclonal antibody (dilution 1: 1000 Chemicon), the incubation lasted overnight under gentle agitation at 4°C. Incubation with the secondary antibody (sheep antirabbit IgG from IGN) lasted 1 h.

Metal determinations
Soft tissues were first thawed and dried at 50°C to a constant weight. The digestion of samples (whole soft body, n = 6 for each station) was performed in a microwave oven (CEM-MDS 81D) in high-pressure vessels with concentrated nitric acid. Cadmium, copper and zinc concentrations were determined by atomic absorption spectrophotometry with flame for copper and zinc and with a graphite furnace for cadmium. Deuterium background correction was used when necessary. The analytical procedure was checked using Standard Reference Material (lobster hepatopancreas TORT-2) provided by the National Research Council of Canada. Our results (in µg of metal per g dry weight) are in good agreement with the certified values.

PAH analyses in mussels
Soft tissues were separated from the shell, freeze-dried and homogenized. Assay samples (1 - 2 g dry weight) were extracted using microwave procedure with a 50:50 acetone:petroleum ether mixture. The extract was cleaned up by passing it through a cyanopropyle/silica column (CN/SIOH cartridge) and concentrated down to 1 ml. The considered PAHs were : naphthalene, anthracene, phenanthrene, acenaphtene, fluorene, pyrene, chrysene, fluoranthene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)anthracene, dibenzo(a,h)anthracene, benzo(a)pyrene, benzo(g,h,i)perylene, indeno(1,2,3,cd)pyrene. PAHs were analyzed using high pressure liquid chromatography HPLC 1100 (Agilent) combined with variable wavelength fluorometer (HPLC/fluorometer). The quantification limit of the method was 0.001 ng. A standard reference material IMR-109 purchased from the Commission of the European Communities was also analysed and results are within the range given by the supplier.

PCB analyses
Preparation and extraction procedures are the same as for PAH determination. The extract is purified on a sodium sulphate/silica impregnated with silver nitrate (NAN cartridge). PCBs were analyzed as individual congeners, PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153, PCB 180. Samples are analysed on a gas chromatograph 6890 Agilent 5973 MSD equipped with an ^{63}Ni electron capture and mass spectrometry detection. The quantification limit was 0.001 ng. In order to test the accuracy of the quantification method, the sediment (Harbour sediment 536), provided by the International Atomic Energy Agency of Monaco (IAEA), was analyzed as a standard reference material. Results are in good agreement with the range of values given by IAEA.

IBR calculation
A method for combining all the measured biomarker responses into one general ''stress index'' termed ''Integrated Biomarker Response'' (IBR; Beliaeff and Burgeot, 2002) was applied for each experiment (performed in 2003, 2004 and 2005). The basis of the calculation is described here briefly. For each biomarker: (1) Calculation of mean and S.D. for each station. (2) Standardisation of data for each station: $x_i' = (x_i - mean\ x)/s$, where: x_i' is the standardized value of the biomarker, x_i : the mean value of a biomarker from each station, mean x : the mean of the biomarker calculated

for all the stations, and *s* : the standard deviation calculated for the station-specific values of each biomarker. Result: variance=1, mean=0. (3) Using standardized data, *Z* was computed as + x_i' in the case of an activation and - x_i' in the case of an inhibition, then the minimum value for all stations for each biomarker was obtained and added to Z. Finally the score *B* was computed as $B = Z + |min|$ where $B \geq 0$ and *|min|* is the absolute value. For all the biomarkers treated this way: calculation of star plot areas by multiplication of the obtained value of each biomarker (B_i) with the value of the next biomarker, arranged as a set, dividing each calculation by 2 and summing-up of all values. The corresponding IBR value is: $\{[(B_1 x B_2)/2] + [(B_2 x B_3)/2] + \ldots [(B_{n-1} x B_n)/2] + [(B_n x B_1)/2]\}$.

Statistical analyses
The variations of each biomarker were tested by one-way analysis of variance considering cage site as a variable. When an ANOVA was significant, post-hoc pair comparisons between sites were done using Student's t test to determine which values differed significantly.

RESULTS

In 2004, the experiment lasted from May 7th to June 8th, during this period water temperature ranged from 15 ± 1 to 20 ± 1 °C for all caging sites. The second experiment in 2005 began later on May 17th and lasted six weeks until June 27th with a temperature range of 17 ± 1 to 21 ± 1 °C. The third experiment took place from September 1st to September 29th 2005 (temperature range 21 ± 1 °C to 20 ± 1 °C). During all the experiments, water salinity reached 37 ± 1 psu at all sites except at ES and EP, where salinity decreased to 23 ± 1 psu. Mortality is negligible in the caging experiments (less than 3%).

The mean lethal time of transplanted animals was evaluated (Table 1); it was always small in animals placed in VP compared to the other sites.

Biomarker and contaminant results
Mean enzymatic activities and TBARS contents obtained in the June 2004 and the June 2005 transplanted mussels (whole soft bodies) are summarized in Fig. 2.

The mussels from VP are often different from the others with lower AChE activity (for instance in 2005: 6.44 ± 0.62 versus 9.10 ± 0.60 nmol/min/mg protein at IL), higher TBARS content (in 2004: 3.06 ± 0.18 versus 1.5 ± 0.06 nmol/mg protein at IL) and higher GST activity (in 2004: 450 ± 22 versus 226 ± 43 nmol/min/mg protein at IL). In 2004, high TBARS concentration was observed in the mussels from PC (3.79 ± 0.5 nmol/mg protein). Catalase activities (Fig. 2) were generally higher in 2005 compared to 2004. In 2004, the mussels caged at VP (18.91 ± 1.1 µmol/min/mg protein) and PC (18.55 ± 3.9 µmol/min/mg protein) presented higher catalase activity than at the other sites. In 2005, the mussels transplanted at PC and ES (56.07 ± 2.35 µmol/min/mg protein) had higher catalase activities than those at VP and IL (33.34 ± 1.89 µmol/min/mg protein). MT determinations were performed on a single pool of ten digestive glands (no standard deviation is shown in Fig. 2), the mussels transplanted at PC showed lower concentrations (99 µg MT/g) compared to those caged at other sites (ca 120 µg MT/g). Metal concentrations in the whole soft bodies of mussels transplanted during the experiments of 2004 and 2005 are shown in Fig. 3.

Table 1. Stress on stress experiment: mean lethal time (in days) in mussels after transplantation (n = 40 in each case) at the four sites (VP, PC, ES and IL) in the Bay of Cannes (NW Mediterranean).

Cage location	VP	PC	ES	IL
2004	6.05	8.05	8.22	8.75
2005	10.39	14.87	13.83	14.44

In 2005, the lowest cadmium concentrations were found in VP (for instance 0.36 ± 0.04 µg/g dry weight) and the highest in PC (1.03 ± 0.07 µg/g in 2004) and in ES. The highest copper concentrations in transplanted mussels were found in VP compared to the other sites. Moreover, copper concentrations in the mussels caged at VP were higher in 2005 than in 2004. Zinc concentrations ranged between 100 and 300 µg/g and did not show significant inter-site differences in 2004 and 2005.

GST and Catalase expression
Western blots were performed in mussels caged in September 2005 in the Bay of Cannes (two cages: at VP and IL) and in the Bay of Nice (EP, HN, St. 4, St. 5 and St. 6). Results are shown in Fig. 7; proteins were visualized using Coomassie blue reagent.

The enzymatic activities are given below the western blots. In the case of catalase activity, the mussels transplanted at HN (harbour of Nice) show a low expression whereas enzymatic catalase activity was the highest observed among the stations, this may be due to interaction between pollutants evoking induction of catalase. Moreover, the used antibody did not come from a marine invertebrate organism. In the case of GST, the highest activity is found in mussels from VP which present the most intense band and the lowest GST activity is found in mussels from IL which show the less intense band. There is therefore a good agreement between GST protein expression and enzymatic activity.

DISCUSSION

The translocation of sentinel species, mainly mussels from a clean site (Théoule-sur-mer in the present paper) to the study areas has been demonstrated as a useful strategy for the assessment of water quality in coastal and estuarine environments, either through accumulation or biomarker analysis (De Kock and Kramer, 1994). Caged mussels facilitate the investigation of areas where native specimens are absent, and/or, reduce the influence of genetic differences, which can attenuate the capacity of biomonitoring to discriminate differences levels of environmental disturbances.

PAHs are classified according to their molecular weight from the very light and soluble naphthalene (two aromatic rings) to the heavy and insoluble benzo(g,h,i)perylene. The highest total PAH concentration is found in the mussels from ES (ΣPAHs = 0.819 mg/kg dry weight) and the lowest in those from IL (0.159 mg/kg); the sites VP and PC show a moderate PAH concentration (0.293 and 0.230 mg/kg, respectively). The predominant PAHs are phenanthrene (0.185 mg/kg at ES), acenaphtene (0.156 mg/kg at VP) and naphthalene (0.351 mg/kg at ES) and benzo(b)fluoranthene (0.045 mg/kg at PC). The results of the seven PCB congeners are displayed in Fig. 5.

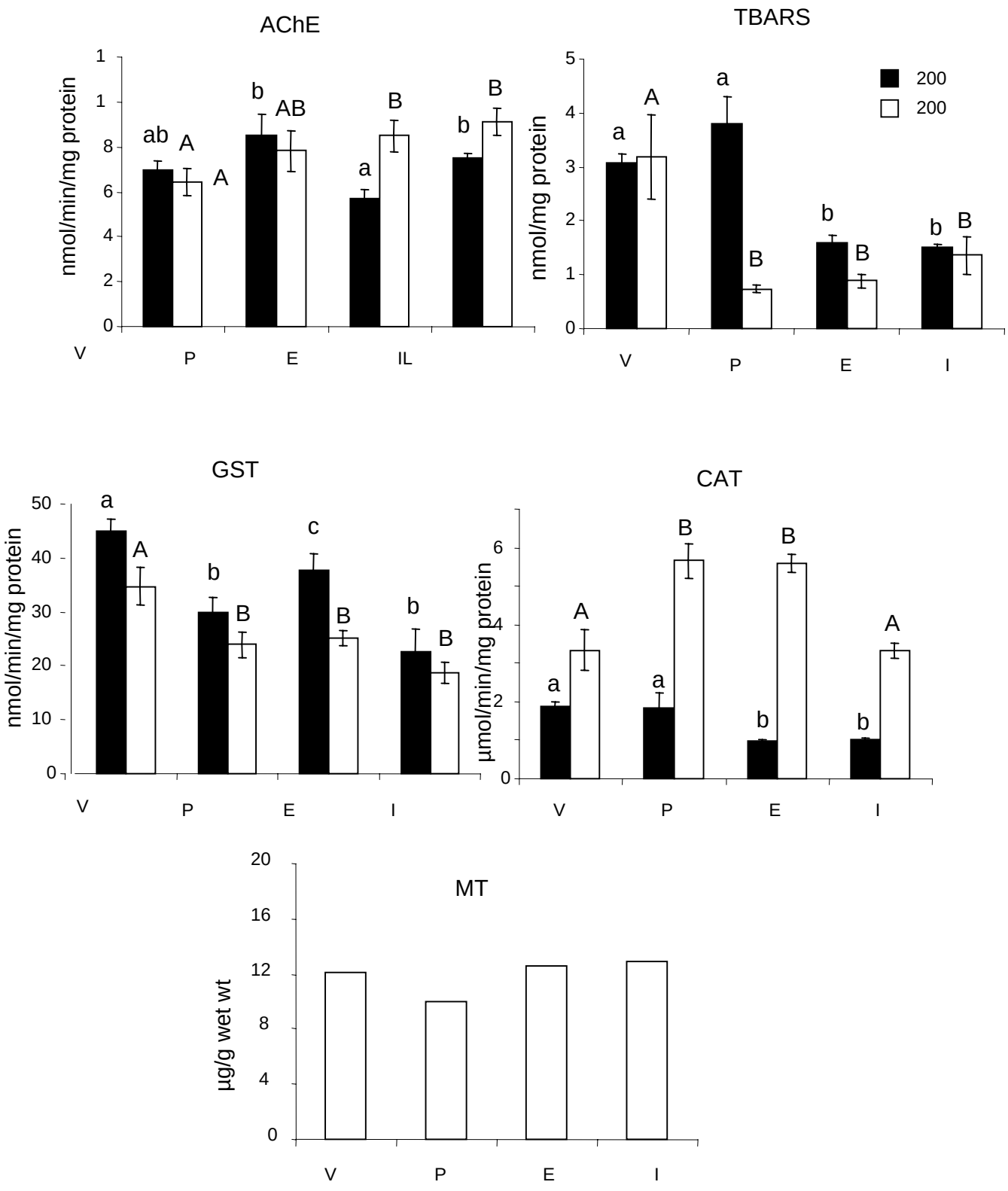


Fig. 2. AChE, GST and CAT activities and TBARS and MT concentrations in mussels transplanted at the four sites (VP, PC, ES, IL) in the Bay of Cannes in June 2004 and June 2005. The bars with the same superscript (small letters in 2004 and capitals in 2005) are not significantly different ($p > 0.05$).

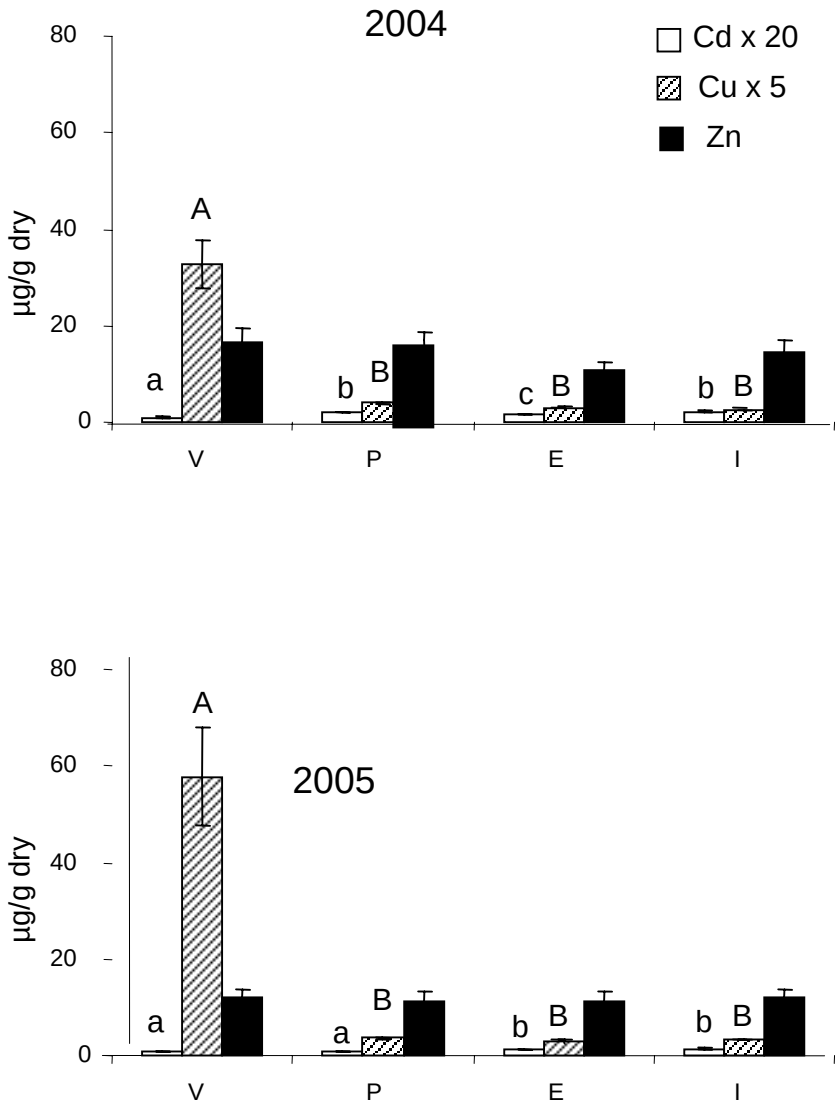


Fig. 3. Mean cadmium, copper and zinc concentrations in the mussels transplanted at the four sites (VP, PC, ES, IL) in the Bay of Cannes in June 2004 and June 2005. The bars with the same superscript (small letters in 2004 and capitals in 2005) are not significantly different ($p > 0.05$).

The predominant PCB is CB 153 (0.104 mg/kg dry weight at PC and VP) followed by CB 138 (0.095 mg/kg at PC). The mussels from PC and VP present higher total PCB concentration (0.437 and 0.364 mg/kg dry weight, respectively) compared to ES (0.206) and IL (0.173 mg/kg).

Integrated biomarker response

The integrated biomarker responses were evaluated (MT concentrations were not taken into account) and are shown in Fig 6 a, b for 2004 and 2005, respectively.

PAH concentrations in the June 2004 mussels transplanted are depicted in Fig. 4.

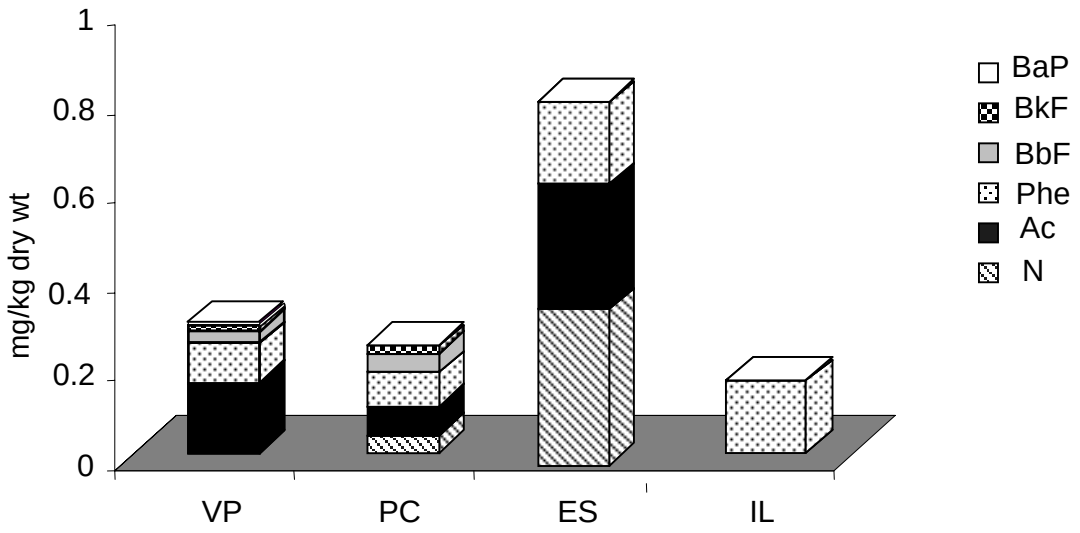


Fig. 4. Concentrations of individual PAHs in mussels transplanted in June 2004 (mean values of 2 determinations in mg/kg dry weight). Abbreviations are the following : Naphthalene: N; Acenaphtene: Ac; Phenanthrene: Phe; Benzo(b)fluoranthène: BbF; Benzo(k)fluoranthène: BkF; Benzo(a)pyrene: BaP. Nine PAHs were below the detection limit: Fluorene, Anthracene, Fluoranthene, Pyrene, Benzo(a)anthracene, Chrysene, Dibenzo(a,h)anthracene, Benzo(g,h,i)perylene, Indeno(1,2,3-cd) pyrene.

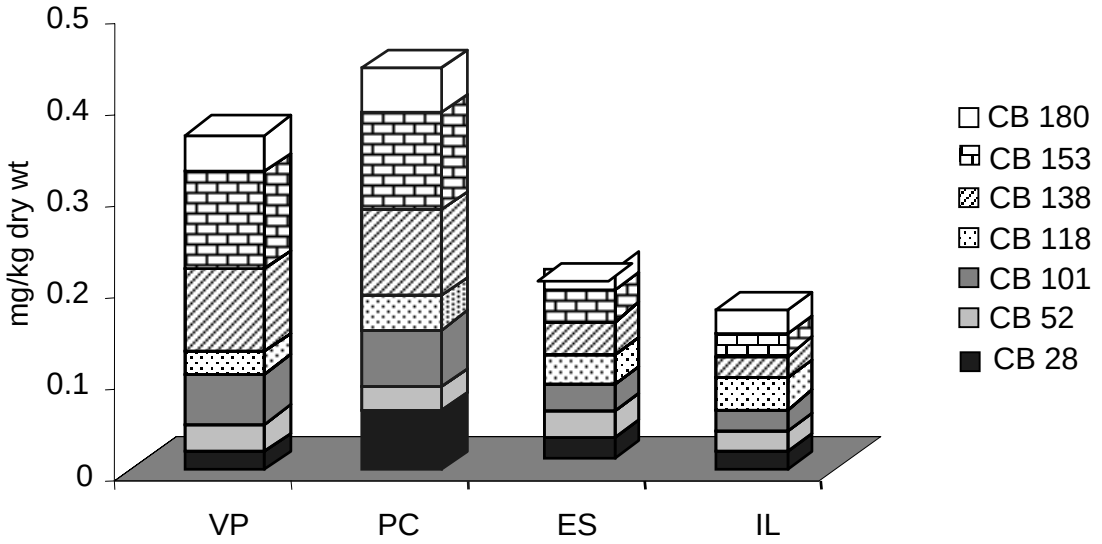
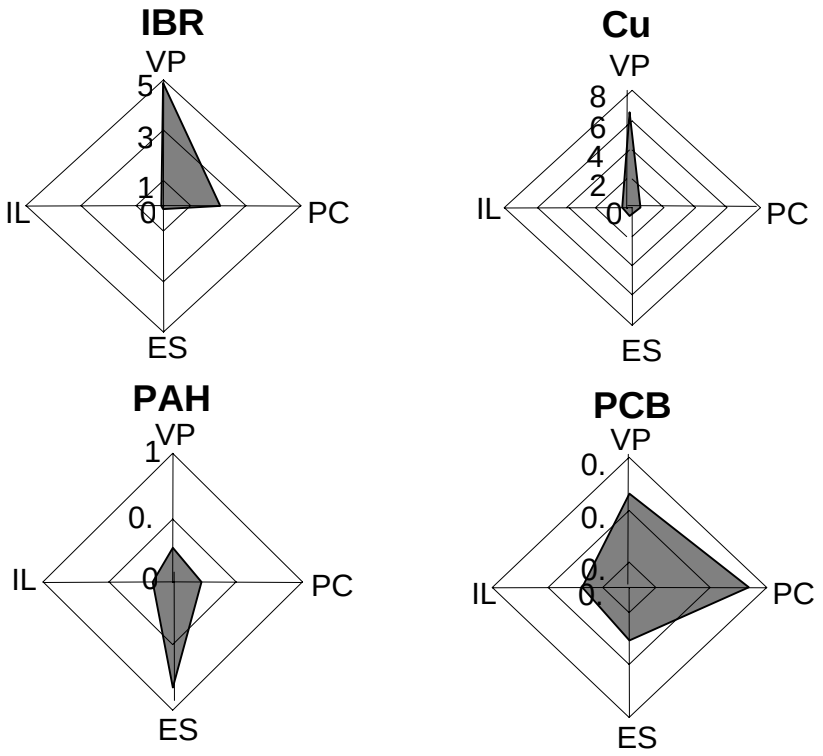


Fig. 5. Concentrations of individual PCB congeners in mussels transplanted in June 2004 (mean values of 2 determinations in mg/kg dry weight).

a)



b)

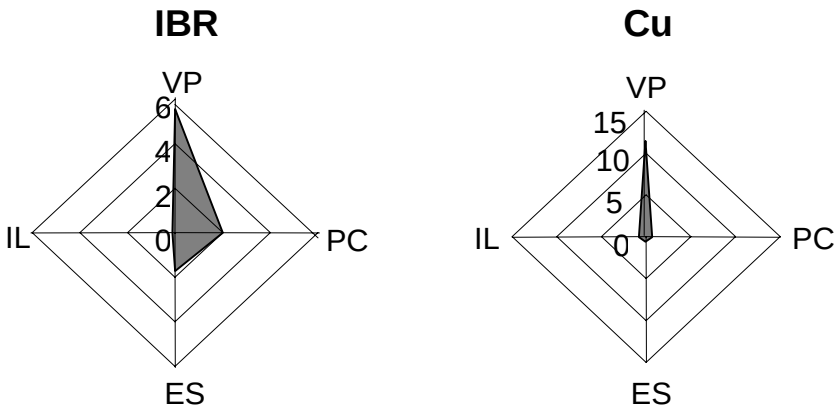


Fig. 6. Integrated biomarker response (IBR) and pollutant star plots: a) IBR, copper (Cu in µg/g), polycyclic aromatic hydrocarbons (PAHs in µg/g)) and polychlorobiphenyls (PCBs in µg/g) star plots in mussels transplanted in spring 2004 at four sites in the Bay of Cannes : VP (Old harbour), PC (Canto harbour), ES (mouth of the Siagne River) and IL (Lérins Island); b) IBR and copper star plot (µg/g) measured in mussels transplanted in spring 2005 at four sites in the Bay of Cannes.

The area in black integrates the IBR for each transplantation site and is represented as star plot. Copper star plot, expressed in µg/g, is shown as well as PAH and PCB star plots, expressed in mg/kg, for 2004. In general, our IBR values show a large range of variation, in 2004 from 0.04 (IL) to 4.85 (VP) and in 2005 from 0.1 (IL) to 1.7 (ES), 1.96 (PC) to 5.52 (VP).

The mussels caged in VP show the lowest survival time "stress on stress" (Table 1) indicating that the physiological status of animals was reduced compared to mussels transplanted elsewhere.
In 2004, copper concentrations in transplanted mussels (65.2 µg/g, Fig. 3) are higher in VP compared to those from the other sites (concentrations < 10 µg/g). This is likely due to the antifouling paints containing copper since the banning of TBT. The variations of cadmium and zinc concentrations are close to the median values reported in IFREMER (2003). Low molecular weight PAHs (such as phenanthrene) found in oils and fuels (RNO, 2002) are accumulated in the transplanted mussels (Fig. 4). High molecular weight PAHs such as benzo(b)fluoranthene and benzo(k)fluoranthène, due to oil combustion coming from an intense maritime traffic (R.N.O., 2002), are present only in the mussels transplanted in harbour areas (VP and PC, Fig. 4). The mussels caged at ES are particularly submitted to oil and fuels, compared to the other mussels. The lowest total PAH concentration is found in the mussels from IL, the "reference" site, this value is close to the median value 0.140 mg/kg determined in the mussels collected from the French coasts (IFREMER, 2003). Superficial sediments collected at ES present relatively high total PAH concentrations: 0.76 mg/kg (Agence de l'Eau Rhône-Méditerranée-Corse: http://eaurmc.fr) compared to the other sites of the Cannes Bay where the sediment concentrations reach ca 0.340 mg/kg (RNO, 1998). Fig. 5 clearly indicates that VP and PC are submitted to PCB contamination. The mussels transplanted at ES and IL are characterized by PCB congeners present in almost equal proportions whereas the distribution in the mussels from VP and PC is dominated by CB 153 followed by CB 138 (highly chlorinated congeners). IFREMER (2003) reported median CB 153 concentration of 0.0209 mg/kg in the mussels from the French coasts; this value is exceeded in the mussels from VP and PC. RNO (1998) reported that the concentrations of total PCBs in superficial sediments collected from the Bay of Cannes are higher than 0.005 mg/kg, concentration which is a reference value for low to non-contaminated sediments (Marchand et al., 1990). As the higher chlorinated congeners are specifically bound to the smaller fraction of the sediment (Piérard et al., 1996), it is possible that this could be the source of PCBs for the water-column bivalves such as mussels. The smaller particles of the sediment are more likely to be re-suspended by water movements and other disturbances of the sediment floor and, thus, made available to the bivalves in the water column (Thompson et al., 1999). According to RNO (1998), the pollution of sediments by PCBs in the North-Western Mediterranean Sea seems to be caused by urban waste waters. In the studied area, the Bay of Cannes, over 300,000 inhabitants are recorded.

In 2004, the biomarker response may be associated with the pollutant (metals, PAHs or PCBs) contents in the mussels: the decrease of AChE was noted in VP and ES, likely due to copper in VP and other pollutants in ES. AChE activity in mollusc bivalves was shown to increase when salinity decreases (Bebianno et al., in press; Pfeifer et al., 2005). Salinity, which is lower in the mouth of Siagne River (ES), has no impact on AChE activity measured in animals transplanted there. The anti-oxidant enzyme catalase presents high activity in VP and PC due to oxidative stress which did not prevent the peroxidation of lipid membranes since TBARS were especially elevated in VP and PC. This oxidative stress may be due to copper or PCBs. Michel et al. (1998) observed a positive relationship between PCB concentrations in sediments and TBARS levels in mussels collected together with sediments along the North-Western Mediterranean coast. The suggested mechanism of action implicates increased production of oxygenated radicals due to cytochrome P-450 induction (phase I enzymes) by PCBs (an endogenous source of active oxygen); subsequent reactions involve phase II enzymes such as GSTs (Stegeman et al. 1992). On the other hand, GST activities, higher in VP and ES than in the other sites, may be due to copper and hydrocarbons, respectively. Nevertheless, Stegeman et al. (1992) observed that the inducibility of GST activity, in various aquatic species, with PAH-type inducing agents gives inconsistent results. GST activity in mollusc bivalves was also shown to increase when salinity decreases (Bebianno et al., in press), the increase in GST activity in mussels from ES may also be assigned to salinity decrease.

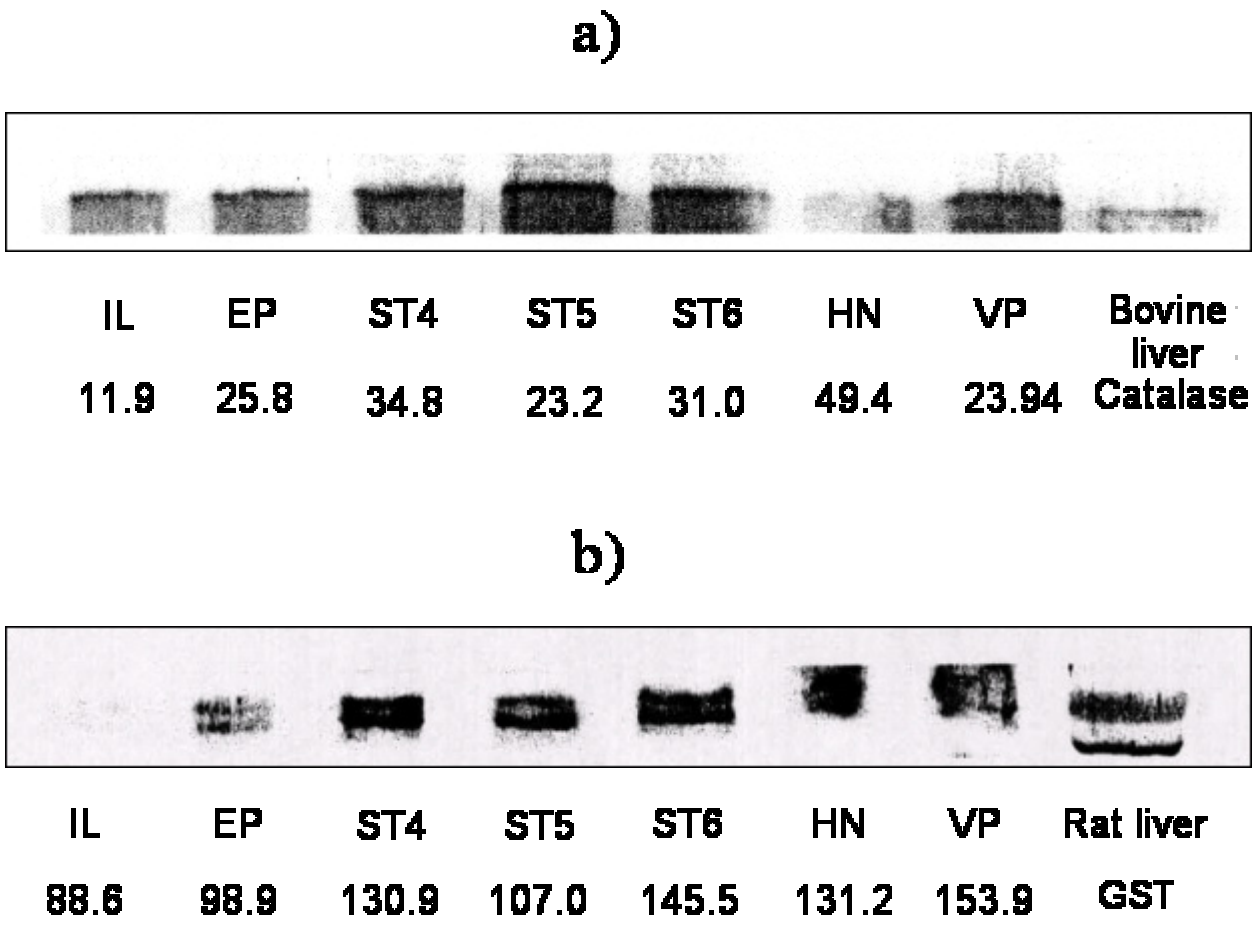


Fig. 7. Western blots of a) catalase and b)GST expressions in the gills of transplanted mussels in the Bay of Cannes at VP (Old harbour), IL (Lérins Island) and the Bay of Nice at EP (mouth of Paillon River), HN (Nice harbour), St. 4, St. 5 and St. 6 (stations located near HN). Samples were subjected to SDS PAGE and immunoblotting with antibodies described in materials and methods. Protein standards (Sigma) came from bovine liver for catalase and from rat liver for GST. Mean enzymatic activities are written below the blots, catalase is expressed as µmol/min/mg proteins and GST as nmol/min/mg proteins.

Fig. 6 a shows a visual reasonable agreement between the copper gradient, the PCB gradient measured in the encaged mussels and the IBR variation whereas the PAH gradient does not seem to contribute to the IBR.

In 2005, the low AChE activitiy and the high GST activity, recorded in the mussels from VP, may be related to copper influence. Copper concentrations are higher than those previously reported for the same site confirming the presence of this metal in the harbour area; cadmium and zinc concentrations are in the range reported by IFREMER (2003). Catalase activity is generally

enhanced compared to 2004, particularly for the animals transplanted in PC and ES, two sites likely impacted by contaminants inducing oxidative stress, the enzyme efficiently prevents lipid peroxidation by neutralizing oxyradical leading to low TBARS concentration at PC and ES. Moreover AChE activity is relatively high at PC and ES, demonstrating a good physiological state (Leiniö and Lehtonen, 2005). Water temperature may also increase the catalase response as suggested by Cancio et al. (1999) for *Mytilus galloprovincialis*. In June 2005, mussels were transplanted later in the season and kept longer in the medium (six weeks) compared to 2004. Metallothionein concentrations in the digestive gland of mussels are of the same order of magnitude as those reported by Viarengo et al. (1997) for wild mussels collected in the NW Mediterranean. MT concentrations are not especially high in mussels from VP with high copper concentrations. It is well known that the induction of MT in mussels is not as good with copper as with cadmium and copper excess may be eliminated by the formation of a Cu-MT insoluble complex (Viarengo et al., 1988).

IBR for 2005 (Fig. 6 b) shows that, other contaminants, besides copper, are present in VP, PC and ES compared to the "reference" station IL. Comparing IBR from 2004 (Fig. 6 a) and 2005 leads to the same assumption. Bocquené et al. (2004) used the integrated biomarker response index to combine four biomarkers (AChE, GST, CAT activities and MDA concentrations, this last biomarker is termed TBARS in the present paper) and quantify the impact of "Erika" oil spill on the common mussel (*Mytilus edulis*) collected at different sites of the coast of Brittany (France). The IBR was found to be strongly related to MDA levels. Their results show that mussel populations were affected by the oil spill during the first year after the event. Broeg and Lehtonen (2006), measuring IBR in eelpout (*Zoarces viviparous*) and blue mussel (*Mytilus sp.*) populations of the Baltic Sea, reported good accordance between IBR and tissue levels of organochlorine compounds.

In the present work, stress on stress responses identified the highest polluted transplantation station but could not discriminate among other locations. IBR values allow a better discrimination and are in good agreement with copper and PCB concentrations in transplanted mussels but not with PAH concentrations. Beliaeff and Burgeot (2002) underlined that the selection of an appropriate battery of biomarkers can avoid false–negative responses obtained with a single biomarker and allow information to be summarized in the form of a multivariate data set. According to Broeg and Lehtonen (2006), due to its mathematical basis, the IBR becomes more robust when the number of biomarkers increases. The number of biomarkers included in the calculation of the IBR plays an important role affecting the "relative weight" of each biomarker in the final index value. When the set of biomarkers is relatively large, e.g. 6-8, the weight of one factor is markedly reduced compared to cases when 3-4 biomarkers are used.

In future studies, other biomarkers could be measured together with those already taken into consideration. Biomarkers responding to PAH exposure could be included in the set, EROD is the most common response to PAHs in fish, but according to Cajaraville et al. (2000) this cytochrome P450 enzyme is not as powerful in molluscs (and mussels in particular) as in fish. In this context, MXR (multi-xenobiotic resistance) protein induction could be an interesting biomarker. These proteins can be induced by polycyclic aromatic hydrocarbons such as benzo(a)pyrene BaP (Kurelec et al., 1996). A MXR protein cDNA sequence was partially cloned from *Mytilus galloprovincialis* in our laboratory (G. Damiens: MXR1 GenBank accession number bankit864536 EF137955). Semi-quantitative PCR experiments showed that the transcript expression (relative to actin) of MXR1 was increased (two-fold compared to the control) in the digestive gland of *M. galloprovincialis* exposed for one week at 100 µg BaP/L. Besides, laboratory and field experiments demonstrated that GST-pi transcripts could also be used as biomarkers of organochlorine substances in the digestive gland *of M. galloprovincialis* (Hoarau et al., 2006); a CDNA partial sequence of a GST-pi protein from *M. galloprovincialis* was cloned in our laboratory (P. Hoarau: GST-p GenBank accession number Gi:22094808 and AF527010). Nevertheless, working with biomarker gene transcript is already a sophisticated approach to biomonitoring and more simple tools such as stress on stress response and lysosomal membrane stability (Viarengo et al., 1995) may be used in a biomonitoring approach together with the biomarkers used in this study.

CONCLUSIONS

Integrated biomarker response was used in this study as one possible tool for visualization of biological effects of contaminants on mussels transplanted at different sites. This integrated approach takes the variations contained in the data set obtained in mussels transplanted in the Bay of Cannes (NW Mediterranean Sea) into account. Nevertheless, our multi-biomarker approach could not fully respond to PAHs. Combining more biomarker data may allow obtaining a higher level of integration to obtain a compact means of representing data, which could be useful to decision makers.

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Data management of biomarkers: integration of MPI as a tool for environmental pollution monitoring

by

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Biomarkers in Biomonitoring programmes

The aim of using biomarkers is to relate toxic-chemical presence in the environment to effects on living organisms. The hazard that chemicals pose to organisms is related to the toxicity of the chemical (or suite of chemicals) involved, and the degree to which the organism has been exposed (i.e. the dose which it has been subjected to over a period of time). The net result of exposure and toxicity is an effect (i.e. an endpoint), which is measurable in the case of a biomarker. Thus the most important potential function of biomarkers is to provide an early warning to impending environmental problems. When challenged by an environmental stressor or a toxic insult, organisms may respond, resulting in observable structural and/or functional changes. Ideally, results of the biomarker responses should accurately reflect the differing status of the organism, thus providing a detailed picture of its health and also the status of the surrounding environment. It is obvious that more than one biomarker is needed to accurately monitor such a situation and these data can be useful to scientists to evaluate the specificity of the responses to natural or anthropogenic changes. However, it is very difficult for the environmental manager to interpret rough results of biomarker measurements (such as increasing or decreasing enzymatic activity), to discriminate among biomarkers of exposure and biomarkers of effects and to select relevant biomarkers among a list of possible biomarkers arising from research labs. Except for some examples in limited areas and time, the translation of biochemical data into environmental information is limited, due to the difficulty to interpret the temporal and spatial extent of biomarker variation.

Monitoring programmes have generally focused on traditional methodologies for assessing contamination but biomarkers are used increasingly to assess the environment. Many studies have, however, focused on single test organisms or limited arrays of biomarker responses and, in particular, there is little published work documenting the systematic use of multiple biomarkers to assess the health condition of complex ecosystems (Adams and Ryon, 1994). Thus, the multi-biomarker approach may be considered to be similar to common procedures in human epidemiology where many responses are interpreted to diagnose disease. Different data management systems were developed in order to provide useful tools to environmental managers and to integrate biomarkers in the routine methodology for environmental monitoring. Among aquatic species, marine mussels are commonly used as sentinel organisms for the detection of environmental pollution in coastal waters due to their capacity to accumulate several organic and inorganic contaminants (Livingstone, 1991). Changes in biological parameters can be useful to scientists to evaluate the specificity of the responses to natural or anthropogenic changes, but it is very difficult for the environmental manager to interpret increasing or decreasing changes in biomarker data. Except for some examples in limited areas and time (Bayne et al., 1988; McCarthy et al., 1990), the translation of biochemical data into environmental information was limited.

Scientists from Plymouth University (UK), have developed a rapid assessment of marine pollution (RAMP) programme (Wells *et al.*, 2001). The assessment included biomarkers of cellular (cell viability, lysosomal integrity) and physiological (heart rate, condition index) status, measures of genotoxicity (micronucleus formation) and immunotoxicity (spontaneous cytotoxicity). The relationships between biological responses and environmental data were investigated in Europe and South America. The RAMP programme and its procedures have been disseminated to many countries (Moore *et al.* 1999; Bui *et al*., 2000).

A more sophisticated example of integrated biomarker data management is the utilisation of the ***Expert System***, recently developed at Di.S.A.V. (University of Alessandria, Italy) by Dagnino et al. (in press) also in the framework of the BEEP (Biological Effects of Environmental Pollutants) EU programme.

The function of the Expert System is to rank the level of the pollutant-induced stress syndrome by integrating the data obtained from early warning biomarkers of stress, biomarkers of exposure and biomarkers of toxic effects. Moreover, the expert system takes into account possible interferences among the different biomarkers.

The MPI approach

The practical approach carried out by the official agencies in charge of environmental surveys, is to establish indexes of environmental quality taking into account chemical or biological criteria, in order to classify the sites being monitored in a scale from “clean” to “highly polluted” (usually 4 for microbiological criteria, or 5 levels for chemical criteria, C.B.A.G. 1996). Rough set analysis was first used to classify sites and identify important biomarkers for specific questions (exposure, effects, environmental signification). This type of analysis is particularly useful since it is a simple and efficient method for classifying multivariable biomarker data and, furthermore, it is free from distributional assumptions. In order to standardize this approach and to give decision makers enough information expressed in a simplified form, we have developed a scoring approach (***Multi Marker Pollution Index, MPI***, Narbonne *et al.,* 1999) for biomarkers of exposure. MPI for field application was developed during the last twenty years, and specifically from the first international workshop on integrated biomarker use (UNESCO-IOC/ICES Oslo GEEP Workshop held in 1986). Design and validation of practical approaches were carried out during national or international programmes in marine waters (BIOMAR, BEEP).

An innovative approach for the organization of biomonitoring programmes that utilizes molluscs as sentinel species may be based on the use of caged organisms for a better standardisation of the results and reduction in sample variability. Caged mussels should be maintained for 4 weeks at about 4 m depth at selected sites. This period of time is sufficiently long so that the biological effects of pollutants can be revealed, at the same time minimizing possible differences in gonad development that may arise in animals maintained at different sites due to exposure to local changes in environmental parameters, such as temperature and food availability. A specific design was recently applied in the Loire river estuary.

The MPI approach is based on a simple scoring approach to provide a relative comparison among sites that exhibited multiple biomarker responses. MPI for each site is calculated as the sum of each Biomarker pollution index (BPI). Finally a pollution scale is established including five levels (from lightly to highly contaminated). The MPI of each site is converted in pollution level and associated to a colour (red, orange, yellow, green and blue for classes from 5 to 1). These colours may be reported on the map of the collected sites in order to visualize easily hot spots and the temporal changes in effects of pollution.

Biomarker selection was in part based on ANOVA analysis used to determine statistical significance of the individual biochemical variables among sites. Tukey’s test was used to determine significance for individual variables between sites to determine the integrated response of mussel to the environmental conditions at each sampling site. All the individual biomarkers were considered jointly within a multivariate context using a canonical discriminant analysis procedure (Statistica software 6.0 StatSoft, Inc. Ed., 2002). A variable selection procedure (Adams *et al*., 1999) was also used to identify and select those variables that contributed most to the discrimination among the integrated biomarker response for each site. Moreover, a scale based on biomarkers of response must be able to be sensitive to different types of pollution. Therefore five biomarkers of exposure must be selected for the scoring approach, among 5 clusters.

Contamination by Heavy metals: AChE, AOE, MT
Contamination by PAHs: EROD, BPH, GST
Contamination by Organic chemicals: GST, AOE
Contamination by Pesticides: AChE, GST, AOE
Non specific stress: NRR, Lysosomal parameters

From each cluster, biomarkers must be selected on the discriminatory power base. Thus, for each biomarker a discriminatory power was calculated by ranking analysis. As a result of discriminatory analysis, the first five powerful biomarkers GSTg, CATdg, AChEg, BPHdg, GSTdg were selected for the MPI calculation.

In field studies, mussels were collected from coastal stations either by grab or by skin divers (in water depth up to 40 m). For *in situ* field bioassays with caged bivalves, cages (500 mussels per cage) were deployed at selected sites by use of boat facilities. From our experience, the optimal exposure period was around one month.

Statistical procedure and indices calculation

Tukey's test was used to determine significance for individual variables between sites to determine the integrated response of mussels to the environmental conditions at each sampling site. All the individual biomarkers were considered jointly within a multivariate context using a canonical discriminant analysis procedure (Statistica software 6.0 StatSoft, Inc. Ed., 2002). A variable selection procedure (Adams *et al*., 1999) was also used to identify and select those variables that contributed most to the discrimination among the integrated biomarker responses for each site.

Multimarker pollution index (MPI) for each site is calculated as

$$MPI_i = \sum_{j=i} BPI$$

Where $_i$ = site, $_j$ = biomarker, BPI=Biomarker pollution index from the table of conversion (Table 1) for individual mean (X_i), related to discriminatory factor (DF) of the measure.

$DF = X_{max} – X_{min} + CI / CI$
Where X_{max} = Mean max, X_{min} = Mean min, CI = confidence interval given by Tukey's test.

Finally a pollution scale was established including five levels (from lightly to highly contaminated). The global biomarker index of each site and for each cruise was converted in pollution level and associated to a colour (red, orange, yellow, green and blue for classes from 5 to 1). These colours were reported on the map of the collected sites in order to visualize easily the temporal changes in effects of pollution.

The MPI classification scale from 1 to 5 (Narbonne *et al*., 1999) was first applied in the European programme BIOMAR.

Table 1: Index given for each biomarker response according to their rank in a scale related to the discriminatory factor.

	Discriminatory factors				
Number of levels	1	2	3	4	5
Index of response	4	10			
	3	6	12		
	2	4	7	12	
	1	2	4	8	14

Example of MPI calculations

The MPI classification scale from 1 to 5 (Narbonne *et al*., 1999) was first applied in the European BIOMAR programme in the Mediterranean Sea (Greece, France, Italy and Spain) and the Baltic Sea (Germany and Poland) (Narbonne et al. 2001), and validated during the BEEP EU programme (Italian, French and Spanish working sites; Narbonne et al., 2005). MPI was also used for monitoring the impact of ERIKA oil spill in Brittany coast during 3 years. Moreover this procedure was applied to fresh water systems both in mesocosm and field conditions, by using both molluscs (corbicula and dressenia) and in fish, in connection with Water Agencies in order to be applied in the context of the Water Framework Directive (FISH BIO in France) (Basseres el al., 2004; Vidal et al., 2001).

The MPI procedure has been now disseminated to other countries, especially from North Africa (Morroco and Tunisia), by using different mollusc species (Ruditapes or Donax in beaches ecosystems) or different biomarkers (stress on stress, lysosomal stability) (El Hamidi et al., 2003; Banaoui et al., 2004; Moukrim et al., 2004; Banni et al., 2005).

In the same way, Beliaeff and Burgeot (2002) describe a simple method summarizing biomarker responses, thereby aiding interpretation. These authors used star plots to display results for a range of biomarkers and integrated response was computed as the star plot area. The integrated response was then used to investigate spatial and temporal variation in contaminant exposure. The approach was applied to Baltic Sea and English Channel sites and ERIKA oil spill monitoring (Bocquene et al., 2004).

Relationship between biomarker of exposure (MPI) and biocenotic indexes was recently studied in fresh water mesocosms. Preliminary results indicated good correlation between early biomarker responses (at 7th day of exposure) and changes in biocenotic indexes (i.e abundance of invertebrates) at 30 days of exposure.

Practical applications

An important point in the organisation of a large biological “mussel watch” is the need to reduce the cost of the programme. It is in fact not feasible to ask environmental agencies to utilize a large number (10-20) of different biomarkers of exposure and effects on mussels sampled from each site. Moreover, the periodicity of sampling should be taken into account. The profile and number of biomarker measures may be different for weekly, monthly or annual frequency. Therefore, a 3 tiers approach is suggested:

Tier 1 - Utilization, as a first screening approach, of mortality index and a sensitive and low-cost stress biomarker such as lysosomal membrane stability and/or lysosomal lypofuscin accumulation.
Tier 2 - Utilization of a set of simple enzyme activities (AChE, GST, CAT, BPH), namely, biomarkers of exposure, associated to a biomarker of general stress. This is the base for MPI calculation.

Tier 3 - Utilization of the full battery of 10-20 biomarkers, and quantification of the stress syndrome by using the expert system. This battery includes biomarkers of exposure and biomarker of effects in order to express the biological effects of pollutants related to the accumulation of toxic chemicals in the organisms.

Conclusions and recommendations

The use of biomarkers is relatively new when compared to traditional chemical monitoring. Even today in developed nations those biomarkers that are considered well understood often still lack historic track records and simple data management adequate for routine risk assessment and monitoring. Furthermore, despite the important principle underlying the biomarker concept (that is, response should lead to ecological effects), there are still few examples where biomarker measurements have been directly linked to community level responses.

Adequate data management is now available leading to an integrated approach for biomarker selection and integration in site classification related to chemical stress, pollution level (MPI) and pollution impact (Expert System). Thus, the MPI procedure appears to be particularly adequate for monitoring trends in low or moderately polluted sites at monthly frequency. The integrated approach by Expert System is able to integrate batteries of biomarkers of exposure and toxic effects measured at different levels (from genes to tissues) and may constitute a general methodology for multimarker data management in specific areas, hot spots and pollution gradients.

In the actual situation, biomarkers provide, in a monitoring programme, what other methodologies, such as, the traditional measurement of chemicals in the environment or organisms, cannot. By their very nature, many biomarkers give information on response to pollution and this gives the environmental manager, tools for site classification related to biological responses (i.e. classification from blue to red with MPI or from A to E with Expert System). Moreover, biomarker measurements provide data that may help restrict chemical analysis only to those contaminants of concern. New tools in data management may now be possible in biomarker-based monitoring programmes and risk assessments, so that financial resources are used wisely and cost effectively. These tools are not yet applied in the MED POL programme. Reference laboratories were selected (Italy, France and Spain) for technology development and practical training. Focus laboratories in southern Mediterranean countries (actually Morocco and Tunisia) are able to perform such biomarker monitoring programmes.

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Results of the BEEP monitoring programme along the NW Mediterranean Sea using biomarkers in sentinel mussels and red mullets

by

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ABSTRACT

In the framework of the EU-funded BEEP project a biomonitoring programme was carried out along the NW Mediterranean Sea using mussels and red mullets as sentinel organisms. A battery of effect and exposure biomarkers were selected for the assessment of biological effects of pollution, namely, lysosomal responses, metallothionein induction and peroxisome proliferation. Additionally, gamete development stages and gonad index were determined with the aim of assessing possible disturbances in reproduction. The purpose of the study was to evaluate the overall health status of the organisms along three pollution gradients. As revealed by principal component analysis (PCA), the use of selected biomarkers in mussels was able to discriminate polluted and reference locations, providing an integrated view of pollutant effects. In red mullets, the reproductive status explained most of the variations in biomarker responses. Additionally, biomarker data interpretation for red mullets was more difficult than for mussels probably because mussels were collected closer to pollution sources than red mullets and due to the migration capacity of red mullets. Therefore, the use of fish caging strategies and collecting fish out of the spawning season are recommended for future biomonitoring programmes. In conclusion, the biomarker approach followed by PCA provided relevant information that can be incorporated into environmental impact assessment of the NW Mediterranean Sea.

INTRODUCTION

The goal of the European BEEP project (Biological Effects of Environmental Pollution in marine coastal ecosystems) was to evaluate the use of biomarkers in marine sentinel organisms for the assessment of biological effects of environmental pollution. With this purpose, thirty cooperating participants investigated three different types of coastal European environments (Baltic Sea, North Atlantic Sea and Mediterranean Sea). In the NW Mediterranean Sea a biomonitoring programme was carried out using Mediterranean mussels (*Mytilus galloprovincialis*) and red mullets (*Mullus barbatus*) as sentinels. Different biomarkers were investigated in mussels, including acetylcholinesterase activity (Beliaeff and Bocquené, 2004), CYP1A- and CYP3A-immunopositive protein levels (Shaw et al., 2004), malondialdehyde and benzo(*a*)pyrene hydroxylase, catalase and glutathione *S*-transferase activities (Narbonne et al., 2005) and responses of the immune system (Auffret et al., 2006). In red mullets, endocrine disruption endpoints were assessed (Martin-Skilton et al., 2006). In our laboratory, a battery of effect and exposure biomarkers recommended by UNEP/MAP (2005) was applied both in mussels (Zorita et al., 2007a) and in red mullets (Zorita et al., in press). Lysosomal membrane stability and lysosomal structural changes were measured as effect biomarkers, metallothionein (MT) induction was selected as biomarker of metal exposure and peroxisome proliferation, measured in terms of acyl-CoA oxidase (AOX) activity induction, was evaluated as biomarker of exposure to organic xenobiotics. Additionally, gamete development stage and gonad index (GI) were determined as supporting parameters with the aim of assessing possible disturbances in reproduction. Quality assurance (QA) received special attention. First, biomarker measurements were made using blind-labelled samples. Second, our research team

participated in the preparation of well-defined protocols and standard operating procedures (SOP) for biomarker measurements in the BEEP project (BEEP Final report, 2004). Third, different methods available for MT determination were compared and intercalibrated (Zorita et al., 2005) and we participated in several intercalibration exercises such as UNEP/MEDPOL intercalibration exercise for MT levels, ethoxyresorufin-*O*-deethylase (EROD) activity, lysosomal membrane stability, and neutral lipid and lipofuscin accumulation. In the present work we give an overview of the results obtained in the BEEP monitoring programme along the NW Mediterranean Sea. Further, with the aim of integrating results obtained with the different biomarkers, principal component analysis (PCA) was applied.

STUDY AREA AND MONITORING PROGRAMME

Four sampling campaigns were carried out on the French oceanographic vessel "L'Europe" in September 2001, May and September 2002 and May 2003 in three areas of the NW Mediterranean Sea. The cruises followed a route starting from the Gulf of Fos/Marseille area in France towards the Ligurian Sea in Italy and finishing in the Catalonian coast (Spain). Subtidal mussels, *M. galloprovincialis*, were hand collected by scuba divers in 12 stations selected according to an expected pollution gradient in France (Aragnon -reference site-, Lavera and Fos harbour), Italy (Portofino -reference site-, Voltri and inside Genova harbour) and Spain (Cala Montjoy -reference site-, Fangar, Alfacs and Barcelona harbour). In addition, Cortiou (which receives sewage effluents from Marseille) in France and outside Genova harbour (site where the oil tanker "The Haven" sank in 1991) in Italy were chosen as sites of special interest (Table 1). The three pollution gradients were confirmed by chemical analysis (Zorita et al., 2007a). Red mullets, *M. barbatus*, were collected by trawling in intervals of 15 min in 7 locations, being Portofino in Italy and Roses in Spain selected as reference stations (Table 1). No fish were collected in the Spanish area in May campaigns due to technical problems.

RESULTS OF MONITORING IN MUSSELS

Details of chemical and biomarker data obtained in mussels have been published recently (Zorita et al., 2007a) and are given in Table 2. Briefly, for lysosomal responses no significant differences were observed between May and September samplings. The differences recorded between stations were consistent with the pollution gradients. In general, mussels from urban-related areas like Fos, Genova and Barcelona harbours presented reduced labilization period (LP) values accompanied by high lysosomal volume density (Vv) and low lysosomal surface to volume ratio (S/V) values indicating disturbed health. Significant correlations were found between lysosomal Vv and LP (R^2 = -0.691, $p < 0.01$) and lysosomal Vv and size (R^2 = -0.880, $p < 0.01$) and this supported the idea that an increase in Vv, accompanied by the formation of pathologically enlarged lysosomes, is associated with membrane destabilization (Lowe et al., 1981; Moore, 1988; Regoli, 1992; Domouhtsidou and Dimitriadis, 2001). Significant correlations were also found between lysosomal responses and polychlorinated biphenyl (PCB), polycyclic aromatic hydrocarbon (PAH), Cu and Pb concentrations measured in the whole soft tissue of mussels collected in the same locations in May 2003 (Zorita et al., 2007a). In addition, lysosomal responses in mussels were consistent with results obtained with other biomarkers measured in the BEEP project, namely, CYP-immunopositive proteins (Shaw et al., 2004) and immunotoxic responses (Auffret et al., 2006).

Table 1: Description of sampling sites of mussels and red mullets in the NW Mediterranean Sea.

				Description
France	Aragnon	Mussel	43º18'70 N 5º04'26 E	Clean seawater, reference station
	Lavera	Mussel	43º25'00 N 4º53'25 E	Moderately polluted, near oil refineries
	Fos harbour	Mussel	43º25'00 N 4º53'25 E	Polluted, refineries and oil tankers
	Cortiou	Mussel	43º11'50 N 5º23'00 E	Sewage effluents from Marseille
	Fos-sur-mer	Red mullet	43º19'50 N 4º57'70 E	Oil refineries, shipping traffic
	Cortiou	Red mullet	43º11'76 N 5º23'24	Sewage effluents from Marseille
Italy	Portofino	Mussel	44º18'62 N 9º12'80 E	Marine reserve park
	Voltri	Mussel	44º25'12 N 8º46'65 E	Moderate industrial input, oil tankers
	Genova inside	Mussel	44º24'73 N 8º54'99 E	Harbour. Intense maritime traffic, industrial effluents
	Genova outside	Mussel	44º23'44 N 8º55'83 E	Shipwreck of oil tanker "The Haven" in 1991
	Portofino	Red mullet	44º18'36 N 9º15'93 E	Marine reserve park
	Arenzano	Red mullet	44º23'93 N 8º42'74 E	Petrochemical activities
Spain	Cala Montjoy	Mussel	42º15'00 N 3º14'50 E	Clean seawater, touristic area in summer
	Fangar	Mussel	40º46'40 N 0º45'60 E	Rice agriculture, marine farms, fishing
	Alfacs	Mussel	40º36'70 N 0º36'30 E	Rice agriculture, marine farms, fishing
	Barcelona harbour	Mussel	41º22'55 N 2º11'80 E	Intense maritime traffic, industrial effluents
	Altafulla	Red mullet	41º06'48 N 1º20'61 E	Agrochemical run-off
	Delta of Ebro	Red mullet	40º31'18 N 0º36'50 E	Agriculture and aquaculture activities
	Roses	Red mullet	42º13'47 N 3º09'94 E	Slightly urbanized, leisure area

In agreement with previous studies in the NW Mediterranean Sea, MT levels did not reveal significant differences between urban-related and clean areas possibly due to the generally low concentrations of metals in these coastal waters (Viarengo et al., 1997; Stien et al., 1998; Mourgaud et al., 2002). The most remarkable metal pollutants in some stations were lead and copper (Zorita et al., 2007a) but the inducibility of MT expression by these two metals is questionable (Raspor and Pavicic, 1991; Mourgaud et al., 2002; Dondero et al., 2005; Zorita et al., 2007b). The lack of differences in MT levels may be partly due to physiological changes caused by gamete development and food abundance in spring which contribute more to changes in MT levels than the bioavailable metal concentrations (Raspor Table 2: Results of selected biomarkers in mussels (*M. galloprovincialis*) for each station at each of the three sites during the campaigns in the Mediterranean Sea between 2001 and 2003. Data from Zorita et al. (2007a).

			LP (min)	Vv_L x 10^3 (μm^3 x μm^3)	MT (µg/mg prot)	AOX (mU/mg prot)	GI (a.u.)
Site	Station	Campaign	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
FRANCE	Aragnon	Sep 2001	21.75 ± 5.7	3.83 ± 1.9	31.5 ± 20.3	0.97 ± 0.3	1.38 ± 0.7
		May 2002	19.75 ± 1.8	3.39 ± 1.1	122 ± 50.9	3.26 ± 0.4	2.13 ± 1.8
		Sep 2002	18.25 ± 1.9	3.37 ± 1.1	30.2 ± 1.6	1.87 ± 0.6	0.86 ± 0.6
		May 2003	15.25 ± 2.1	4.66 ± 2	50.1 ± 4.5	1.82 ± 0.5	3.13 ± 0.6
	Lavera	Sep 2001	13.75 ± 1.2	3.25 ± 0.7	32.3 ± 11.1	0.96 ± 0.1	1 ± 0.5
		May 2002	9.2 ± 2.1	1.77 ± 0.8	49 ± 18	1.92 ± 0.4	3 ± 1.4
		Sep 2002	11.25 ± 2.8	4.3 ± 1.4	26 ± 2.6	0.65 ± 0.09	1.38 ± 0.9
		May 2003	8.5 ± 2.2	4.9 ± 2.2	20 ± 7.9	0.65 ± 0.09	4.44 ± 1
	Fos harbour	Sep 2001	7.75 ± 2.4	3.55 ± 0.8	24.1 ± 6.1	0.61 ± 0.3	1.88 ± 0.3
		May 2002	6.55 ± 0.9	3.67 ± 2.6	52.6 ± 39.4	1.72 ±0.1	3.7 ±0.6
		Sep 2002	6.05 ± 1.9	8.67 ± 2.4	24.2 ± 2.9	0.96 ± 0.1	1.78 ± 0.6
		May 2003	4.3 ± 1.8	11.5 ± 2.2	29.5 ± 20.5	0.96 ± 0.1	3.56 ± 0.7
	Cortiou	Sep 2001	15.75 ± 3	1.57 ± 0.3	42.9 ± 27.1	1.48 ± 0.6	2 ± 0
		May 2002	12.25 ± 2.5	1.66 ± 0.5	nd	2.81 ± 0.3	4.22 ± 1.6
		Sep 2002	12.5 ± 3.1	2.57 ± 1.1	34.9 ± 3.7	2.08 ± 0.09	0.2 ± 0.4
		May 2003	nd	nd	nd	nd	nd
ITALY	Portofino	Sep 2001	14.75 ± 5.7	3.23 ± 1.1	23 ± 11.6	0.91 ± 0.3	0.75 ± 0.8
		May 2002	20.5 ± 2.8	2.75 ± 0.5	nd	2.95 ± 0.4	4.89 ± 0.3
		Sep 2002	20 ± 1.5	1.47 ± 0.6	23.7 ± 3.9	1.87 ± 0.2	0.1 ± 0.3
		May 2003	18 ± 3.1	2.27 ± 0.3	90.1 ± 10.4	1.87 ± 0.3	3.4 ± 0.5
	Voltri	Sep 2001	9.75 ± 1	3.32 ± 0.9	23.1 ± 5.3	0.64 ± 0.1	0.4 ± 0.5
		May 2002	15.25 ± 2.7	3.43 ± 1.2	41.2 ± 27.3	2.51 ± 0.7	3.33 ± 0.5
		Sep 2002	7.25 ± 3.3	6.98 ± 5.7	35.2 ± 2.6	1.19 ± 0.1	0.75 ± 0.7
		May 2003	10.25 ± 3.2	5.94 ± 1.8	54.4 ± 9.2	1.19 ± 0.2	3.8 ± 0.6
	Genova inside	Sep 2001	10.75 ± 3.6	7.04 ± 2.4	40.6 ± 21.7	0.76 ± 0.2	1.44 ± 1
		May 2002	7.15 ± 1.1	3.44 ± 1.5	30.3 ± 5.7	1.81 ± 0.5	4 ± 0.8

		Sep 2002	6.75 ± 3	8.42 ± 3.5	30.8 ± 15.2	0.79 ± 0.1	1 ± 1.2
		May 2003	5.25 ± 2	6.82 ± 3.8	62.2 ± 26.4	0.79 ± 0.1	4.2 ± 0.7
	Genova				35.5 ±	0.42 ±	0.63 ±
	outside	Sep 2001	16.25 ± 3.6	2.54 ± 1.1	13.5	0.04	0.5
		May 2002	14 ± 3.4	2.84 ± 0.4	48 ± 17.3	3.46 ±0.5	4.3 ± 0.8
		Sep 2002	11 ± 3.8	3.97 ± 1.2	36.6 ± 3.3	1.15 ± 0.2	0.6 ± 0.7
		May 2003	10 ± 3	2.85 ± 0.7	76 ± 45.6	1.15 ± 0.2	3.78 ± 0.8
	Cala					0.95 ±	
SPAIN	Montjoy	Sep 2001	29.25 ± 7.6	1.97 ± 0.7	40 ± 1.3	0.09	nd
		May 2002	nd	nd	nd	nd	nd
		Sep 2002	27.1 ± 4.4	1.2 ± 0.2	25 ± 3.4	1.93 ± 0.5	1.22 ± 0.4
		May 2003	18.25 ± 1.8	1.41 ± 0.4	51.6 ± 8.4	1.93 ± 0.6	4.22 ± 0.6
	Fangar	Sep 2001	17.25 ± 1.8	2.26 ± 1.1	46.2 ± 3.7	0.99 ± 0.1	0.57 ± 0.7
		May 2002	nd	nd	nd	nd	nd
		Sep 2002	20 ± 2.9	3.11 ± 1.2	24.5 ± 3.3	0.59 ± 0.07	1.86 ± 0.3
		May 2003	18.25 ± 2.4	2.18 ± 0.5	127 ± 105	0.59 ± 0.07	4.7 ± 0.4
	Alfacs	Sep 2001	14.25 ± 2.5	1.83 ± 1	32 ± 21.4	0.86 ± 0.2	0.89 ± 0.6
		May 2002	nd	nd	nd	nd	nd
		Sep 2002	13.5 ± 2.4	4.77 ± 1.1	25.7 ± 4.6	0.79 ± 0.2	0.67 ± 0.8
		May 2003	16.75 ± 2.2	2.1 ± 0.4	78.8 ± 17.5	0.79 ± 0.2	4.6 ± 0.5
	Barcelona				35.6 ±	0.73 ±	0.88 ±
	harbour	Sep 2001	8.25 ± 1.8	5.39 ± 2	11.1	0.09	0.8
		May 2002	nd	nd	nd	nd	nd
		Sep 2002	5 ± 2.8	6.97 ± 3.7	36.5 ± 2.6	0.81 ± 0.1	0.5 ±0.8
		May 2003	5.7 ± 1.3	6 ± 1.4	33.3 ± 17.6	0.81 ± 0.1	4.7 ± 0.4

LP: labilization period, Vv_L: lysosomal volume density, MT: metallothionein content, AOX: acyl-CoA oxidase activity, GI: gonad index, a.u.: arbitrary units, nd: not determined, SD: standard deviation

et al., 2004). Accordingly, MT levels were significantly correlated with gonad index (R^2 = 0.454, p < 0.01) indicating that MT levels fluctuated following the same pattern as gamete development. The analysis of expression of specific MT isoforms could give rise to more reliable data as it is known that specific MT isoforms may accomplish distinct functions and may be differentially regulated in presence of different metals (Zorita et al., 2007b).

Peroxisomal AOX activity did not significantly differ between May and September samplings although a clear seasonal fluctuation with maximum values in spring was observed in mussels from the Gulf of Biscay (Cancio et al., 1999). The highest levels of AOX activity were found in the reference sites and in Cortiou (Table 2) whereas the lowest AOX activities were recorded in stations with moderate/high concentrations of PAHs and PCBs, such as Fos, Genova and Barcelona harbours. Interestingly, AOX values were negatively correlated with Cu (R^2 = -0.750, p < 0.05) and Hg (R^2 = -0.718, p < 0.05) levels measured in mussels collected in May 2003, which may suggest that metals found in polluted stations might inhibit the activity of peroxisomal AOX (Zorita et al., in 2007a). Alike, these two metals are known to produce inhibitory effects on EROD activity (Oliveira et al., 2004). Similarly, Narbonne et al. (2005) reported decreases in several enzyme activities in most polluted stations. Thus, the observed inhibition of AOX activity in polluted stations could be due to complex interactions occurring in the Mediterranean environment among mixtures of pollutants.

As to gamete development stages, mussels sampled in May showed mature-spawning-post-spawning gonads while mussels collected in September showed gonads in resting and gametogenesis stages (Zorita et al., 2007a). No gross gonad alterations were observed suggesting undisturbed reproductive potential of mussels in the studied areas.

RESULTS OF MONITORING IN RED MULLETS

Results obtained in red mullets have been described in detail (Zorita et al., in press) and are given in Table 3. Lysosomal membrane LP values did not differ between May and September samplings but significant differences were recorded among stations. In general, fish collected in Fos-sur-mer, Cortiou, Arenzano and Delta of Ebro showed reduced LP values indicating disturbed health (Table 3). The low LP values in Fos-sur-mer, Cortiou and Arenzano could be attributed to the impact of pollutants from urban and industrial activities common in the area (Burgeot et al., 1996; Piccardo et al., 2001; Bodin et al., 2004), while the low values recorded in Delta of Ebro could be linked to agricultural and aquaculture activities (Burgeot et al., 1996; Baumard et al., 1999). In the case of fish from Portofino, considered a reference area, a significant decrease of the lysosomal membrane stability was observed in the last two samplings suggesting signs of cellular damage (Zorita et al., in press).

MT levels did not differ significantly in organisms sampled in different stations (Table 3). Except for the high MT values found in Portofino in May 2003, MT levels could be considered low. Generally, MT values obtained in May were higher and more variable than in September (Table 3). The higher MT concentrations observed in May could be linked to the drastic increase in hepatic Zn levels during vitellogenesis, which is followed by the up-regulation of MT transcription (Olsson et al., 1987; Werner et al., 2003) suggesting that the protein is involved in Zn regulation during vitellogenesis (Olsson et al., 1989). This hypothesis is further supported by the significant correlation observed between MT levels and GI (R^2 = 0.628, p < 0.01).

AOX activity was generally higher in May than in September in all stations except in Cortiou, which showed high values all along the study indicating a high bioavailability of organic xenobiotics in red mullets from this station (Burgeot et al., 1996; Porte et al., 2002; Martin-Skilton et al., 2006). AOX activity was significantly correlated with GI (R^2 = 0.534, p < 0.05) suggesting that peroxisomal AOX activity fluctuated according to the reproductive status of

Table 3: Results of selected biomarkers in red mullets (*M. barbatus*) for each station at each of the three sites during the campaigns in the Mediterranean Sea between 2001 and 2003. Data from Zorita et al. (in press).

			LP (min)	MT (µg/mg prot)	AOX (mU/mg prot)	GI (a.u.)
Site	Station	Campaign	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
FRANCE	Fos	Sep 2001	nd	6.32 ± 5.6	2.49 ± 0.5	3 ± 1.1
		May 2002	11 ± 3.8	15.9 ± 7.9	3.53 ± 0.6	3.33 ± 0.5
		Sep 2002	15.75 ± 3	6.75 ± 2.2	3.22 ± 0.4	3.42 ± 0.9
		May 2003	nd	nd	nd	nd
	Cortiou	Sep 2001	14 ± 3.8	5.81 ± 4.4	6.64 ± 1.8	3.11 ± 1
		May 2002	9 ± 1.6	10.9 ± 2.72	5.89 ± 0.1	4.14 ± 0.6
		Sep 2002	9.75 ± 2.9	7.96 ± 1.1	6.21 ± 0.6	2.44 ± 1
		May 2003	4.75 ± 1.2	17.1 ± 10	5.35 ± 1.4	3.25 ± 0.4
ITALY	Portofino	Sep 2001	18.75 ± 1.9	3.81 ± 1.9	3.50 ± 0.7	2.66 ± 0.8
		May 2002	24 ± 5.1	36.6 ± 24.2	6.77 ± 1.6	4 ± 0
		Sep 2002	7.5 ± 0.8	5.63 ± 1.3	4.28 ± 0.2	2.1 ± 0.3
		May 2003	10.5 ± 1.4	108.48 ± 69.5	5.36 ± 0.5	3.68 ± 0.2
	Arenzano	Sep 2001	nd	4.17 ± 2.6	2.82 ± 0.3	2.87 ± 0.6
		May 2002	9.5 ± 1.4	34.5 ± 25.8	6.41 ± 0.8	4 ± 0
		Sep 2002	14.25 ± 4.2	4.38 ± 0.5	4.78 ± 0.3	2.5 ± 0.8
		May 2003	9.5 ± 2.1	34.0 ± 18.8	5.69 ± 1	3.5 ± 0.2
SPAIN	Altafulla	Sep 2001	21 ± 4.2	3.99 ± 1.1	2.62 ± 0.1	2.33 ± 0.5
		Sep 2002	25.25 ± 6.8	8.25 ± 0.4	4.68 ± 0.4	2.44 ± 0.8
	Delta of Ebro	Sep 2001	12.5 ± 2.6	3.60 ± 1.2	2.39 ± 0.2	2.85 ± 0.3
		Sep 2002	9.75 ± 1.6	6.20 ± 1.4	3.29 ± 0.4	2 ± 0
	Roses	Sep 2001	nd	3.72 ± 2.4	3.04 ± 0.4	nd
		Sep 2002	25.25 ± 5.4	6.12 ± 0.6	3.15 ± 0.4	2.2 ± 0.6

LP: labilization period, MT: metallothionein content, AOX: acyl-CoA oxidase activity, GI: gonad index, a.u.: arbitrary units, nd: not determined, SD: standard deviation

fish. Season related variations in the peroxisomal compartment have been detected in fish and have been attributed to variations in the reproductive cycle and food availability (Rocha et al., 1999).

Delayed gamete maturation in females and high prevalence of melanomacrophage centers in male testis was observed in fish from Cortiou and Fos-sur-mer (Zorita et al., in press). These results are in agreement with those of Martin-Skilton et al. (2006) who partly attributed these changes to the high levels of alkylphenols, PCBs and other organochlorinated compounds measured in red mullets (Porte et al., 2002; Martin-Skilton et al., 2006).

PRINCIPAL COMPONENT ANALYSIS

With the aim of integrating results obtained with the different biomarkers and to discriminate between the studied stations, principal component analysis (PCA) was applied using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Mean values for each variable (biomarker) and experimental group were used in the analysis. Eight variables were taken into consideration for mussels: GI, MT, lysosomal Vv, Sv, S/V, and Nv, LP and AOX whereas for fish 4 variables were examined: GI, MT, LP and AOX. Since the unit rank of measurements of the variables differed, data were standardized using the *Z* score method prior to the analysis.

For mussels, the first two components from PCA explained 66.72% of the total variance as shown in Fig. 1. Component 1 explained 42.13% of total variance and was characterized mainly by high factorial weight of lysosomal Vv, Sv, size and membrane stability (Table 4). Component 2 explained 24.59% of total variance and was characterized by high factorial weight of GI and lysosomal Nv (Table 4). Overall, component 1 appeared to indicate health condition of musssels and distributed the studied stations according to the pollution gradient (Fig. 1). Indeed, most of the lysosomal responses followed the pollution gradient and were significantly correlated with PAH, PCB, Cu and Pb concentrations determined in mussel tissues (Zorita et al., 2007a). Component 2 appeared to indicate reproductive condition of mussels and separated the localities according to the sampling season, mussels collected in May campaigns being easily separated from those sampled in September campaigns (Fig. 1).

Thus, PCA was a powerful tool able to discriminate impacted sites from reference sites, as previously shown by other studies (Pampanin et al., 2005; Kopecka et al., 2006). On the other hand, PCA indicated that lysosomal responses (lysosomal Vv, Sv, S/V and lysosomal membrane stability) were the most discriminating among selected biomarkers. Beliaeff and Boquené (2004) reached the same conclusion in a study including data on acetylcholinesterase activity, malondialdehyde and our data on lysosomal membrane stability. Similarly, in the 2-tier biomarker approach proposed recently for ranking of environmental health status, lysosomal membrane stability is given a central role as first screening tool to obtain general health information (Viarengo et al., 2000; Broeg et al., 2005). In this respect, our results support the use of indexes based on lysosomal responses since they are able to discriminate the stations according to the pollution gradient indicating disturbed health at the most impacted sites.

Table 4. Factor analysis: component matrix for mussel data.

Biomarker	Component 1	Component 2
GI	0.035	-0.823
MT	0.380	-0.547
Vv	-0.939	-0.124
Sv	-0.887	0.244
S/V	0.807	0.476
Nv	-0.096	0.801
LP	0.802	0.141
AOX	0.503	-0.166

GI: gonad index, MT: metallothionein content, Vv: lysosomal volume density, Sv: lysosomal surface density, S/V: lysosomal surface to volume ratio, Nv: lysosomal numerical density, LP: lysosomal membrane labilization period, AOX: acyl-CoA oxidase activity

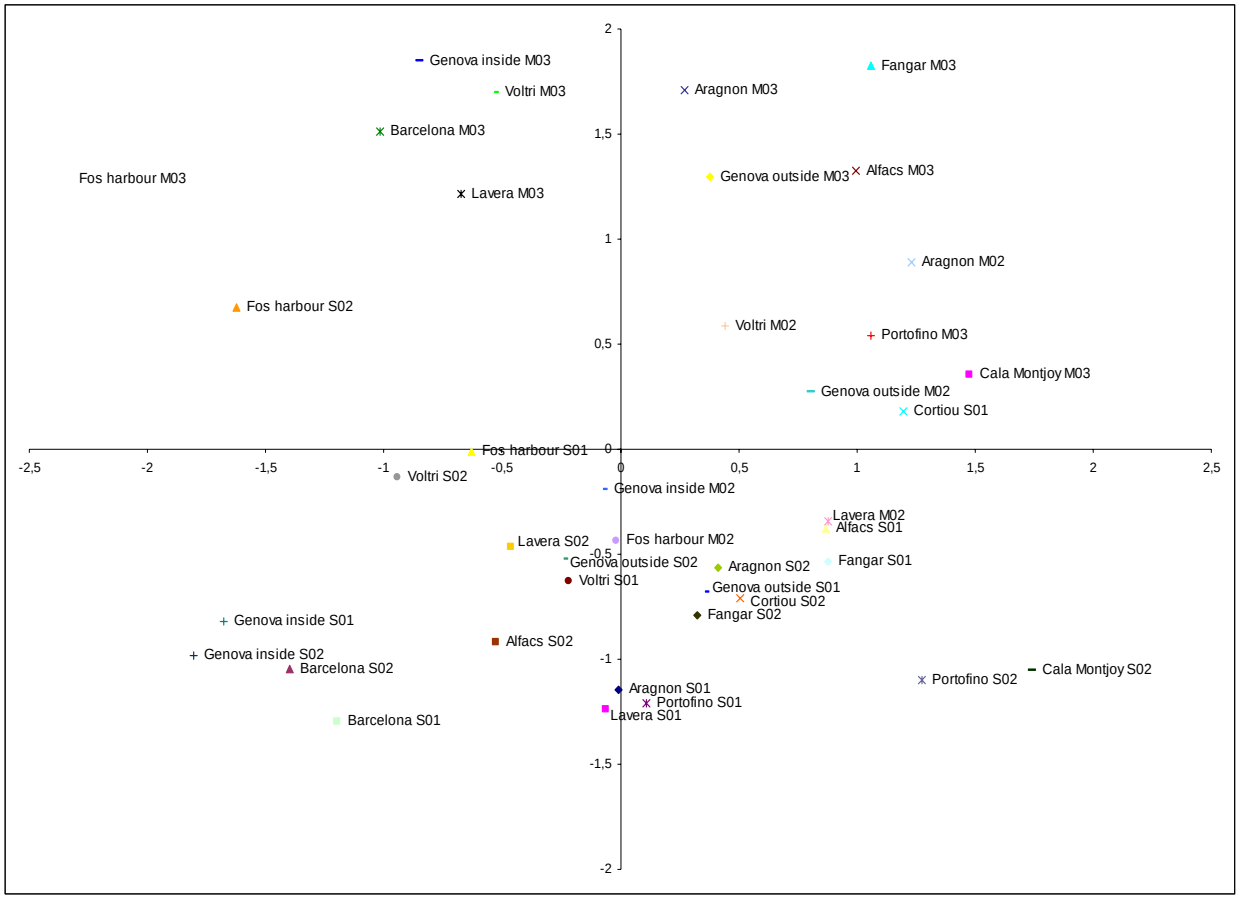

Fig. 1: Principal component analysis of the biomarker data set for mussels. The factorial weights of the biomarkers are plotted against the first two components which explain 66.72% of the total variance. Note that reference and polluted stations are well separated along component 2. Component 1 appears to discriminate May and September samplings. S01: September 2001, M02: May 2002, S02: September 2002, M03: May 2003.

When data on red mullets were analysed only one component was extracted from the PCA and therefore component plots were not produced. This component explained 52.45% of the total variance and GI was the variable producing the highest factorial weight (Table 5). This result indicated that biomarkers in red mullets were more affected by the reproductive status than by the pollution gradient suggesting that more reliable data could have been obtained by collecting fish in other periods. In this sense, it has been proposed to collect fish out of the spawning season and if possible with gender discrimination (Zorita et al., in press) because these physiological variables may affect biomarker responses (Rocha et al., 1999; Marijic and Raspor, 2006). Thus, comparing mussel and fish results, it appears that biomarker data is more difficult to interpret in fish than in mussels. This could be partly explained by the fact that mussels were collected closer to pollution sources whereas red mullets were sampled in open coastal waters, where pollutant concentrations could be diluted. In addition, the migratory capacity of fish needs also to be taken into consideration. Overall, there is a need for further studies on the assessment of biological effects of contaminants in coastal marine ecosystems with the use of caging strategies. These strategies are a general trend in the design of biomonitoring programmes in the last years not only for fish (Teles et al., 2004; Vermeirssen et al., 2005) but also for mussels (Stien et al., 1998; Regoli et al., 2004; Orbea and Cajaraville, 2006).

CONCLUSIONS

In this study carried out along the NW Mediterranean Sea, differences observed in a battery of biomarkers in mussels were caused mainly by differential exposure to pollutants among locations. Lysosomal responses were the most discriminating biomarkers and indicated disturbed health at the most polluted sites. Seasonal variations, especially those related to

Table 5. Factor analysis: component matrix for fish data.

Biomarker	Component 1
GI	0.848
MT	0.743
LP	-0.454
AOX	0.788

GI: gonad index, MT: metallothionein content, LP: lysosomal membrane labilization period, AOX: acyl-CoA oxidase activity.

gamete development, also contributed partly to observed differences in biomarker data. For red mullets, biomarkers were influenced more by the reproductive status than by the pollution gradient. These differences between mussels and red mullets probably reflect that mussels were collected closer to pollution sources than red mullets and could be also due to the migration capacity of fish. This, in addition to difficulties for native fish sampling, makes it recommendable the use of fish caging strategies and collecting fish out of the spawning season for future biomonitoring programmes. Finally, PCA constitutes an easy and useful tool to integrate biomarker results and to identify main sources of variability in studied data.

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Lessons learned from Beep and MED POL on the biomarker use: application on the Adriatic and Algerian coasts

by

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INTRODUCTION

The health status of coastal marine environments and transitional waters, exposed more than others to the consequences of domestic and industrial discharges, is a major concern at international level. At the same time, the monitoring of these ecosystems is rather difficult because of the high variability of chemical and physical parameters that also limits the powerness of chemical and ecotoxicological analysis. For these and other reasons, the scientific community suggests new tools for environmental monitoring, addressed to as biomarkers. These are biochemical, physiological or histological indices that, measured in sentinel organisms exposed to a contaminated environment, reflect the exposure or the effects of pollutants at the molecular, cellular or organism level. Biomarkers do not substitute but integrate the more conventional analysis, providing an early warning of environmental contamination. Moreover, they give a specific contribution for the identification of toxicity due to a mixture of contaminants, although each present at very low concentration.

Scientific research contributed to the identification of biomarkers and sentinel organisms. The lessons learned from international environmental biomonitoring programmes such as MED-POL (UNEP/MAP, 1997; http://www.unepmap.gr/) and BEEP [http://www.lptc.u-bordeaux.fr/beep] indicated that different animals have different sensitivity to the same stress stimuli, and that not all of the observed alterations are consistent with the extent of environmental contamination. As a consequence, some animals better than others can be used as sentinel organisms; similarly, only a number of selected biochemical, physiological or histological alterations can be addressed to as biomarkers.

As a result of many years of research and application, biomonitoring through biomarkers has become a reliable tool for the systematic evaluation of environmental health (Cajaraville et al., 2000). The *Expert System* for data interpretation recently developed (Viarengo et al., 2000; Viarengo et al., 2007 in press) has contributed towards this.

The Adriatic coast experience

Many reports are indeed available which confirm the successful application of biomarkers in fresh and seawaters. However, their suitability in changeable environments such as coastal lagoons is poorly documented (Nasci et al., 2002). Therefore, a battery of biomarkers selected from those suggested by the BEEP and MED-POL experience was applied to evaluate the quality of a coastal lagoon, the Pialassa Baiona.

The Pialassa Baiona is a brackish marsh located in the north-eastern part of Ravenna (Italy). It consists of small, shallow ponds and deeper artificial channels connected to the North Adriatic Sea through the Candiano Channel (Fig. 1). The basin receives fresh waters from five main channels draining agricultural run off and effluents from industrial and municipal treatment plants. Generally, deposition and remobilisation of pelitic material are promoted by weak water dynamics. The actual geomorphology of this lagoon is mainly the result of anthropogenic activity and its survival depends on a delicate balance between preservation issues (e.g., Ramsar Convention) and human impacts on the area (Amorosi et al., 2002). During the period 1957-1977 about 100-200 t of mercury were delivered in this area, coming from an industrial discharge channel which inflows in the southern

part of the lagoon. Previous investigations (Miserocchi et al., 1994; Fabbri et al., 1998; Trombini et al., 2003) showed the presence of high levels of mercury in surface and suspended sediments, despite the expected burial of the toxic element based on a crude estimation of sediment accumulation rate. High levels of polycyclic aromatic hydrocarbons (PAH) and heavy metals are also found in the sediments (Fabbri D et al., 2006; Matteucci et al., 2005).

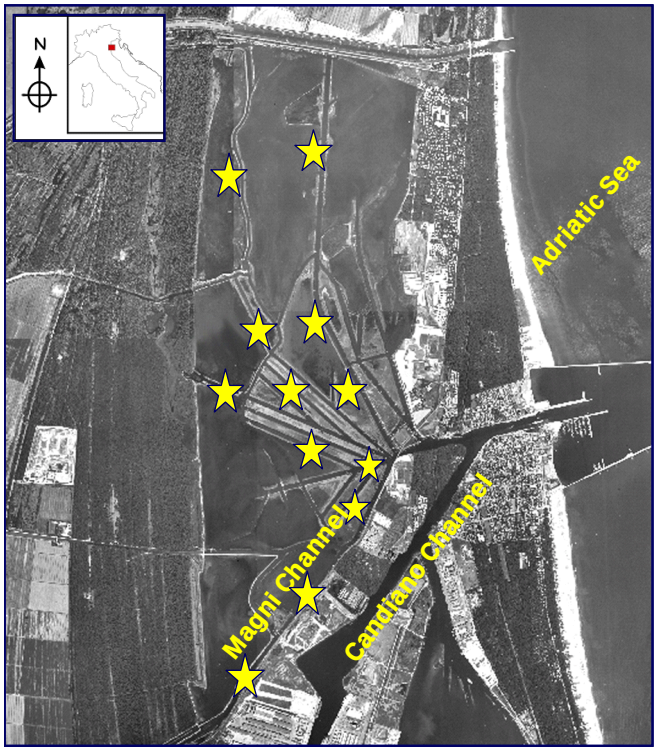

Figure 1: The Pialassa Baiona (Ravenna, Italy). White stars identify the sites of sampling.

Mussels (*Mytilus* spp.) have been employed for biomonitoring purposes for many years (Viarengo and Canesi, 1991; Romeo et al., 2003) but it was interesting to test their suitability within the peculiar environment of the Pialassa Baiona, even though resident mussels are present only in selected areas of the lagoon. On the other hand, Manila clams *Tapes philippinarum* are widely distributed within the lagoon, and partially exploited for commercial purposes; the possibility that these clams could be used as sentinel organisms for biosurveillance programmes was also investigated. Furthermore, the biomarker response was assessed in the clam *Chamalea gallina*.

Three surveys have been carried out every two years since 2000; partial results have already been reported in Caselli et al. (2003b) and Caselli and Fabbri (2006). Transplanted organisms (*M. galloprovincialis* and *C. gallina*) were exposed to the lagoon environment for 4 weeks in a total of 12 sites along the main channels. Mussels collected along the coast of the North-Western Adriatic Sea by professional fishermen were selected on a size basis (5 – 6 cm length) and transferred into cages. Cages were built for the purpose with steel and plastic nets and had two levels, one to keep clams in contact with the sediments, another one to keep mussels at a distance of about 50 cm from the sediments.

The digestive glands and gills of reference organisms were excised immediately after their collection at sea for *M. galloprovincialis* and *C. gallina*, then stored at –80°C until analysed. *T. philippinarum* were resident in the lagoon. From previous surveys, no clean site was identified within the lagoon to be used as internal reference site.

The battery of biomarkers selected was composed as follows: two biomarkers were selected according to specific problems affecting the lagoon, i.e. metallothioneins, induced by heavy metals (Viarengo et al., 1997) and acethylcholinesterase (AChE) activity, inhibited by pesticides (Valbonesi et al., 2003). Three further biomarkers were chosen among the most sensitive responses to generic stress factors i.e. not induced by a specific class of contaminants: lysosomal membrane stability, lipofuscin and unsaturated neutral lipid accumulation (Domouhtsidou and Dimitriadis, 2001; Marigomez et al., 2005). To contribute to a question recently posed within the BEEP Project regarding the possible relationship between the cAMP levels and the environmental pollution (Dailianis et al., 2003), we also evaluated the adenylyl cyclase activity in the gills of the bivalves used as potential sentinel organisms. The methodologies for biomarker analysis were in general those recommended by the MED POL Programme for the biomonitoring of Mediterranean coastal areas (Manual UNEP/RAMOGE, 1999). AChE activity was assessed spectrophotometrically as reported by Valbonesi et al. (2003). The adenylyl cycase activity was assessed as recently reported (Fabbri and Capuzzo, 2006).

According to Miserocchi et al. (1990, 1994) contamination by mercury is widely distributed within the lagoon, with maximum levels of about 160 mg/kg of superficial sediments, and up to about 1100 mg/kg at 80 cm of depth, in the southern area (Magni channel). A north – south pollution gradient was described by several authors (Fabbri et al., 1998; Trombini et al., 2003). Various studies reported the sediment distribution of other heavy metals such as As, Pb, Cd, Cr, Cu, and Zn (Ballardini et al., 1994), and the presence of polycyclic aromatic hydrocarbons (Fabbri et al., 2006).

As an overall, the battery of biomarkers analysed in *M. galloprovincialis* transplanted for four weeks in different sites of the Pialassa Baiona provided evidence for the low environmental quality of the lagoon. In particular, mussels from the surveys performed in different years highlighted the critical health status of the southern part of the lagoon, namely Canale Magni.

Lysosomal membrane stability was lower in transplanted mussels with respect to reference mussels, and in particular the value decreased in agreement with the north-south increasing pollution gradient (Hg, PAH) measured in previous surveys (Fabbri et al., 1998; Trombini et al., 2003), reaching minimum values of 3-5 min *vs* reference values of 20-25 minutes. Neutral lipid and lipofuscin accumulation showed similar trends, with a higher accumulation in the southern sites. Animals transplanted along the Magni Channel showed a high percentage of mortality up to 100% in the sampling of September 2003. These responses were not surprising, since this channel collects and carries to the Pialassa wastewaters from the industrial area of the town of Ravenna. On the other hand, metallothionein levels in the digestive glands of transplanted mussels were generally higher than those found in reference mussels apparently without a direct correlation with the reported north-south pollution gradient. Recent data on sediment geochemistry indicated that heavy metals (Al, Cu, Cr, Mn, Mg, Ni, Pb, Zn, etc.) are diffused throughout the lagoon without following a specific gradient (Donnini et al., submitted). Levels of AChE activity were in general lower during the late spring samplings, however, more data are needed to establish a true correlation between the enzyme inhibition and the possible pesticide discharge (Caselli et al., 2003a).

As to the use of *T. philippinarum* as a sentinel organisms, the same suite of biomarkers was utilized, and resident clams were collected from four sites within the lagoon. Unexpectedly, AChE activity of *T. philippinarum* was very low, ranging from 0.3 to 0.8 nmol min^{-1} mg^{-1} at the average concentration of acetylthiocholine tested, 1 mM. This low activity was reported by our laboratory in

different tissues of *T. philippinarum* (gills, adductor muscle, digestive gland, mantle and haemolymph), in the presence of different concentrations of three substrates (acethylthiocholine, butyrylthiocoline and propionylthiocholine), and assessed at various protein concentrations, pH values and incubation times (Valbonesi et al., 2003; Caselli and Fabbri, 2006). A maximum ChE activity of 2 nmol min^{-1} mg^{-1} protein was detected in extracts from the adductor muscle (Valbonesi et al., 2003). Considering that the effect of pesticides on this enzyme is inhibitory, a well detectable basal activity is essential for this biomarker. So, from these data it appears that AChE activity cannot be used as a biomarker in a hypothetical survey using *T. philippinarum* as sentinel organism. Among the biomarkers related to the lysosome activity, *T. philippinarum* digestive glands did not display accumulation of neutral lipids, other important biomarker not suitable in this case; the content of lipofuscin appeared instead in the average. The levels of metallothioneins did not change among samples from different sites, from north to south; however. digestive glands of *T. philippinarum* resident in the lagoon showed an average content of metallothionein of about 70 µg/g tissue, while individuals collected at sea showed levels of about 40 µg/g tissue (Caselli at al., 2003b). The areas being rather different, the increase in metallothionein content cannot be ascribed to the heavy metals present in the lagoon. Taken together, these results led to the conclusion that the use of *T. philippinarum* for biomonitoring programmes is indeed limited by the low or ungraduated responses to certain stimuli, at least when employing the present battery of biomarkers.

Searching for another potential sentinel organism living in direct contact with the sediments, we studied the responses of C*. gallina* exposed to the lagoon environment. Only one paper to our knowledge deals with the use of *C. gallina* for biomonitoring (Viarengo et al., 1998). In agreement with the suggestions from MED-POL and BEEP, animals were collected at sea, caged and exposed to the lagoon for four weeks. Metallothioneins levels did not increase significantly with respect to the reference clams (not exposed to the lagoon). This result was unexpected since high levels of contaminants were reported in the lagoon sediments, in particular in the southern area. The basal AChE activity was about 2 nmol min^{-1} mg^{-1} therefore lower than that of other bivalves such as mussels or oysters (Valbonesi et al., 2003), although higher than that evaluated in *T. philippinarum* (Caselli and Fabbri, 2006); individuals transplanted in different sites did not show different activities when compared to the reference organisms. Neutral lipid and lipofuscin accumulation measured in digestive glands of *C. gallina* transplanted in the lagoon were significantly reduced with respect to the reference animals. In particular, it seemed that the response was the opposite of that of mussels, i.e. in those sites where the mussels showed significant accumulation of neutral lipids and lipofuscins with respect to reference mussels, *C. gallina* showed a significant reduction of their content.

A great number of studies reported that lysosomal membrane stability is the biomarker that better accounts for the quality of the environment. Also in this case, the responses of mussels and clams were not in agreement, in particular the lysosomal membrane stability evaluated in the digestive glands of clams was medium-high (15 min) in sites where the LMS of mussels was low (5 min). It is not surprising that the response of the two species is different since clams mostly reflect the sediment quality and mussels the water quality. We also have to keep in mind that the two species utilize different feeding mechanisms and display a number of other physiological and behavioral differences. However, we observed that according to the diagnosis based on the *C. gallina* responses the Pialassa Baiona environment would appear to be of a better quality than that indicated by the mussels, and not so related to the situation indicated by numerous chemical and geochemical surveys carried out in the lagoon.

Adenylate cyclase activity was measured in the gills of mussels and clams, exposed or not exposed to the lagoon (see Table I). It was observed that the enzyme activity was similar in exposed and reference organisms and that exposing organisms to different sites of the lagoon did not significantly modify it. The intracellular levels of cAMP are the result of equilibrium between synthesis (adenylyl cyclase) and degradation (phosphodiesterase) enzymes. Therefore, the present data are not in disagreement with those reported along the Greek coast (Dailianis et al., 2003) since we cannot exclude that cAMP levels are influenced by environmental stress factors independently from adenylyl cyclase. Since cAMP has an important role in several physiological

processes in bivalves, especially in the modulation of energetic resources for gonad development (Diaz Enrich et al., 2003), the possible impairment of its levels in contaminated environments deserves further investigations.

Species	Tissue	Basal	Average in exposed organisms
M. galloprovincialis	Gills	5.3 ± 0.8 n=20	4.8 ± 0.7 n=40
T. philippinarum	Gills	9.8 ± 1.1 n=20	9.6 ± 1.9 n=40
C. gallina	Gills	8.3 ± 0.7 n=20	9.0 ± 1.8 n=40

Table I - The adenylate cyclase activity in bivalves exposed to the Pialassa Baiona environment. (Adenylate cylcase activity is expressed as pmoles cAMP formed/10 min /mg protein)

The present data, integrated with those from chemical and geochemical surveys carried out in the lagoon, indicate that the mussel *M. galloprovincialis* is a suitable sentinel organism that may provide information in a rapid and clear manner about the quality of a brackish coastal lagoon, as it does in marine waters (Romeo et al., 2003). In particular, lysosomal membrane stability decreased following the well demonstrated increasing north-south levels of organic pollutants, reaching the minimum values in the southern part of the lagoon where also episodes of thermal stress have been registered. The Pialassa Baiona is in fact the site of discharge of industrial cooling water (500-600 million cubic meters per year) from two power plants. Temperatures as high as 35°C have been reported in the southern part of the lagoon (Bazzi, 1991) along the Magni channel, and recent surveys demonstrated that the average temperature in the southern area is maintained up to 10°C higher than in the surrounding areas of the lagoon (Donnini et al., 2003).

Due to the scanty information about the use of *T. philippinarum* as sentinel organism for biomarker analysis, it can only be assumed that this species has a low sensitivity to the environmental contamination. This clam is an allochthonous bivalve species that rapidly expanded along the North Adriatic Sea (Italy). Among other reasons, its rapid development has been ascribed to a high tolerance to environmental changes, resistance to parasites and to pollutants (Sorokin and Giovanardi, 1995). It may be hypothesised that some adaptive mechanisms may reduce the environmental effects on biochemical and cytological responses of this organism. However, only limited attention has been given to the understanding of the regulation of physiological functions possibly related to the ecological success of the clam *T. philippinarum*, and therefore more work is needed in this regard.

As to *C. gallina*, more information is needed before this organism can be used for the biomonitoring of coastal lagoons. A previous report (Viarengo et al., 1998) highlighted the suitability of *C. gallina* for analysing the environmental quality of marine environment; but on the basis of our data it can be speculated that the peculiar features of the brackish lagoon influence the responses of this organism so as to be inconsistent with the contamination reality.

These results fully support the conclusions reached by the BEEP and MED POL Programmes, i.e. that caged mussels represent the most suitable sentinel organisms and that the reduction of lysosomal membrane stability is indeed the biomarker to take into consideration as the first evidence of environmental degradation.

The Algerian coast experience

Some laboratories of CIRSA (University of Bologna, Italy) are presently involved in the Project AMIS (Algerian coast Management through Integration and Sustainability) financed by the European Union within the framework of the Short and Medium term Action Programmes (SMAP) for the protection of the Mediterranean Environment. The SMAP Programme ideally started at the Conference of Barcelona (1995) when a new Partenariat was established between the European Community and 12 East/South Mediterranean Countries (Algeria, Cyprus, Egypt, Israel, Jordan, Lebanon, Malta, Morocco, Syria, Tunisia, Turkey, Palestine Authority*)* recognizing the importance of matching the economical development with the environmental protection. Definitively approved at the Euro-Mediterranean Conference on the Environment held in Helsinki (1997), the SMAP Programme led so far to three calls for funding, SMAP I, II and III. Five action priorities are identified within the SMAP Programme, which represent some of the most important environmental challenges, namely Water management; Waste management; Hot spots, Integrated Management of Coastal Zones and Desertification. SMAP I and II Projects were addressed to all the above priorities; differently the SMAP III Projects are focused entirely on the Integrated Management of Coastal Zones (ICZM) to take advantages from the simultaneous action carried out by the 8 Projects financed by the EU on the east-southern Mediterranean coast, strengthening the Euro-Mediterranean collaboration and the cross-fertilization among projects, and hopefully the capacity building at the regional scale.

The overall objective of the action of the Project AMIS is to contribute to the protection of the natural environment, to a sounder use of resources, and a more sustainable development of coastal zones in the Mediterranean through the development of an ICZM plan for the Wilaya d'Alger. The development of an ICZM plan is a complicated issue that involves several components of the environment (Fig. 2) that are indeed explored in the AMIS Project implementation.

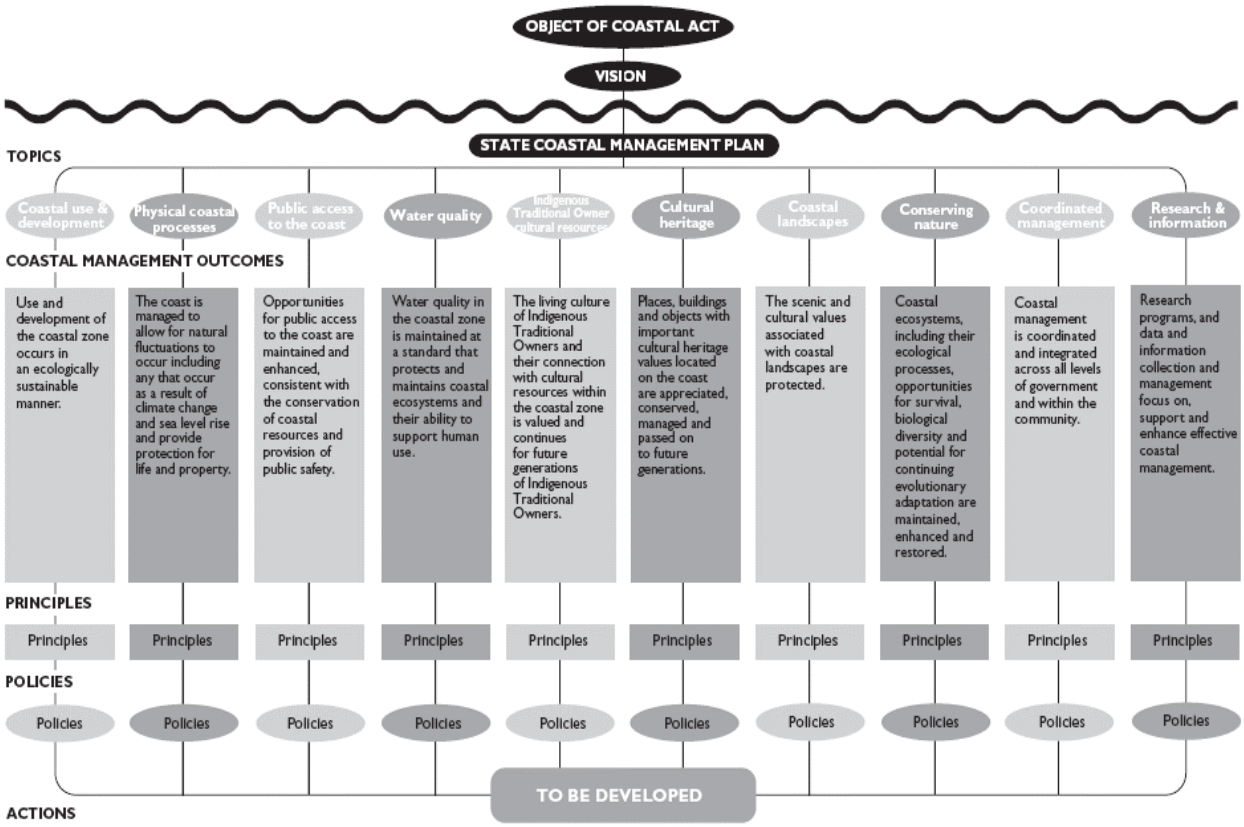

Figure 2 – Example of Coastal Management Policy framework (from State Coastal Management Plan – Queensland's Coastal Policy; http://www.epa.qld.au/environmental_management/)

One of the aspects concerns the availability of environmental quality data, either from pre-existing sources as well as from newly developed monitoring strategies. Plans and methodologies for assessing *Posidonia oceanica* distribution, phytoplankton and toxic phytoplankton, and invasive species have been developed and applied through the first-year project in collaboration with the local partner (A.P.P.L., Algiers). Furthermore, a biomonitoring strategy through sentinel organisms and biomarkers started. According to the lessons learned from BEEP and MED-POL, caged mussels are used as sentinel organisms and exposed to the selected sites on the Wilaya d'Alger coast for 4 weeks. The battery of biomarker includes:

1) lysosomal membrane stability measured through Neutral Red Retention assay in mussel haemocytes
2) stress on stress
3) percent mortality
4) acethylcholinesterase activity
5) catalase activity
6) glutathione S- transferase activity
7) metallothionein levels

The biomarkers were selected from the list suggested by MED-POL; however the choice of the biomarkers included in the battery mainly depends on the availability of the required appropriate equipment locally. The lack of cryostat made impossible the use of the set of biomarkers tested on tissue slices; as to the metallothioneins, the lack of a high-speed centrifuge (30.000xg) required an adjustment of the original method lowering the speed of centrifugation. However, a laboratory collaborating with the MED-POL programme has recently observed that a lower speed of centrifugation for a higher time allows obtaining supernatants suitable for the metallothionein determination (Lazo P, oral communication at the 2006 MED-POL Meeting, Italy).

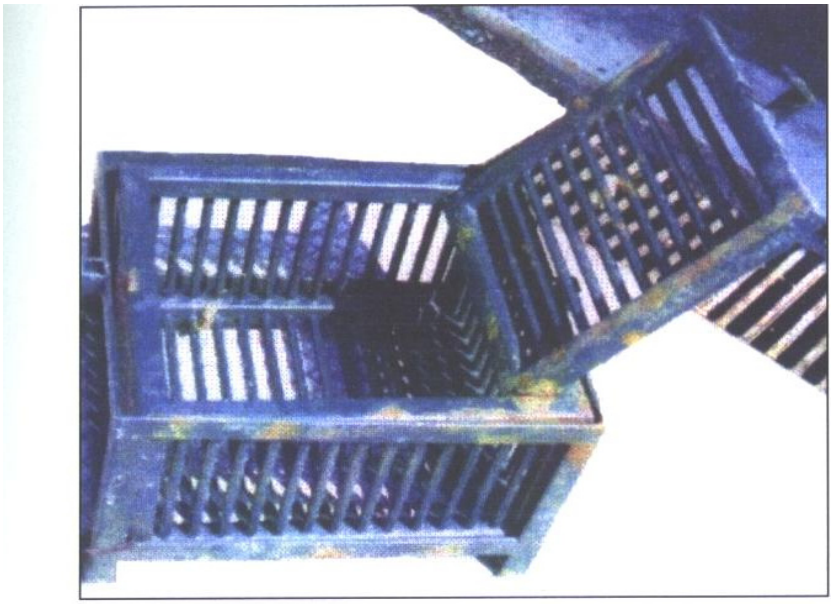

Figure 3. Cage for mussels transplantation to biomonitoring sites. Cages (marine steel, 43x48x25cm) have been built by La.Met., Lucca, Italy.

Marine steel cages were constructed (Fig. 3) and fixed to the substrate in two sites of study area. More sites of sampling are foreseen in the next future. A 10-day training course on Biomarker Analysis has been performed at CIRSA in November 2006 to transfer to the Algerian collaborators the theoretical knowledge and practical methodologies on the biomarker use, including those with the use of cryostat. The Manual UNEP-RAMOGE (1999) and the related video were used, and the most important papers on the same topics distributed. The concept behind monitoring through

biomarkers as disseminated by BEEP and MED-POL has been used by the Project AMIS as well. Italian personnel from CIRSA worked at the Laboratory of Biology and Animal Physiology of the Ecole Normal Superior de Kouba to transfer the methodologies and the basic concepts also to the personnel of the Institution.

The first survey was carried out from October 21 to November 20, 2006. Eighty mussels selected by size (5-6 cm length) were collected from a Mussel Culture Centre and transplanted in the same area (Wilaya de Tipaza, reference site) and in a polluted site (Wilaya d'Alger, Algiers' Harbour) (Fig. 4). Mussels were collected and immediately transported to the laboratory in aerated cold seawater. Resident mussels were also collected from the Algiers' Harbour. The size of these individuals was much lower (maximum 3 cm) than the farmed mussels. We noticed also that their valves were closed in a much tighter manner, and the fluids collected contained parasites that were apparently absent in the mussels transplanted from the reference to the same polluted site. The percentage mortality of caged mussels was about 10% of the initial amount in the polluted site.

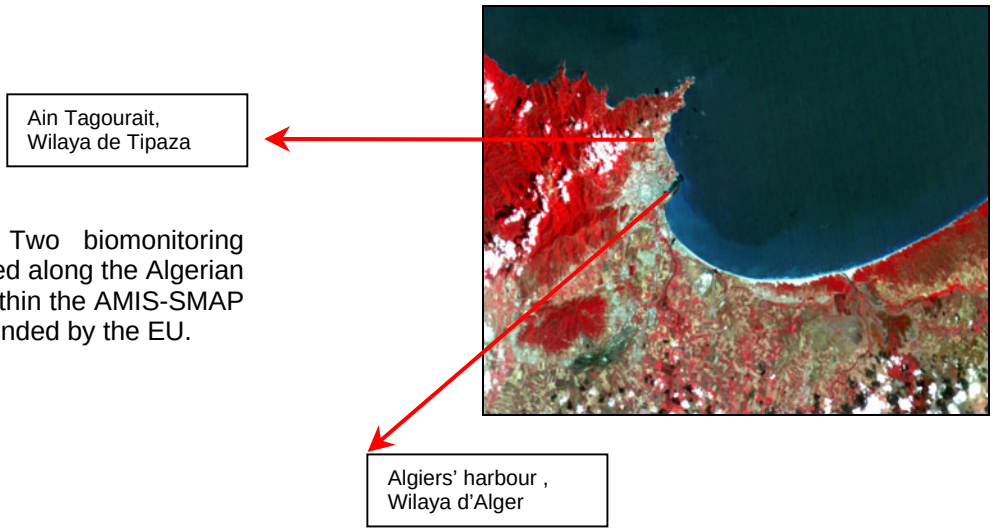

Figure 4. Two biomonitoring sites selected along the Algerian seacoast within the AMIS-SMAP III Project funded by the EU.

As to the biomarker responses, only the lysosomal membrane stability was assessed so far, mainly because some chemical reagents needed for the enzymes and metallothionein analysis are difficult to find on the Algerian market. Lysosomal membrane stability was measured in reference, transplanted and resident mussels collected at the same polluted site. The microscope slides were prepared as indicated in the Manual UNEP-RAMOGE (1999) and observed under the microscope (Axiocam 100 Zeiss equipped with videocamera). The NRRT was about 120 min in reference mussels and 45 min in transplanted and resident mussels from the Algiers' Harbour. These preliminary results confirm once again the validity of this biomarker to highlight the low environmental quality. Further biomarker analysis will be performed in tissues kept frozen in liquid nitrogen, and further samplings are planned seasonally for 2007.

In conclusion, the problems faced are those encountered by MED POL when disseminating these methodologies in countries where such approaches were not utilized, i.e. the limited availability of specific instruments or reagents. The present experience fully supports the conclusions of the recent MED POL Meeting (December 2006, Alessandria, Italy). A two-tier approach was suggested for the future biomonitoring approaches, where the first step is performed on caged mussels analysed as to the NRRT, the % mortality and the stress on stress response. These indices can easily be determined at low cost and can be applied in less equipped laboratories.

Acknowledgments

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Mussel Watch in estuarine waters: the Venice lagoon experience

by

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The Venice lagoon (Italy) is the largest lagoon in the Mediterranean basin, covering an area of about 550 km^2 with an average depth of about 0.5 m. It is a complex system characterized by a network of channels and shallow water flats, which are influenced by tidal flows, coming from the three inlets (Lido, Malamocco and Chioggia) that connect it to the Adriatic Sea (Fig. 1). Due to its position between the land and the sea, the lagoon of Venice has been subject to anthropogenic inputs of nutrients and pollutants that have increased with industrial and agricultural development. Inorganic and organic micropollutants enter the lagoon from industrial point sources (Porto Marghera industrial area), municipal wastewater discharges (Venice and Mestre urban areas), oil tanker traffic, agricultural drainage, atmospheric deposition and illegal dumping (UNESCO, 2000).

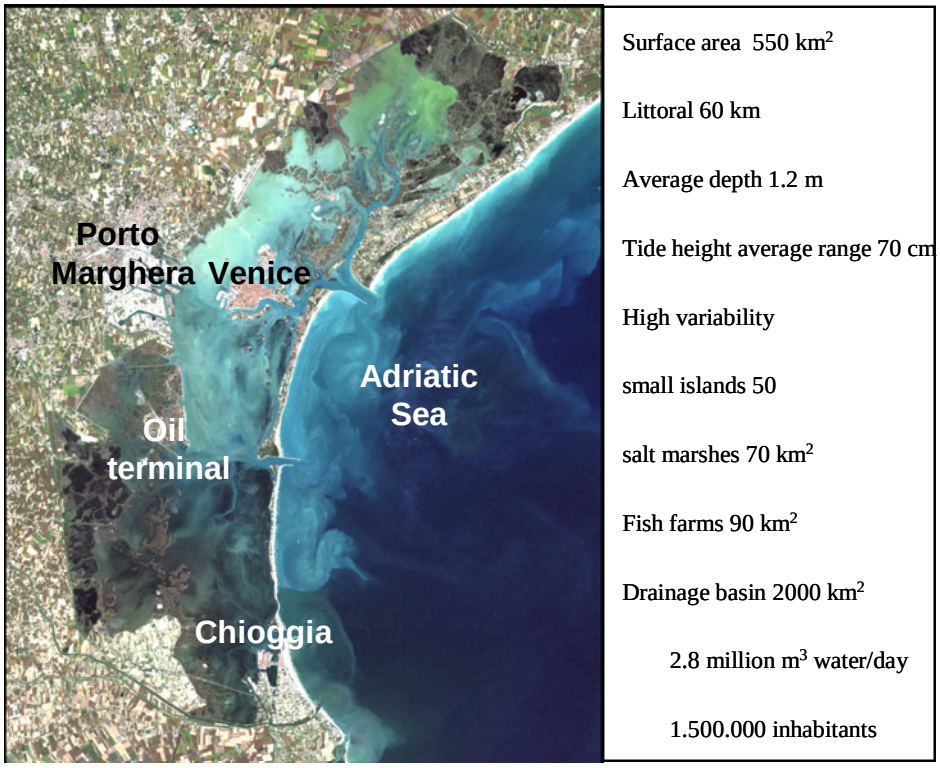

Fig. 1. Venice lagoon (satellite image) and its main features (Barbanti et al., in press)

The contamination of the Lagoon has been studied and described in several studies and with different approaches integrating chemical and biological measurements.

Since the 1970s, indicator organisms have proved to be useful tools in identifying spatial and temporal variations of chemical contamination in coastal waters. Molluscs of the genus *Mytilus* have been widely used as sentinel organisms for water monitoring to assess contamination of coastal or estuarine systems by trace metals and organic micropollutants both in national and international programmes such as the International Mussel Watch and MED POL biomonitoring programme (Goldberg et al., 1978).

The sessile filter-feeding mussel, *Mytilus galloprovincialis*, is an autochthonous species widely distributed in the Venice lagoon both as wild and cultured populations supporting a commercial fishery producing about 2500 t annually (Da Ros et al., 2000). It grows on pilings and rocks in the upper water layer and it is able to bioaccumulate different kinds of substances in its tissues from water and through ingestion of food and suspended particulate materials.

Starting from the 1970s, within the framework of local 'mussel watch' investigations, several studies based on chemical analysis have investigated the distribution and the level of contaminants in mussels of the Venice lagoon (Fossato and Siviero, 1974; Fossato and Craboledda, 1979; Campesan et al., 1980; Nasci et al., 1989; Zatta et al., 1992; Lowe et al., 1995; Nasci et al., 1998; Lowe and Fossato, 2000; Fossato et al., 2000; Giusti and Zhang, 2002; Wetzel and Van Vleet, 2004). The primary objective of these studies (chemical monitoring) was to assess the level of organic (PAHs, PCBs, AH, DDTs) and inorganic (metals) micropollutants in mussels' tissues, as index of lagoon environment quality (Table 1).

Table 1. Temporal comparisons of hydrocarbon and chlorinated hydrocarbon content in mussels. Mean ± SD in ng/g wet weight (from Fossato et al., 2000).

Platform (Adriatic Sea)	1974-1976	1977-1980	1986-1988	1991-1993
AH*10^3	6.5 ± 2.3	–	–	58 ± 39
ΣHCH	2.4 ± 1.7	1.2 ± 1.0	0.4 ± 0.1	0.6 ± 0.5
ΣDDT	9.5 ± 5.7	13.0 ± 7.0	2.8 ± 0.9	1.4 ± 0.6
ΣPCB	56 ± 24	41 ± 27	22 ± 6	13.8 ± 5.2
Salute (Venice urban area)	1974-1976	1977-1980	1986-1988	1991-1993
AH*10^3	45 ± 19	109 ± 50		151 ± 82
ΣHCH	2.9 ± 1.0	5.0 ± 2.3		1.0 ± 0.8
ΣDDT	11.2 ± 4.7	31 ± 14		6.6 ± 3.7
ΣPCB	130 ± 52	268 ± 99		141 ± 101
CVE (Industrial area)	1974-1976	1977-1980	1986-1988	1991-1993
AH*10^3	33 ± 14	–		133 ± 54
ΣHCH	2.5 ± 0.9	3.0 ± 1.5		2.0 ± 1.3
ΣDDT	12.2 ± 6.4	21 ± 10		5.6 ± 2.1
ΣPCB	107 ± 25	100 ± 35		121 ± 49

It has been generally accepted that the assessment of ecosystem "health" should be performed adopting a combination of physico-chemical and biological measurements in order to couple cause and effect. Starting from the 1980s the so-called "biomarker approach" has been proposed to evaluate the level of the stress syndrome induced by pollutants in sentinel organisms, integrating chemical and biological markers in ecotoxicological monitoring. An important issue of this approach is the selection of the most appropriate set of biomarkers to be determined at different degrees of biological organization from biochemical to physiological levels.

In the '90s this approach has been applied within the framework of a large research programme (UNESCO-MURST Venice Lagoon Ecosystem Project) to study the structure and the functions of the Venice lagoon ecosystem. In this context, the concentrations of pollutants originating from anthropogenic activities and their effects at different levels of biological organization have been studied on indigenous mussels (Livingstone et al., 1995; Fossato et al., 2000; Livingstone and Nasci, 2000; Lowe and Da Ros, 2000; Widdows and Nasci, 2000).

Biochemical (antioxidant enzymes, MFO system), cellular (lysosomal membrane stability, digestive cell lysosomal enzyme activities, atrophy and lipidosis in digestive tubules) and physiological (scope for growth; Fig.2) biomarkers were determined in mussels occurring naturally (passive biomonitoring) in sites of the Venice lagoon characterized by different levels of anthropogenic inputs. The results were in agreement with sediment, water and tissue chemistry, indicating that the animals from the "positive control sites" were the most impacted and confirming the use of mussels as good sentinel organisms in ecosystem "health" monitoring programmes.

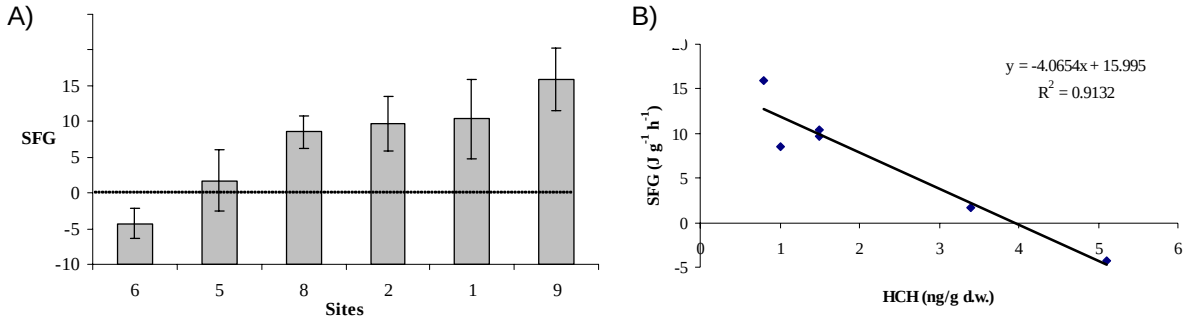

Fig. 2. A) Scope for growth (SFG; J g^{-1} h^{-1}) of mussels (*M. galloprovincialis*) collected from 6 sites in the Venice lagoon. Mean ± 95% C.I. *n* = 16. Sites: 6 – Canale Vittorio Emanuele (industrial area); 5 – Punta Salute (Venice urban area); 8 – Alberoni (Malamocco inlet); 2 – Isola di Crevan (northern lagoon); 1 – Lio Grande (near Lido inlet); 9 – Chioggia (urban area) (from Widdows et al., 1997). B) Relationship between Scope for Growth (SFG, J g-1 h-1) and hexachlorocyclohexane (HCH) accumulated in the tissues of mussels (*Mytilus galloprovincialis*) (from Widdows and Nasci, 2000).

Transplanting mussels from a reference site to polluted areas (active biomonitoring) can be a more feasible strategy for biomonitoring the effects of pollution impact and environmental changes in coastal zones (de Kock and Kramer, 1994; Nasci et al., 2002; Romeo et al., 2003; Damiens et al., 2007). The active biomonitoring approach is based on the comparison of parameters in organisms from a single population exposed to different environmental conditions. This strategy provides the advantage of ensuring comparable biological samples, reducing the variability of results usually encountered in field sampling programmes (Dame, 1996).

The comparison between passive and active biomonitoring approaches has been performed in the framework of "Orizzonte 2023"project (MAV-CVN, 1998-2000) and CORILA project (2000-2003). The results of the active biomonitoring highlighted more significant correlations between chemical and biological markers and a better discrimination between reference site and polluted areas. In particular, NADPH-cytochrome c reduttase and lysosomal membrane stability showed positive significant correlations with organic pollutants (DDT, PAHs, PCBs, HCB) accumulated by mussels. These studies demonstrated the need to apply a battery of biomarkers in order to correctly define animals' stress syndrome and the importance of the transplantation approach in marine coastal monitoring (Da Ros et al., 2002; Nasci et al., 2002).

The effects of anthropogenic pollution in Venice historical centre were studied using passive and active biomonitoring strategies (WATERS project, CORILA, 2002). In this context, a spatial and temporal survey was undertaken to evaluate estrogenic and general stress syndrome in indigenous mussels. Animals were collected at a reference area (Treporti) and at various sites located in the 'canals' of Venice historical centre (S. Tomà, Salute, Tre Archi), where urban wastewaters are discharged directly into the canals (Pampanin et al., 2005a). At the same time, mussels were transplanted at one location in the main canal (S.Tomà) and at reference site (Treporti) for 30 days. The applied battery of biomarkers included the evaluation of: micronuclei frequency, metallothionein content, survival in air, condition index, total oxyradical scavenging capacity (TOSC assay), alkali-labile phosphate levels. The Principal Component Analysis (PCA) of biomarkers and chemical data measured in indigenous mussels produced a two dimensional pattern which explained 73% of the total variance and showed a spatial and seasonal separation of sampling sites (Fig.3).

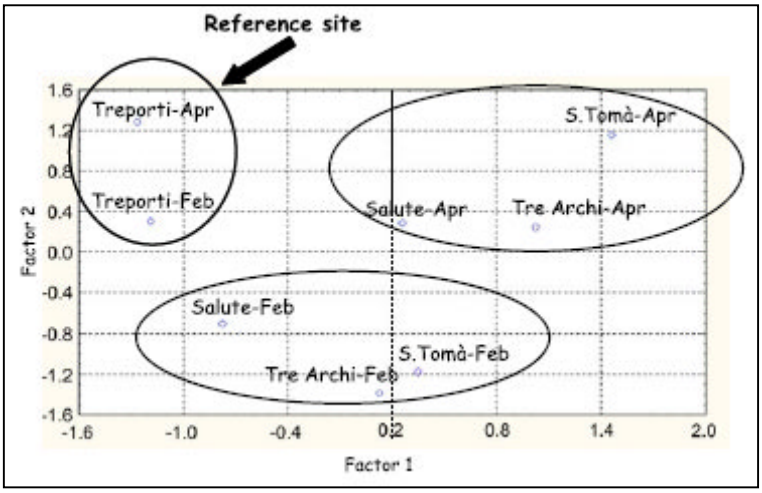

Fig. 3. The Principal Component Analysis (PCA) of biomarkers and chemical data. Spatial and seasonal separation of sampling sites is shown. Data referred to passive biomonitoring (from Pampanin et al., 2005a).

The results of transplantation experiments highlighted a gradient in bioaccumulation and stress syndrome advancement during the time frame of exposure (1, 2 up to 4 weeks). In addition to this, relationships between biological parameters and pollutants concentrations allowed to link exposure to stress effects.

In both biomonitoring strategies, biomarker results showed an impairment of the general health condition in mussels from the urban area, in agreement with organic and inorganic micropollutants accumulated in animals' tissues.

More recently the active monitoring strategy has been adopted in the ICSEL project, 'Monitoring water and sediment pollution in the Venice Lagoon integrating chemical and biological approaches' (MAV-CVN, 2003-2006). The project focused on the evaluation of the ecotoxicological impact of water and sediment pollution in the Venice lagoon in order to: develop tools for monitoring programmes of environmental quality; support interventions of environmental recovery with experimental and site specific evaluations (e.g. site-specific sediment quality criteria); address further investigation and studies on specific issues, matrices, contaminants, organisms. To achieve these objectives, chemical and ecotoxicological monitoring strategies have been integrated.

During the three year monitoring activities, transplantation experiments of caged mussels were performed at different lagoon locations and biological and chemical markers evaluated in exposed organisms. In order to follow a cost-effective biomonitoring strategy, a two-tier approach has been adopted. In the initial screening (Tier 1) a reduced set of biomarkers, based on the evaluation of the lysosomal membrane stability, survival in air (stress on stress) and lipofuscin content, has been applied in mussels transplanted in selected sites (10 locations). In Tier 2 the level of stress syndrome has been quantified by means of a full battery of biomarkers (metallothionein content, lipofuscin lysosomal content and neutral lipid accumulation, lysosome/cytoplasm ratio, Ca+2-ATPase activity, condition index, total oxyradical scavenging capacity assay, catalase activity, malondialdehyde content) in mussels transplanted in a reduced number of sites (5 locations), where an alteration of animals' health has been established in the screening phase.

The overall data, obtained from the full battery of biomarkers, have been integrated by an " expert system", a tool for biomarker data integration and interpretation, developed within the 2001-2004 EU BEEP framework to obtain a mussel stress syndrome classification (Viarengo et al., 2000; Dagnino et al., 2007). The application of this approach has demonstrated a good site ranking, highlighting the development of a stress syndrome in mussels transplanted at most impacted sites.

Moreover, biomarkers and pollutant bioaccumulation results have been integrated with chemical, toxicological and benthic community structure data into a weight of evidence approach, providing more information on the possible classes of contaminants which cause adverse biological effects.

In conclusion, the overall biomonitoring studies undertaken in the Venice lagoon indicate the active biomonitoring by transplanting caged mussels as the most suitable strategy, making it easier to standardize the results and allowing a better discrimination among differently impacted sites. It is important to emphasize the need of a good reference site as a “clean site” in the lagoon has not yet been adequately identified and the variability of environmental conditions in the lagoon makes it difficult to define. As a final point, it is crucial the choice of the suitable biomarkers to be utilized in molluscs in order to provide a clear insight in the pollutant-induced stress syndrome advancement and to make the biomonitoring tool a cost-effective strategy.

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Biomonitoring of environmental pollution in the Israeli marine coastal ecosystems

by

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ABSTRACT

The aim of the research was to bio-monitor the Israeli Mediterranean coast through the evaluation of the physiological status of local fish and limpet by determining the values of selected biological parameters (biomarkers) that are known to respond to the toxic effects of pollutants. Time course of cytochrome CYP1A1 induction was characterized in liver of the mullet *Mugil capio* after treatment with Aroclor 1254. The induction of CYP1A1 in the Mugil was a slow process, noticeable in the liver only five days after treatment and reaching maximal level only 10 days after treatment with Aroclor 1254. EROD activity in the liver of Aroclor 1254 treated Mugil followed well CYP1A1 induction, but was much more pronounced. The highest induction of CYP1A1 and EROD activity was detected in Mugil sampled from Ashdod in the south and Qiryat Yam near Haifa; both locations are in the vicinity of harbour and heavy industry including refineries and power plants.

Significant metallothionein induction in the hepatopancreas of *Patella caerulea* from Haifa-Shemen beach indicated the presence of pollution with heavy metals in those sites. GST activity in haemolymph of the Patella (D-GST) was tested as a novel biomarker of exposure to heavy metal ions in sampling sites along the Israeli Mediterranean coast. The biomarker D-GST was not dependent on the reproductive cycle of the Patella. High D-GST activities were detected in Patella from Qiryat Yam and Shemen beach confirmed that Haifa–Shemen site suffers from chronic pollution. The appearance of GST protein in haemolymph was an additional support to the kinetic results. GST protein was detected in haemolymph of Patella from Haifa-Shemen, Shikmona and Hadera sites that are close to coal power plants. The relatively low AChE activity recorded in samples of Patella from Haifa–Shemen and Shikmona may be a result of nonspecific anti acetylcholinesterase action of pollutants such as heavy metal ions apart from low levels of residues of OP and CB compounds.

INTRODUCTION

Cytochrome P450-dependent monooxygenases (CYP) have been studied in one or more species in over 10 families of fish (Miranda et al., 1990; Klotz et al., 1986; Goksoyr, 1985; Zhang et al., 1991). Many of CYP proteins bio-transform hydrophobic chemicals often present as pollutants in the environment. Lipophilic organic xenobiotics that were found as marine pollutants, such as polynuclear aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCBs), are metabolized by the CYP1A1 gene subfamily (Nebert and Gonzalez, 1987). Two different CYP1A1 genes, CYP1A1 and CYP1A12, are found in mammals (Gonzalez, 1989) and are thought to have originated by gene duplication event perhaps 250 million years ago (Nebert and Gonzalez, 1987). In lower vertebrates such as reptile and birds two CYP1A1 genes have been immunodetected (Yawetz et al., 1998). In fish, only a single gene has been identified in some species (Morrison et al., 1995) while in others multiple CYP1A1s occur (Berndtson and Chen, 1994), the result of a very recent divergence. CYP1A1 in fish are apparently induced by Ah receptor-dependent mechanisms similar to those in mammals (Whitlock, 1990; Stegeman and Hahn, 1994). The regulation and function of this CYP may determine effects of chemicals on organisms. Induction of ethoxyresorufin O-deethylase (EROD) activity in vertebrates is generally accepted as an indicator of CYP1A1 induction and is largely specific to CYP1A1.

The polychlorinated biphenyls are industrial chemicals and major environmental pollutants, their residues abundant in tissues of fish, birds and humans. They are known to induce hepatocellular carcinoma in rats and birds, and are suspected of being carcinogenic to humans (Dubois et al., 1995). The coplanar polychlorinated biphenyl congeners, that are present in trace amounts in the commercial preparations - Aroclors, are responsible for much of the biological activity of these chemicals.

The glutathione s-transferases (GST) are a group of enzymes whose main function is to convert endogenous, and xenobiotic electrophilic compounds to water-soluble intermediates that may be eliminated. Soluble glutathione S-transferases (GSTs) are proteins with typical molecular masses of around 50 kDa, each composed of two polypeptide subunits. GSTs catalyze the transfer of the tripeptide glutathione to substrate containing a reactive electrophilic centre to form a polar S-glutathionylated reaction product (R-SG). Each soluble GST is a dimmer of approximately 26 kDa subunits, typically forming a hydrophobic 50-kDa protein with an iso-electric point in the pH range 4-5.
The glutathione S-transferases (GST) represent a major group of detoxification enzymes. All eukaryotic species and many bacteria possess multiple cytosolic and membrane-bound GST isoenzymes, The cytosolic enzymes are encoded by at least five distantly related gene families designated as class alpha, μ (mu), π (pi), σ (sigma), and θ (theta) GST. The biological control of these families is complex as they exhibit sex-, age-, tissue-, species-, and tumor-specific patterns of expression. In addition, GST are regulated by a structurally diverse range of xenobiotics and, to date, at least 100 chemicals have been identified that induce GST.
Many of the compounds that induce GST are themselves substrates for these enzymes, or are metabolized (by cytochrome P-450 monooxygenases) to compounds that can serve as GST substrates, suggesting that GST induction represents part of an adaptive response mechanism to chemical stress caused by electrophiles. It also appears probable that GST are regulated in vivo by reactive oxygen species (ROS), because not only are some of the most potent inducers capable of generating free radicals by redox-cycling, but H_2O_2 has been shown to induce GST in plant and mammalian cells induction of GST by ROS would appear to represent an adaptive response as these enzymes detoxify some of the toxic carbonyl-, peroxide-, and epoxide-containing metabolites produced within the cell by oxidative stress.

Many environmental contaminants including certain metals and organic xenobiotics are sequestered in lysosomes under certain conditions (Moore, 1985). These may in some circumstances be accompanied by lysosomal damage. One category of lysosomal disturbance involves membrane permeability that can be investigated cytochemically. Time course of retention of the cationic probe neutral red within the lysosomal compartment was used, for mussel's blood cells, as a measure of damage to the lysosomal membrane (Lowe, Soverchia and Moore, 1995).

The hypothesis that led us in developing the novel biomarker – D-GST - Displaced Glutathione S Transferase activity in haemolymph is close to that of the biomarker, Lysosomal Stability. The instability found for the lysosomal membrane, after exposure to xenobiotic pollutants, may very well be exemplified also by cell membranes in various tissues. Cytosolic enzyme such as GST may, in molluscs exposed to pollutants, leak into the haemolymph. Presence of displaced GST activity in haemolymph may indicate exposure of the mollusc to pollutants, as this enzymatic activity is not normally found in the haemolymph of unexposed molluscs. The method was tested with heavy metals and chlorinated hydrocarbons.

Displaced GST activity (D-GST) in haemolymph of *Patella caerulea* was found to be an extremely sensitive index of cellular condition. D-GST in haemolymph was very low in untreated *Patella*. Upon exposure of the *Patella* to heavy metal ions D-GST activity appeared in haemolymph of treated limpets. The dose-response was not linear but polynomial, indicating that the cell membrane structure changed gradually up to a given ion concentration where the membrane underwent massive disruption and the cytosolic enzyme was poured to the haemolymph. The first stage of the impact of metal ions on cell membrane, resulting in moderate permeability changes, was probably mediated by active oxygen species causing lipid peroxidation cascade or modulation of the activities of enzymes and ion transporters contained in membranes by oxidization of

sulfhydryl groups of these proteins. The permeability changes of the plasma membrane developed to subsequent extensive breakage of the membranes. The displaced GST activity leakage from tissue cells to haemolymph may thus be metal-ions-induced membrane disintegration that resulted in enzyme leakage and finally death of cells in the limpet organs.

The response was selective and dependent on the metal ion to which the limpet was exposed. Thus, the response to the highly toxic Hg^{+2} and to Cu^{+2} ions appeared at very low concentrations while the reaction to Ni^{+2} and Pb^{+2} was moderate. The response to Cd^{+2} was more pronounced and thus this metal served as a model stressor in this work. The biomarker D-GST activity in haemolymph did not compel killing of the test animal and it was possible to draw haemolymph from each individual several times. Study of the response of the D-GST biomarker in other mollusc species included, till now, only the limpet *Cellana rota,* where D-GST activity in haemolymph of *Cellana* exposed to 2 ppm Cd^{+2} ions was about 73% of the value recorded for exposed *Patella*. The response of the biomarker to chlorinated hydrocarbons in the form of Aroclor 1254 was very low. Metallothioneins are low molecular mass (6-7kDa) non-enzymatic proteins with biological functions ranging from homeostatic regulation of essential metal ions (Cu, Zn) and detoxification of heavy metals (Cd, Hg) to free radicals scavenging. The metallothionein was found to be effective antioxidant agent and thus have defensive role in the protection of the cells against oxidative stress (Viarengo et al, 2000). The metallothionein content represents a biomarker of exposure that is often used for evaluating the effects of heavy metals such as Cu, Hg, Cd, and Ag. Metallothioneins are metal-inducible proteins whose synthesis increases in the cells of organisms that accumulate high concentrations of contaminant metals. Metallothioneins are usually considered as a specific stress index for heavy metal contamination, but recent studies on fish have demonstrated that the *de novo* synthesis of these metalloproteins is not only induced by heavy metal accumulation, but also by different agents such as hormones and reactive oxygen species, whose levels were shown to be enhanced by inorganic and organic pollutants.

We are using a simple and inexpensive technique for the detection of organophosphorus (OP) and carbamate (CB) residues in water. Acetylcholinesterase enzymes from different organs of various fish species were dialyzed, for a defined period of time, against buffer containing known concentrations of the inhibitors. The reduction in gills and brain-AChE activity in exposed fish correlated well with the sum of OP and CB residues detected in the gill tissue using gas-chromatography-mass-spectroscopy. The range of concentrations needed to inhibit half of the activity of any of the enzymes tested by each of the inhibitors were determined and used for the establishment of a model ranking the risk associated with the presence of this nerve poisons in drinking water. Using the criteria supplied by the model for fish mass mortality events, we were able to define cases where there was no apparent exposure to OP or CB, cases with exposure to sub-lethal levels of OP or CB and cases where mortality was definitely caused by OP or CB intoxication. This bio-monitoring technique provides information concerning the extent of penetration of OP and CB pesticides into the aquatic ecosystem in Israel. The AChE bio-monitoring technique was found to be more sensitive than the conventional analytical-chemical method in its capacity to detect minute amounts of OP and CB residues in fresh water.

The aim of the research was to bio-monitor the Israeli Mediterranean coast through the evaluation of the physiological status of local fish and limpet by determining the values of selected biological parameters (biomarkers) that are known to respond to the toxic effects of pollutants. The use of a battery of biomarkers is more advantageous than the use of a single biomarker as it offers an effective early warning system. The biomarkers derive from cell biochemistry and can detect pre-pathological alterations within the cells of the exposed organism before toxicological changes may occur and lead to mortality, population decline and impairment of the ecosystem.

METHODS

Chemicals: Aroclor 1254 was from AccuStandard (Campro Scientific, Veenedaal, NL). Ethoxyresorufin and other biochemicals were from Sigma (Axel. NL). Universal Immunoperoxidase Staining Kit (Murine) was purchased from Sigment Laboratories, Dedham, MA, USA. General chemicals were from Merck (Darmstadt, Germany).

Sampling sites:

Area	Sign	Site	Site description	Mugil	Patella
Haifa	Q.Y.	Qiryat-Yam	Petrochemical industry	+	+
		Haifa-Shemen	Harbour		+
		Shikmona	Recreation site		+
Sharon		Sdot-Yam	Recreation site		+
		Michmoret	Recreation site		+
	CSR	Caesarea	Recreation site	+	+
	HED	Hadera	Power plant	+	+
		Alexander	River	+	
Tel Aviv	S.Ali	Sydney-Ali	Recreation site	+	
	RID	Riding	Power plant	+	
	YRQ	Yarqon	River	+	
	YFO	Jaffa	Recreation site	+	
South	ASD	Ashdod	Harbour, petrochemical industry	+	

Methods for CYP1A1 and EROD determination in the Mugil

Animals and Treatments: Mullets for exposure experiments were from commercial rearing ponds. The dose-response studies were carried out by interperitoneal injections (ip) of Aroclor 1254 dissolved in corn oil. The total doses used for Aroclor 1254 were: 25, 50 and 100 mg/Kg. Each dose was administered by five ip injections and an interval of 3-4 days was set between consecutive injections. Fish were sacrificed 10 days after the last treatment with Aroclor 1254. The volume of the corn oil that was used as carrier did not exceed 0.5 ml per injection. Control fish, treated with carrier alone and examined fish were kept in the laboratory in aquaria with aerated and biologically filtered water and fed *ad libitum*.

Preparation of microsomes: All procedures were carried out at 0-4^{o}C. Livers removed from freshly killed fish, homogenized in 5-10 volumes of 50 mM Tris (tris[hydroxymethyl]-aminomethane) buffer, pH 7.4 containing 0.15 M KCl. The homogenate was centrifuged for 20 min at 10,000 x g. The resulting supernatant was then centrifuged for 20 min at 105,000 x g and the microsomal pellet was carefully scraped from the glycogen that precipitated beneath it and was resuspended in 50 mM Tris buffer, pH 7.4 containing 20% glycerol, 1 mM EDTA (ethylenediaminetetra-aceticacid), and 1 mM DTT (dithiothreitol), at a volume of 1 ml per microsomes derived from 1 g of liver. Microsomal suspensions were kept in liquid nitrogen until used.

Fluorometric analysis of 7-ethoxyresorufin O-deethylase (EROD) activity: The microsomal EROD activity was determined fluorometrically as described by Burke and Mayer, 1974 with some modifications. The substrate 7-ethoxyresorufin dissolved in hot methanol, was added to 7.2 mM KH_2PO_4, 35 mM Na_2HPO_4 buffer, pH 7.4 containing 2.5 mM glucose-6-phosphate and 0.25 mM NADP. The 7-ethoxyresorufin concentration was adjusted to 2 µM by monitoring the absorbance of the solution at 482 nm using extinction coefficient of 22.5 $mM^{-1}cm^{-1}$. The microsomal protein, 50-100 µg in 0.1 ml of 8 mM HEPES buffer, pH 7.4 containing 200 mM sucrose and 20% glycerol, was added to 0.9 ml of the buffered substrate solution. After 2-3 min equilibration at 25^{o}C the

reaction was initiated by the addition of 1 unit of Glucose-6-Phosphate dehydrogenase. The reaction was carried out for 5 min at 25^{o}C and terminated by the addition of 2 ml cold acetone. After centrifugation (5min, 9000 X g), fluorescence of the supernatant was determined using spectrofluorimeter with an excitation wavelength of 537 nm and an emission wavelength of 583 nm. The standard contained 100 pmol of resorufin in 7.2 mM KH_2PO_4, 35 mM Na_2HPO_4 buffer, pH 7.4, containing 2 ml acetone. Blank was made by the addition of 2 ml acetone to 1 ml of reaction mixture at 0 time.

Immonoblot assay: Monoclonal antibodies (Mab 1-12-3) against cytochrome CYP1A1 (scup CYP1A1) were a donation of Prof. John Stegeman, WHOI, Woods Hole, MA. USA. The antibodies were obtained by conventional hybridoma techniques 8 mM HEPES buffer, pH 7.4, containing 200 mM sucrose and 20% glycerol and their preparation and characterization have been described (Kloepper-Sams et al., 1987). Immunoblotting was carried out essentially by the method of Burnette (1981), with some modifications as previously described (Yawetz et al., 1993). The densitometric data for the staining of CYP1A1 were done by scanning the nitrocellulose sheets and analyzing with a computerized densitometric programme based on comparison with the staining of purified scup CYP1A1. Content was expressed as pmoles of CYP1A1 per mg microsomal protein assuming molar equivalency of reaction between scup CYP1A1 and the mullet CYP1A1.

Neutral Red Retention assay (NRR) of the haemocyte lysosomes: The procedure is performed according to (Lowe et al., 1995) with slight modifications. Haemolymph is withdrawn from the posterior adductor muscle of 10 mussels in physiological saline so as to obtain a 50/50 of cell/physiological saline suspension. The physiological saline, pH 7.3 contained 4.77 g/l HEPES, 25.48 g/l NaCl, 13.06 g/l MgSO4, 0.75 g/l KCl, 1.47 g/l CaCl2. Suspensions are spread on slides, transferred to a lightproof humidity chamber, and allowed to attach. Then, 40µl of the neutral red (NR) probe are added to the cell monolayer. After a 15-min incubation period, slides are examined systematically under a light microscope every 15 min. The time after the NR probe application, where there is evidence of dye loss from the lysosomes to the cytosol or of other lysosomal abnormalities in at least 50% of the examined cells, belonging to the granular haemocytes, represented the NRR time for the mussel. The mean NRR times of the 10 mussels corresponded to the NRR time for the sampling station.

Displaced Haemolymph GST Activity (DH-GST)" determination: Suction of small amount (about 100µl) of haemolymph is done by careful insertion of the needle to the mollusc and suction of the haemolymph from the cephalopedal blood sinus surroundings without hurting the adjacent tissues. Spectrophotometric determinations of reduced glutathione S-transferase (GST) activity were made using Jasco UVIDEC 675 double beam computerized spectrophotometer (Japan). GST activity with 1-chloro-2, 4-dinitrobenzene (CDNB) was followed by monitoring changes in absorbance at 340 nm, using 1.0 ml capacity cuvettes with 1.0 cm light path. A complete assay mixture without enzyme served as control. Assays were done at pH 7.0 and at 25^{O}C in 100 mM KH_2PO_4 buffer. The concentration of CDNB was 0.2 mM and reduced glutathione (GSH) 5 mM. Protein concentration in reaction mixture was 10-20 µg of hepatopancreas 105,00 x g cytosol. An extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ was used (Habig et al., 1974).

Evaluation of metallothioneins: The procedure will be the one described by a modification of the method of Viarengo et al. (1999). The digestive glands of mussels is rapidly removed and homogenized in 3 vol. of 0.5 M sucrose, 20 mM Tris–HCl buffer, pH 8.6, containing protease inhibitors 6 µM leupeptine, 0.5 mM PMSF and 0.01% β-mercaptoethanol. Aliquots of the homogenate (3 ml) are stored at −80°C until use. The homogenate is centrifuged at 30000xg for 20 min, and followed by the addition of 1.05 ml cold (−20°C), absolute ethanol and 80 µl of chloroform per 1 ml of the resulting supernatant. Subsequently, the samples are centrifuged at 6000xg for 10 min at 0–4°C. The supernatants are added to 3 vol. of cold ethanol, stored at −20°C for 1 h and centrifuged in a swinging rotor at 6000xg for 10 min. The pellets are washed with ethanol–chloroform–homogenization buffer (87:1:12), centrifuged again at 6000xg for 10 min, dried under a nitrogen gas stream and resuspended in 300 µl of 5 mM Tris–HCl, 1 mM EDTA, pH 7. The re-suspended metallothionein fraction is added to 4.2 ml of 0.43 mM DTNB in 0.2 M phosphate

buffer, pH 8 and after 30 min the concentration of reduced sulfhydryls was evaluated by reading the absorbance at 412 nm, utilizing GSH as a reference standard.

Protein determination: The protein concentration is determined with the Bradford (Bradford, 1976) method using BSA fraction V as a standard.

Data processing and statistical analysis: Statistical analysis was done by analysis of variance, Student - Newman-Keuls aposteriori test for multiple comparisons among means of unequal sample size, the non parametric Kruskal-Wallis test, and test of significance of the product-moment correlation coefficient (Sokal and Rohlf, 1969).

RESULTS

A. Monitoring exposure to petrochemical contamination in several sites along the Israeli Mediterranean using the biomarkers CYP1A1 and EROD induction in the Mugil hepatic tissue

Induction of cytochrome CYP1A1 and EROD activity was characterized in liver of *Mugil capio* after treatment with Aroclor 1254 (Fig. 1). A significant correlation (Rval=0.823, P<0.01) was found between CYP1A1 content and EROD activity in the hepatic microsomes from Mugils treated with various doses of Aroclor 1254 (Fig. 1). The Mugils that were used for the induction experiments were not exposed before to CYP1A1 inducers, as there was almost no CYP1A1 protein or EROD activity that could be detected in their hepatic microsomes. At low inducer concentrations there was a lag between the appearance of the protein to the manifestation of its catalytic activity EROD.

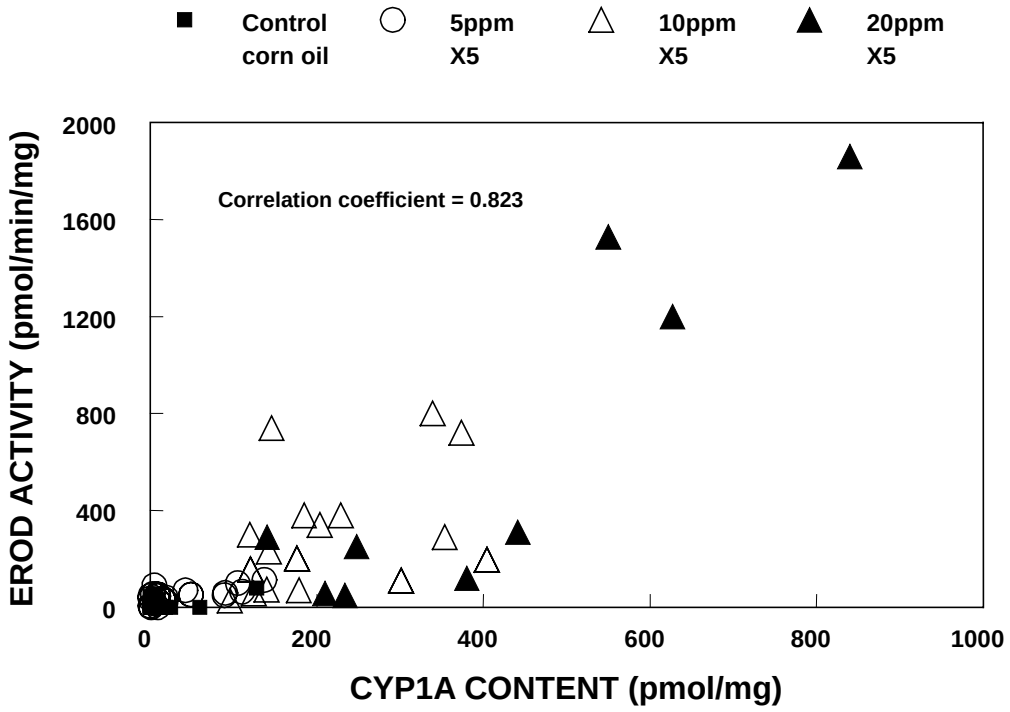


Figure 1. The correlation between EROD activity and CYP1A1 content in the microsomal fraction of Mugil treated with different doses of Aroclor 1254.

As can be seen in Fig. 2 the induction of CYP1A1 in the Mugil was a slow process. Induction appeared in the liver only five days after the fish were treated with Aroclor 1254, a procedure that took by itself two weeks. Even then induction of CYP1A1 was hardly noticed in the Mugil that were treated with 50 mg/Kg while very pronounced in those that were treated with 100 mg/Kg. The time course of induction of CYP1A1 was very different in animals treated with the different doses. In liver of Mugil that were treated with the maximal dose of Aroclor 1254 induction reached much higher levels than in those treated with the lower doses. In higher doses CYP1A1 induction reached maximal level in 10 days after treatment and declined thereafter while in the fish treated with the lower doses induction increased gradually and reached maximal levels 60 days after treatment. Time course of EROD activity (Fig.3) in the liver of Mugil treated with Aroclor 1254 was very much the same as exemplified by the CYP1A1 induction but the difference between the level of EROD induction in the fish that received the 100 mg/Kg dose to that in the fish that received the lower doses was much more pronounced than that exemplified by the CYP1A1 induction. Furthermore, in the fish treated with the lower dose EROD activity was hardly detected till 60 days after treatment. Determination of EROD alone as a biomarker in the Mugil was much less sensitive than that of CYP1A1 content. In order to monitor the contamination of the Israeli Mediterranean shores with Mugil as a test animal it was necessary to apply both biomarkers CYP1A1 content as well as EROD activity.

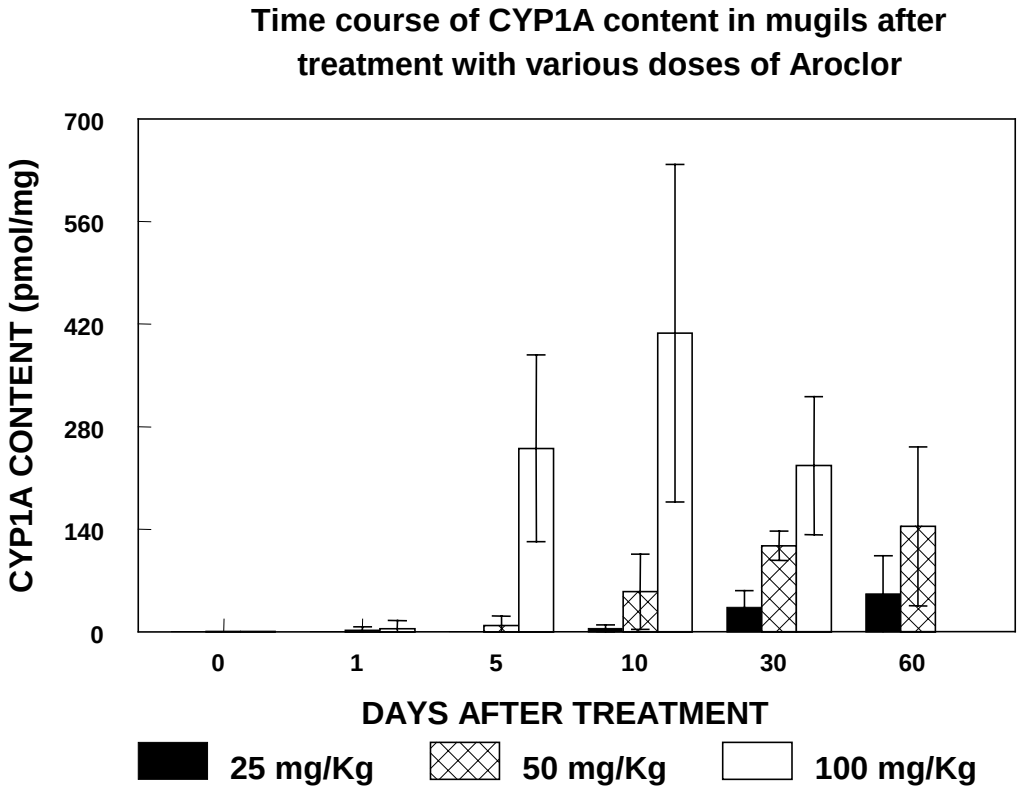


Figure 2. Time course for the induction of CYP1A1 in Mugil by interperitoneal injections (ip) of Aroclor 1254 dissolved in corn oil. The total doses used for Aroclor 1254 were: 25, 50 and 100 mg/Kg. Results are mean ± SD for about 6 determinations.

The biomonitoring results for the Israeli Mediterranean coast are presented in Figure 4. Members of the genus *Mugil* are euryhaline, and thus able to enter estuarine and marine waters where they may be exposed to a variety of foreign compounds that include CYP1A1 inducers. Ashdod in the south and Qiryat Yam near Haifa represent locations that are placed in the vicinity to harbour and heavy industry including refineries and power plants. The highest induction of CYP1A1 and EROD activity was detected in Mugil sampled from those sites. The levels of induction of CYP1A1 and EROD in the Mugil from Qiryat Yam were as high or even higher then that recorded in the Mugils

that were treated with the highest dose of Aroclor 1254 in the laboratory (Fig. 2,3). Riding and Hadera are located very close to power plants and in the liver of Mugils sampled from those sites a moderate exposure to petrochemical was detected. Jaffa (YFO), Yarqon river estuary and Sydni Ali with the relatively low levels of CYP1A1 and EROD induction represent, very probably, the general pollution background that may be typical to locations with defused and unclear sources of pollutants. We considered Caesaria as a clean site. Yet, the low induction of the biomarkers in the Mugils liver may indicate some petrochemical pollution that may originate from the power plant located about 8 Km to the south.

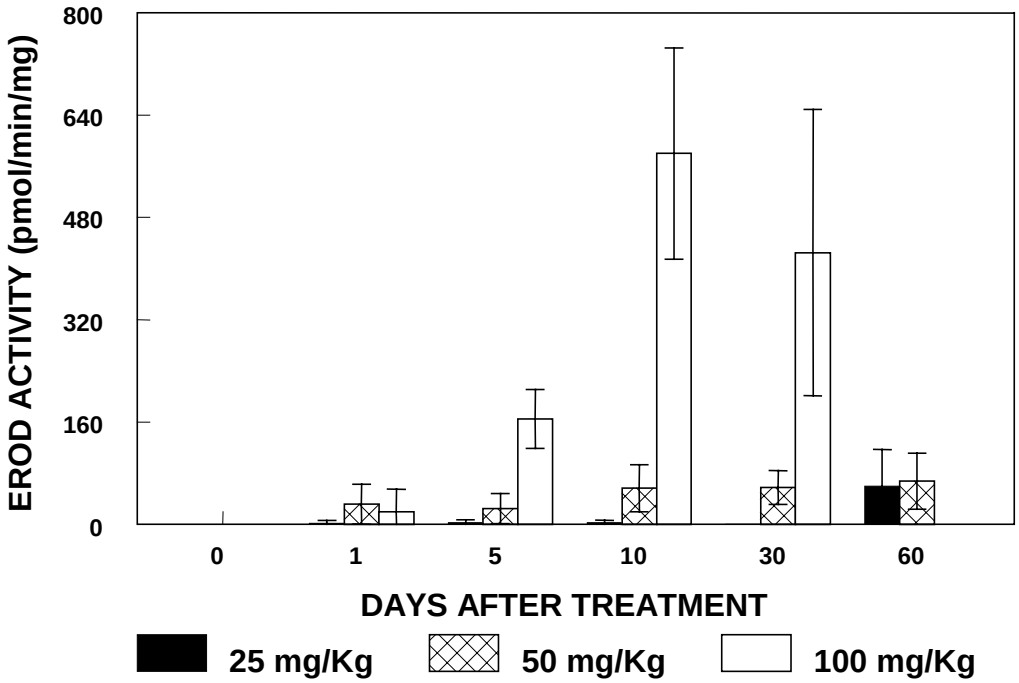


Figure 3. Time course for the induction of EROD activity in Mugil. by interperitoneal injections (i.p.) of Aroclor 1254 dissolved in corn oil. The total doses used for Aroclor 1254 were: 25, 50 and 100 mg/Kg. Results are mean ± SD for about 6 determinations.

The correlation found between CYP1A1 content and EROD activity in the microsomal fraction of Mugil sampled at indicated locations along the Israeli coastline (Fig. 5) was highly significant (Rval=0.726, $P<0.01$). As expected the significance was somewhat lower than that of Mugil exposed by injection of Aroclor 1254 under controlled conditions (Fig. 1).

B. Metallothionein determination in the limpet *Patella caerulea* as a biomarker of exposure to heavy metals ions in sampling sites along the Israeli Mediterranean in the spring.

Metallothionein induction in the hepatopancreas of the Patella was used as a biomarker to monitor exposure to heavy metal ions in selected sites of the marine environment along the Mediterranean coast of Israel. According to the results obtained (Fig. 6) Shikmona and Caesarea were relatively unpolluted with heavy metals ions while some pollution was evident in Hadera and significant pollution with heavy metals was detected in Haifa-Shemen beach ($P<0.05$). The results obtained were as expected and thus MT appears to be a useful and reliable biomarker.

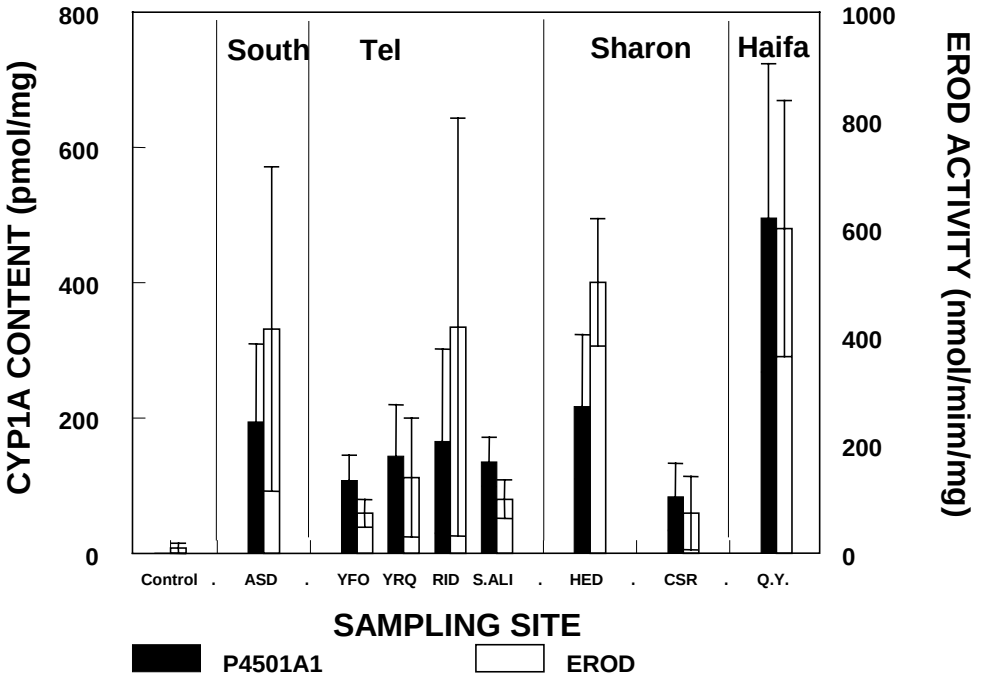


Fig. 4: CYP1A1 content and EROD activity in hepatic microsomes from Mugil sampled at indicated locations along the Israeli coastline. Sampling sites: Ashdod (ASD); Jaffa (YFO); Yarqon (YRQ); Riding (RID), Sydni Ali (S.ALI), Hadera (HDR); Caesaria (CSR); Qiryat Yam (Q.Y.).

C. Displaced Haemolymph GST Activity (D-GST) in the limpet *Patella caerulea* as biomarker of exposure to heavy metals ions in sampling sites along the Israeli Mediterranean

Displaced GST activity (D-GST) in the Patella Haemolymph was applied as a novel biomarker of exposure to heavy metals ions in sampling sites along the Israeli Mediterranean. Summer and winter results are presented in Fig. 7.

The biomarker D-GST was not dependent on the reproductive cycle of the Patella. The results obtained were in good agreement with the expected pollution of the sampling sites. In specimens from Sdot-Yam and Michmoret D-GST activities in haemolymph of the Patella were low as expected to be in clean resort sites (Fig. 7). In Patella sampled from Shemen-beach in Haifa D-GST activities were much higher ($P<0.01$) compared to all other sites. Shemen-beach is located very near to Haifa harbour at the estuary of the Kishon River that carries petrochemical sludge. D-GST activity in Patella sampled from the clean sites Shikmona, Sdot-Yam and Michmoret were low as expected. Qiryat Yam and Shemen beach are located very near to Haifa harbour at the estuary of the Kishon River that carries petrochemical sludge.

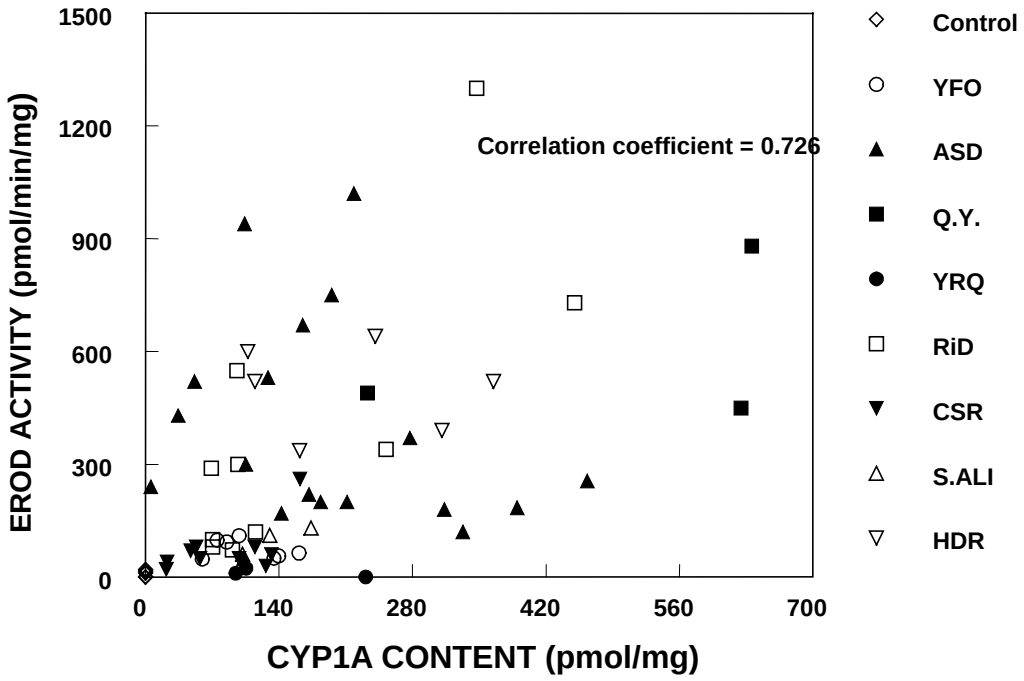


Fig. 5: Correlation between EROD activity and CYP1A1 content in the microsomal fraction of Mugil sampled at indicated locations along the Israeli coastline. Sampling sites: Ashdod (ASD); Jaffa (YFO); Yarqon (YRQ); Riding (RID), Sydni Ali (S.ALI), Hadera (HDR); Caesaria (CSR); Qiryat Yam (Q.Y.).

Sampling of Patella in the spring (Fig. 8) reconfirmed that Haifa–Shemen site is a polluted site as D-GST activity in the Patella haemolymph was significantly higher ($P<0.01$) than that recorded in Patella from the clean site Caesarea as well as Patella from Hadera, site located near a power plant (Figure 8). In Shikmona D-GST activities were found to be high and probably, this site may be subjected occasionally to increased level of pollution. The two sites, Shikmona and Qiryat Yam suffer from incidental exposure to pollution. The source of this pollution in unclear but it may very well be from Ships discharging their balance water before entering Haifa harbour.

Plate I represents typical immunoblot of haemolymph taken from Patella from the main sampling sites. The appearance of GST protein in haemolymph is an additional support to the kinetic results. As can be seen in the immunoblot there was no indication for D-GST in haemolymph of the Patella from Caesarea. As expected D-GST protein appeared in Patella from Haifa-Shemen. D-GST appeared also in Patella from Shikmona as could be expected from the kinetic results (Fig. 8). Hadera site is close to a coal power plant and is also subjected to occasional increased levels of pollution as was exemplified also by the increasing induction of CYP1A1 and EROD in mullets sampled from this area (Fig. 4).

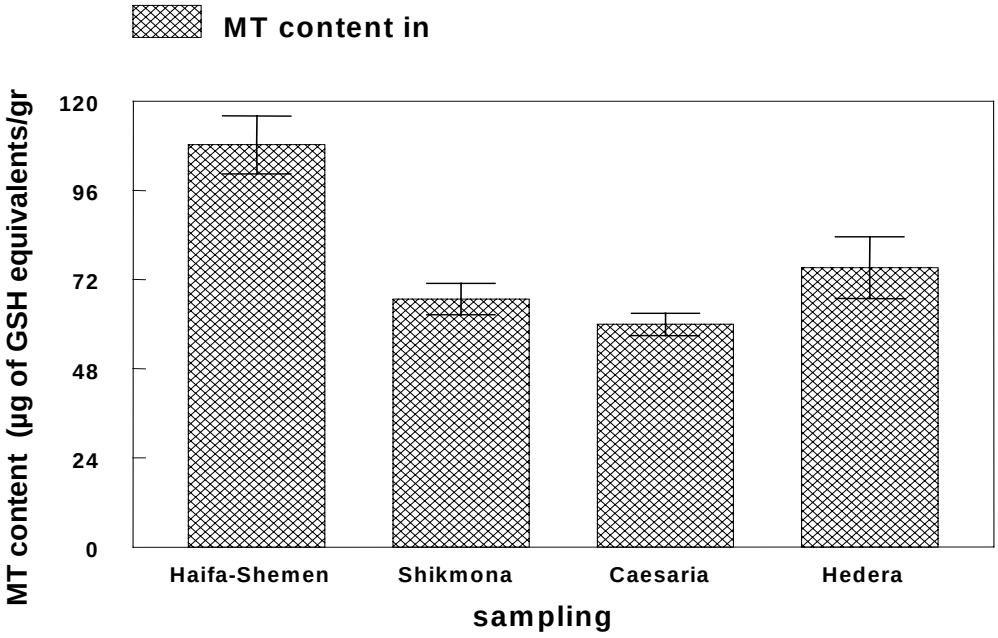


Figure 6. Metallothionein content in hepatopancreas of Patella from different sites along the Israeli Mediterranean coast (n= about 20 specimens from each site).

D. Lysosomal stability as a biomarker in the Patella.

The stability of lysosomes from Patella blood cells was determined in samples of the limpet taken from several sites along the Israeli Mediterranean coastline at different seasons. The results obtained confirmed our early findings that *Patella caerulea* lysosomes were able to retain the dye only between August and October when the gonadal index of the limpet was of a very small value (Fig 7). The rest of the year the gonadal index increased and reached its highest value in March, just before sprawling. The hormonal activity associated with the gonads gross probably decreased the stability of the lysosomal membranes to the point that it was impossible to use the lysosomal stability as biomarker of exposure to pollutants.

E. Inhibition of acetylcholinesterase activity in the gills of the limpet *Patella caerulea* as a biomarker of exposure to organophosphates and carbamate residues in sampling sites along the Israeli Mediterranean coast

Acetylcholinesterase activities were determined in the gills of Patella collected from four sampling sites during the spring (Fig. 9). Mean AChE activities recorded in Caesarea and Hadera were higher than those recorded in Haifa–Shemen and Shikmona but with no statistical significance. The decline in Ache activities recorded in Patella from Haifa–Shemen and Shikmona may be a result of nonspecific anti acetylcholinesterase action of pollutants such as heavy metal ions apart from low levels of residues of OP and CB compounds.

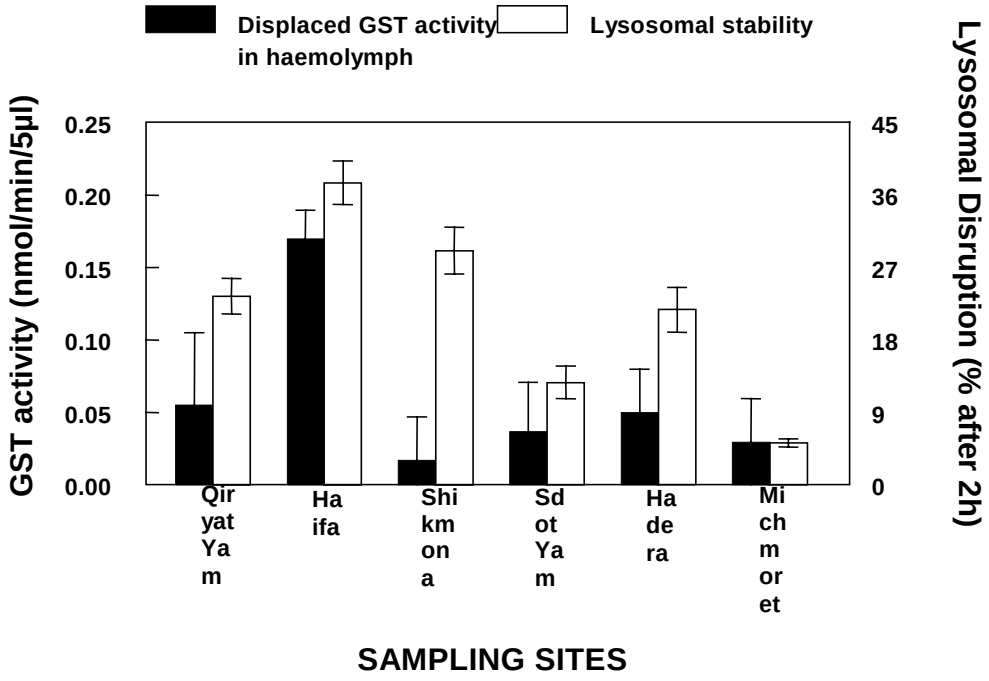


Figure 7. D-GST activity and lysosomal stability in haemolymph of *Patella caerulea* sampled from various sampling sites along the Israeli Mediterranean coast in September. Gonadal index was 1.

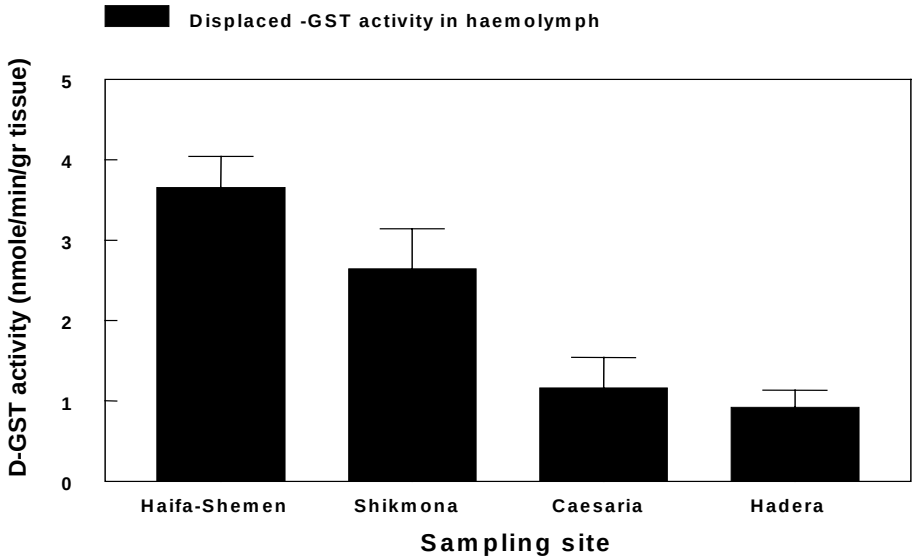
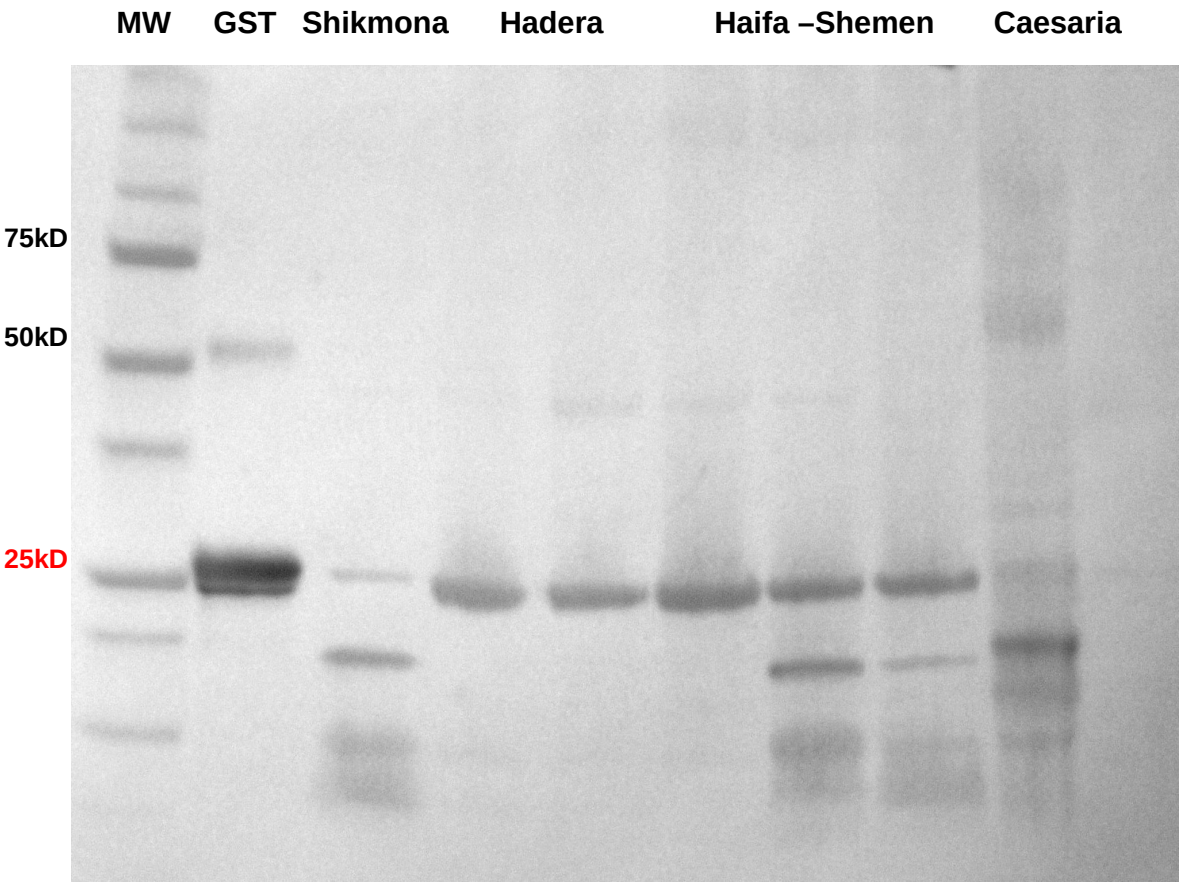


Figure 8. D-GST determination in the limpet *Patella caerulea* as biomarker of exposure to heavy metal ions in sampling sites along the Israeli Mediterranean in the spring Gonadal index was 12.

Plate I. Imunoblots of *Patella caerulea* haemolymph from specimens collected from various sites along the Israeli Mediterranean coast in August 2005. Primary antibody was a rabbit anti-α GST polyclonal antibody.



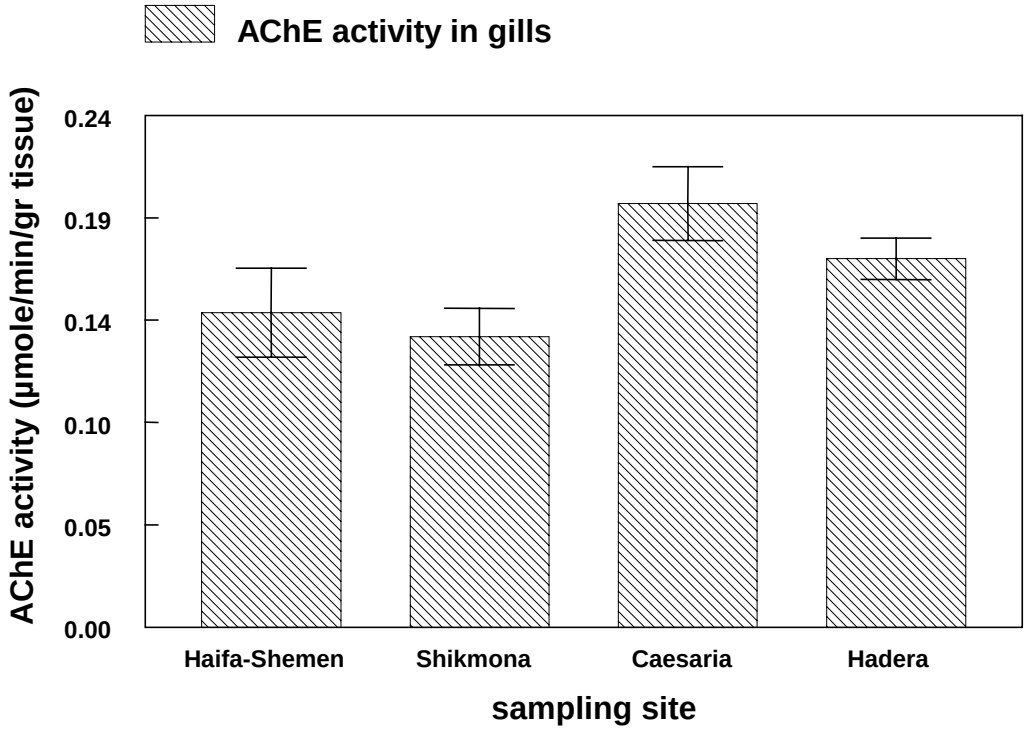


Figure 9. Acetylcholinesterase (AChE) determination in the gills of the limpet *Patella caerulea* as biomarker of exposure to organophosphates and carbamates in sampling sites along the Israeli Mediterranean in the spring.

DISCUSSION

The aim of the research was to bio-monitor the Israeli Mediterranean coast through the evaluation of the physiological status of local fish and limpet by determining the values of selected biological parameters (biomarkers) that are known to respond to the toxic effects of pollutants. The use of a battery of biomarkers is more advantageous than the use of a single biomarker as it offers an effective early warning system. The biomarkers derive from cell-biochemistry can detect pre-pathological alterations within the cells of the exposed organism before toxicological changes may occur and lead to mortality, population decline and impairment of the ecosystem.

Abundant information concerning CYP1A1 protein is available for some fish species but the effects of pollutant exposure on many other fish species that potentially may be candidates for biomonitoring programmes, has not been established. Among fish and other vertebrate groups there are marked species differences in response to inducers. Such differences could result from differences in properties of the Ah receptor, or response of the CYP1A1 gene(s) to activated receptor.

Time course of cytochrome CYP1A1 induction was characterized in liver of the mullet *Mugil capio* after treatment with Aroclor 1254. There was good correlation between CYP1A1 content and EROD activity. The induction of CYP1A1 in the Mugil was a slow process. Induction appeared in the liver only five days after fish were treated with Aroclor 1254, a procedure that took by itself two weeks. Even then induction of CYP1A1 was hardly noticed in Mugils that were treated with 50 mg/Kg while very pronounced in those that were treated with 100 mg/Kg. In higher doses CYP1A1 induction reached maximal level in 10 days after treatment and declined thereafter while in the fish

treated with the lower doses induction increased gradually and reached maximal levels 60 days after treatment.

Time course of induction of EROD activity in the liver of Aroclor-1254 treated Mugil followed well CYP1A1 induction, but the difference between the level of EROD induction in the fish that received the high dose (100 mg/Kg) to that in the fish that received the lower doses was much more pronounced than that exemplified by CYP1A1 induction. Furthermore, in the fish treated with the lower dose, EROD activity was hardly detected till 60 days after treatment.

High Acolor 1254 concentrations were needed to induce CYP1A1 protein in the mullet. This property of low sensitivity to CYP1A1 inducers, although uncommon in other fish species, was reported for other organisms including humans, where analysis of the kinetics and congeners selectivity for PCB metabolism showed that PCB concentration required for the induction of CYP1A1 and the associated toxic effects were greater than 600 mg/Kg (Brown et al., 1944).

Determination of EROD alone as a biomarker in the Mugil was less sensitive than that of CYP1A1 content. In order to monitor the contamination of the Israeli Mediterranean shores, using Mugil as test animal, it was necessary to apply both biomarkers CYP1A1 content as well as EROD activity. Several inducers or substrates of CYP1A1 synthesis can inhibit CYP1A1 catalytic activity. Such inhibition of CYP1A1 catalysis was recorded for EROD or AHH (aryl hydrocarbon hydroxylase) activities in microsomes from fish receiving high doses of benzo[a]pyrene (BaP) (Goddard et al., 1987), β-naphthoflavone (BNF) (Haasch et al, 1993), or planar chlorobiphenyls (Gooch et al., 1989; Miranda et al 1990b; Hahn et al., 1993), even though CYP1A1 protein and/or mRNA levels may remain elevated at those same high doses. There is evidence that inhibition of CYP1A1 catalytic activities occur in fish from sites highly contaminated by polychlorinated biphenyls. The inhibition of P450 catalytic activities may be competitive (Haasch et al, 1993), but other mechanisms such as alkylation of the heme by metabolites of nitrogen heterocyclic compounds (Ortiz de Montellano and Reich, 1986) or thiono-sulfur compounds (Neal, 1980) may also be involved.

Members of the genus Mugil are euryhaline, and thus able to enter estuarine and marine waters where were they may be exposed to a variety of foreign compounds that include CYP1A1 inducers. Ashdod in the south and Qiryat Yam near Haifa represent locations that are in the vicinity to harbour and heavy industry including refineries and power plants.

The highest induction of CYP1A1 and EROD activity was detected in Mugils sampled from those sites. The levels of induction of CYP1A1 and EROD in the Mugil from Qiryat Yam were as high or even higher than that recorded in Mugil that were treated with the highest dose of Aroclor 1254 in the laboratory. Riding and Hadera are located very close to power plants and a moderate exposure to petrochemical waste was detected in livers of Mugil sampled from those sites. Levels of CYP1A1 and EROD induction in Mugil from Jaffa (YFO), Yarqon river estuary and Sydni Ali were relatively low, representing very probably the general background typical to locations where the sources of pollutants are unclear and defused. Caesarea was expected to be a clean site. Yet, the low induction of the biomarkers in the liver of Mugils that were sampled there may indicate that some petrochemical pollution, that probably originate from the power plant located about 8 Km to the south, may casually contaminate this site. The correlation between CYP1A1 content and EROD activity in the microsomal fraction of Mugils sampled from the sampling sites Rval=0.726 was significant ($P<0.01$) but somewhat lower than that recorded for Mugil exposed at the laboratory by injection of Aroclor 1254 under controlled conditions Rval=0.823.

Metallothionein induction in the hepatopancreas of the Patella was used as a biomarker to monitor exposure to heavy metal ions in the marine environment along the Mediterranean coast of Israel. According to the results obtained, Shikmona and Caesarea were relatively unpolluted with heavy metal ions while some pollution was evident in Hadera and significant pollution in heavy metals was detected in Haifa-Shemen beach ($P<0.05$). The results obtained were as expected and thus MT appears to be a useful and reliable biomarker.

Displaced GST activity (D-GST) in the Patella Haemolymph was applied as a novel biomarker of exposure to heavy metals ions in sampling sites along the Israeli Mediterranean. The biomarker D-GST was not dependent on the reproductive cycle of the Patella. The results obtained were in good agreement with the expected pollution of the sampling sites. In specimens from Sdot-Yam and Michmoret, D-GST activities in haemolymph of the Patella were low as expected to be in clean resort sites. In Patella sampled from Shemen beach in Haifa D-GST activities were much higher ($P<0.01$) compared to all other sites. D-GST activity in Patella sampled from the clean sites Shikmona, Sdot-Yam and Michmoret were low as expected. Qiryat Yam and Shemen beach are located very near to Haifa harbour at the estuary of the Kishon River that carries petrochemical sludge.

Sampling of Patella in the spring reconfirmed that Haifa–Shemen site is a polluted site as D-GST activity in the Patella haemolymph was significantly higher ($P<0.01$) than that recorded in Patella from the clean site Caesarea as well as Patella from Hadera site located near a power plant. In Shikmona, D-GST activities were found to be high and probably, this site may be subjected occasionally to increased level of pollution. The two sites, Shikmona and Qiryat Yam suffer from incidental exposure to pollution. The source of this pollution in unclear but it may very well be from ships discharging their ballast water before entering Haifa harbour. In immunoblot of haemolymph taken from Patella from the main sampling sites gave an additional support to the kinetic results. In the immunoblot of Patella from Caesarea there was no indication for D-GST in haemolymph.

As expected, D-GST protein appeared in immunoblot of Patella from Haifa-Shemen. D-GST appeared also in Patella from Shikmona as could be expected from the kinetic results. Hadera site is close to a coal power plant and is also subjected to occasional increased levels of pollution as was exemplified also by the increasing induction of CYP1A1 and EROD in mullets sampled from this area.

The lysosomal stability in Patella blood cells was determined in samples of the limpet taken from several sites along the Israeli Mediterranean coastline at different seasons. The results obtained confirmed our earlier findings that *Patella caerulea* lysosomes were able to retain the dye only between August and October when the gonadal index value for the limpet was very small. The rest of the year, the gonadal index increases and reaches its highest value in March, just before sprawling. The hormonal activity associated with the gonads gross probably decreases the stability of the lysosomal membranes to the point that it was impossible to use the lysosomal stability in *Patella caerulea* as biomarker of exposure to pollutants.

Acetylcholinesterase activities were determined in the gills of Patella collected from four sampling sites during the Spring. Mean AChE activities recorded in Caesarea and Hadera were higher than those recorded in Haifa–Shemen and Sikmona but with no statistical significance. The decrease in AChE activities in Haifa–Shemen and Sikmona could be a result of non-specific anti-acetylcholinesteratic action of pollutants such as heavy metal ions apart from the existence of low level of OP and CB residues in the seawater in those sites.

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Biological effects of contaminants in mussels (Mytilus galloprovincialis) from the Iberian Mediterranean coast

by

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ABSTRACT

The use of mussels (*Mytilus sp*) as bio-indicators of the quality of the marine environment is a valuable approach to monitor pollution and its biological effects. In the framework of the integrated monitoring carried out by the Spanish Institute of Oceanography (IEO) along the Iberian Mediterranean coast, a set of biomarkers were measured in mussels (*Mytilus galloprovincialis*) collected from 19 sites in May-June 2003. Metallothioneins (MTs), lysosomal membrane stability (LMS) and micronucleus (MN) frequency were the biomarkers selected. MT levels showed significant differences between stations and were significantly correlated with Cd and Zn body burdens.

The neutral red retention time assay method (NRRT), used to evaluate the LMS in twelve mussel populations, always showed mean retention times (RT) lower than the RT minimum optimum established to consider that the mussel populations from this coast are healthy. The MN frequencies in gills of the mussels varied ranging from 1.9 to 11.4 MN/1000 cells. The lowest MN values were found in mussels from the reference site, while the highest levels of MN were registered in stations polluted by organic contaminants and metals, indicating genotoxic effects in mussels from these sites.

Thus, the results obtained demonstrate pollution effects in the Iberian Mediterranean coast, and the utility of a battery of biomarkers in the identification of areas where pollution is relevant.

INTRODUCTION

The Mediterranean coastline of Spain, 1.660 km long, has a population of 16 million, representing more than 39 % of the country's population, and urbanization affects the most valuable and fragile coastal biotopes, such as dunes, coastal forests, wetlands and beaches. As the population changes from season to season, due to tourism, and it increases considerably in the summer, the pressure from tourism and the urban development is affecting the coastal area that is still in pristine conditions. Another important economical activity of environmental concern is agriculture. In this coastal area, several pollution hot spots and areas of major environmental concern have been identified near the big cities (Barcelona, Tarragona, Valencia, Benidorm, Palma de Mallorca, Cartagena, Málaga and Algeciras), harbours with industrial areas and river mouths (Ebro, Segura and Jucar) (EEA, 2006). Thus, the costal area under study is mostly affected by land-based pollution sources (urban, industrial and agriculture wastewater) through direct disposal, continental runoff and atmospheric transport. Bivalve molluscs, and in particular mussels, are widely used as sentinels for pollution monitoring in coastal environments. These filter-feeding sedentary species are known to accumulate high levels of contaminants in their tissues, reflecting the environmental conditions. Following the Mussel Watch concept (Goldberg et al., 1978), concentrations of organic pollutants and heavy metals have been determined regularly along the Iberian Mediterranean coast by the Spanish Oceanography Institute (IEO). This monitoring programme covers largely the Mediterranean coastline of Spain with different types of sampling stations: Protected areas,

shellfish waters, river mouths, hot spots, reference zones, etc. Since 1990, monitoring identified the areas contaminated by heavy metals, polycyclic aromatic hydrocarbon (PAHs) and polychlorinated biphenyls (PCBs), and detected the temporal trends of these concentrations. However, from this chemical monitoring little was concluded about the biological effects of the pollutants considered.

In order to fill in this gap, the IEO Chemical Monitoring was improved incorporating, since 2000, a biological effects study that includes the use of biomarkers as an early warning system. Biomarkers consist of biochemical and physiological changes in organisms exposed to contaminants, and represent initial response to environmental perturbations and contamination (Shugart et al., 1992). In contrast to the simple measurement of contaminants accumulating in body tissues, biomarkers can offer a more complete and biologically more relevant information on the potential impact of toxic pollutants on the health of organisms (Van der Oost et al., 1996). Mussels are very suitable organisms to study the biological effects of pollutants. In this context, several biomarkers, recommended in the framework of the MED POL Programme (UNEP/RAMOGE 1999), have been applied and tested in the network monitoring carried out by the IEO to assess deleterious effects in biological systems. This study of biological effects was focused to evaluate specific stress (metallothionein content) and general stress (lysosomal membrane stability and micronuclei frequency) biomarkers.

Metallothioneins (MTs) constitute a family of low molecular weight cysteine rich proteins induced by a variety of metal ions. MT levels represent a specific biochemical indicator for the early detection of potential impact of heavy metals in the aquatic environment (George and Olsson, 1994). MTs play a primary role in the homeostasis of the essential metals Cu and Zn, and are involved in the detoxification of non-essential metals like Cd and Hg (Roesijadi, 1992).

Micronucleus (MN) cell frequency demonstrates objectively the level of genotoxic damage in response to the presence of mutagenic substances. Genotoxic biomarkers represent the primary event of the animal response after environmental exposure to toxic carcinogen and mutagenic compounds (UNEP/RAMOGE, 1999). The MN test has been successfully applied in organisms from marine and estuarine ecosystems.

Lysosomal membrane stability (LMS) is an indicator of general stress, because this biomarker responds to a wide range of environmental impacts, reflecting tissue concentrations of the main classes of organic and inorganic contaminants (Regoli 1992; Lowe and Pipe, 1994; Fernley et al., 2000; Viarengo et al., 2000; Domouhtsidou and Dimitriadis, 2001).

The main objective of the present work was to study the levels of MTs, MN frequency and LMS in mussels from the Iberian Mediterranean coast, in order to assess the quality of the marine environment. The results reported here were obtained in coastal areas under investigation through the chemical monitoring programme from the IEO during 2003. Thus, we have integrated the measurement of the biological effects in the monitoring network of the IEO to identify the exposure and toxic effects of the environmental contaminants.

MATERIALS AND METHODS

Mussels (30 - 42 mm shell length) were collected along the Iberian Mediterranean coast in May-June 2003, in the framework of the annual integrated monitoring programme performed by the IEO (Benedicto et al., 2004; Campillo et al., 2004). These native mussel populations were collected to study the concentrations of organochlorinated compounds, aromatic hydrocarbons and heavy metals in whole soft tissues, and at 19 sites for biomarker analysis (Figure 1). These samples were used to study the response of MTs, LMS and MN frequency.

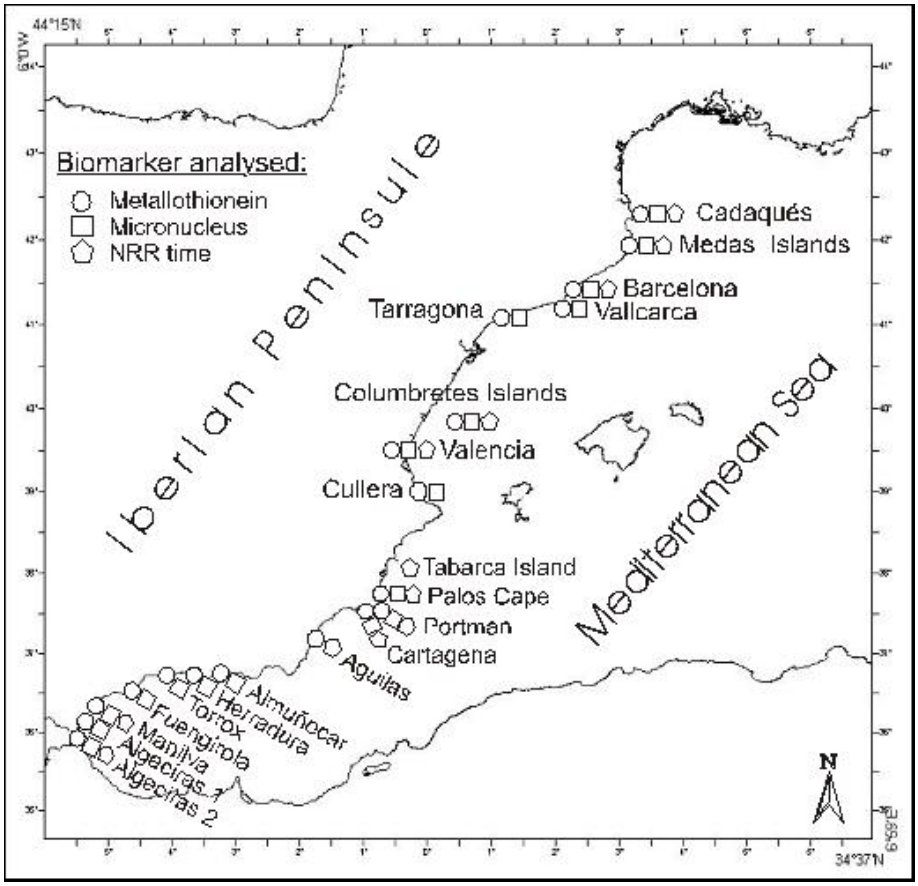

Figure 1. Map of the sites sampled in 2003 along the Iberian Mediterranean coast for the determination of the selected biomarkers.

Mussel digestive glands were rapidly dissected and stored in liquid nitrogen until processed for MT analyses. The mussels used for LMS and MN determinations were transported in refrigerated humid thermo-insulated containers to the laboratory. The mussels used for the LMS were kept overnight in clean aerated seawater (with the same temperature and salinity conditions as the original sampling sites) before testing started.

Metallothioneins

MT concentrations in mussel digestive gland were determined by the spectrophotometric method described by Viarengo et al. (1997). Digestive tissue was homogenized in three volumes of buffer (0.5 M sucrose, 50 mM Tris-HCl, pH= 8.9) containing 0.01% β-mercaptoethanol, 0.5 mM PMSF (phenylmethylsulphonilfluoride) and 3µg/ml leupeptin in a teflon glass potter homogenizer at 4 ºC. The homogenate was then centrifuged at 30.000 g for 20 minutes at 4ºC. To precipitate the high-molecular weight proteins, 1.05 ml of cold (-20 ºC) absolute ethanol and 80 µl of chloroform were added to aliquots of 1 ml of supernatant. The samples were then centrifuged at 6000 g for 10 minutes at 4ºC and the collected supernatant was combined with RNA (1 mg/10 µl) and 40 µl of 37 % HCl and three volumes of cold ethanol (to a final concentration of 87 %). The samples were maintained at –20 ºC for 1 hour and centrifuged at 6000 g for 10 minutes. The MT-containing pellets were washed with ethanol/choloform/homogeneization buffer (87/1/12), centrifuged at 6000 g for 10 minutes and dried under nitrogen gas stream. The pellet was resuspended in acid solution with 0.25 M NaCl, 1N HCl and 4 mM EDTA and 4.2 ml of the DTNB reagent (0.43 mM DTNB, 2 M NaCl, 0.2 M phosphate buffer, pH 8) was added. The sample was finally centrifuged at 4000 g for 5

minutes and the supernatant absorbance was evaluated at 412 nm utilizing reduced glutathione as a reference standard. The concentration of MT protein was expressed in µg/g tissue (wet wt.) and calculated assuming a cysteine content in a mussel MT molecule of 21 residues and a molecular weight of 8600 Da (Mackay et al., 1993). The MT analysis was performed on 6-8 pools of five digestive glands per station.

Micronuclei frequency

The micronucleus test was done in gills of mussels immediately dissected after their arrival to the laboratory. The gills were removed and cut in small pieces with scissors and digested for 10 min a 37 ºC in modified Hank`s Balanced Salt Solution containing a 0.1 mg/ml Dispase I (neutral protease, grade I). The cellular suspension was filtered and after centrifugation at 400 rpm for 10 minutes, aliquots of cellular pellet were fixed in methanol/acetic acid (3:1) for 20 minutes, dropped onto clean glass slides, air-dried and then stained with 3% Giemsa. Before counting cells the slides were coded and scored blind. We analyzed MN in the subpopulation of cells prevailing in gill tissue, the main gill cells (Venier et al., 1997), and at least one thousand cells with preserved cytoplasm per mussel were scored under oil immersion at 1000 x magnification. The criteria for micronuclei identification were the following: not touching the main nucleus, similar or weaker staining than the main nucleus, size <1/3 of the main nucleus, on the same optical plane as the main nucleus, round or oval and not fragmented. MN frequency was measured in six organisms per station and expressed per 1000 cells.

Lysosomal membrane stability

LMS was tested by the neutral red retention time assay method (NRR), described in the biomarkers manual of the MED POL Biomonitoring Programme (UNEP/RAMOGE, 1999) and also recommended by ICES (2004), with minor modifications. A stock of neutral red solution was prepared by dissolving 20 mg of dye in 1 ml of DMSO and a working solution then prepared by diluting 3 µl of the dye stock solution with 997 µl of physiological saline. The NRR assay was made on small granulocytes. 0.2 ml of haemolymph was withdrawn from the anterior adductor muscle into a 1.0 ml hypodermic syringe fitted with a 25-gauge needle and containing 0.2 ml of physiological saline. The suspension, obtained from each mussel, was spread on a slide, transferred to a lightproof humidity chamber to allow the cells to attach. Then, the neutral red working solution was added and after a 15 minutes incubation period, slides were examined systematically under a light microscope every 15 minutes (x 400 magnification). The test for an individual sample was terminated when the lysosomal dye loss was evident in 50% (numerically assessed per field of views and counting 250-300 cells, using a hand counter) of the small granular haemocytes, and the time recorded. Fifteen specimens from each site were taken for the NRR assay.

Data analysis

One-way analysis of variance (ANOVA) was applied to test differences in MT concentrations between the sampling stations. When significant differences were detected ($p < 0.05$), Tukey-b's test was used to determine which locations were significantly different from each other. The relation between metal concentrations and MT levels was tested using Spearman correlation analysis. Differences in MN frequencies were evaluated with the Mann-Whitney U-test.

Once the normality of the NRR data was assumed, the equality of group variances was tested using the Levene statistic (Levene, 1960). Comparisons of NRR mean values between areas were performed by one-way ANOVA. When significant differences were found, Tukey´s test was also applied.

All significance tests were performed at the significance level of $p < 0.05$. Statistical analysis was performed using the SPSS programme (v 11.0).

RESULTS AND DISCUSSION

Metallothioneins

MT induction has been proved in mussels after the experimental exposure to Cd, Cu, Zn and Hg (Bebianno and Langston, 1991; Pavicic et al., 1993; Bolognesi et al., 1999; Viarengo et al., 2001). Low levels of these proteins are present under natural environmental conditions in bivalves' tissues, but anthropogenic changes of trace metal concentrations in seawater induce increases in MT concentrations (Bebiano and Langston, 1991). Field investigations have shown that MTs can be used as a biomarker of exposure for certain metals in mussels (Bebianno and Machado, 1997; Stien et al., 1997; Viarengo et al., 1999; de Lafontaine et al., 2000; Lionetto et al. 2001). More precisely, the digestive gland of mussels has been proposed as the best biological matrix for MT determination (Pavicic et al., 1993; Amiard et al., 1998; Mourgaud et al., 2002).

MT concentrations were measured in indigenous mussels in order to assess the spatial variation of exposure to heavy metals along the Iberian Mediterranean coast. MT levels measured in the digestive gland of mussels, collected from nineteen stations, are shown in Fig. 2. The concentrations of MTs were fluctuating within the range of 70-280 μg/g ww and were similar to the levels measured in mussels collected from other Mediterranean sea areas using the same spectrophotometric method (Pavicic et al., 1993; Stien et al., 1997; Viarengo et al., 1997; Cotou et al., 1998; Bolognesi et al., 1999; Ivankovic et al., 2003; Kalpaxis et al., 2004; Domouhtsidou, et al., 2004) or the Baltic sea (Leiniö and Lehtonen, 2005; Barsiene et al., 2006; Kopecka et al., 2006).

Significant differences in MT expression were observed among stations. Concentrations were significantly higher than at Valencia at four sites (Portman, Palos Cape, Manilva and Columbretes Islands). The higher levels of MTs found in these areas can represent an early warning of possible toxic effects by heavy metals on marine organisms. Among these four sites showing the highest MT concentrations, three also showed the highest concentrations of Cd from the Iberian Mediterranean coast (Columbretes Islands, Portman and Palos Cape). The concentrations of Cd in the whole soft tissues of mussels from these stations ranged from 1.45-1.65 mg/kg dw (data from the IEO chemical monitoring programme).

Two of these sampling stations, Portman and Palos Cape, are located near Portman Bay (Murcia), southeast Spain. The bay was filed up with tailings from 1958 to 1991 and the surroundings contain the most seriously metal-contaminated sediments of the Mediterranean Sea (Cesar et al., 2003). The high level of Cd found at Columbretes Islands Marine Reserve is probably related to the pollution derived from the industrial and urban area of Castellón.

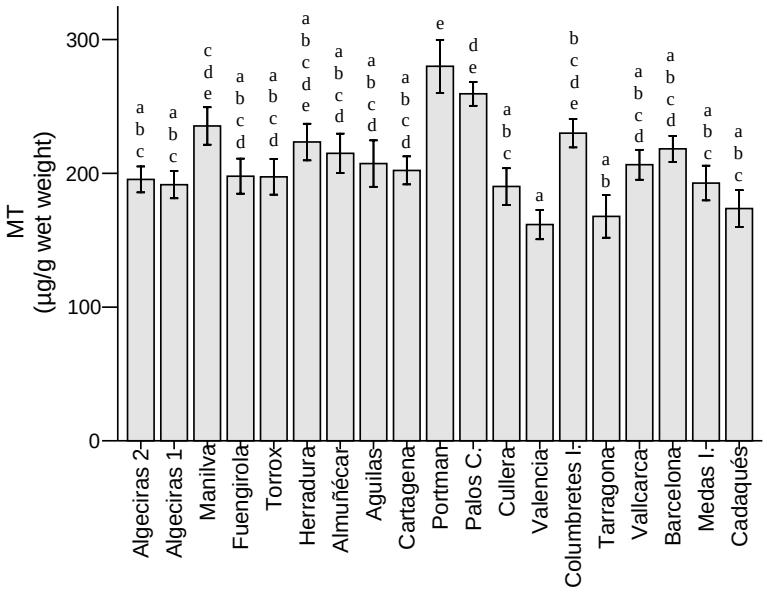

Figure 2. MT concentrations (mean ± S.E.) in digestive glands of mussels. Stations with a common letter subscript were not significant different in a Tukey-b's test following a 1-way ANOVA.

The correlation studies between MT levels and metal concentrations in the whole soft tissues of mussels showed significant relationship with Zn (R= 0.500, $p<0.05$, N=19) and Cd (R=0.679, $p < 0.01$, N= 19) , metals which are considered as potential inducers of MT and/or are able to bind this protein. In contrast, no significant correlations were found for Hg (R= 0.278, p=0.250, N=19) and Cu (R=0.248, p=0.305, N=19), two metals which are also able to induce MT expression. These results are in agreement with other field studied, which have demonstrated the relationship between the environmental exposure to heavy metals and MT levels. Bebianno and Machado (1997) found that MT concentrations in whole soft tissues of native mussel collected from the Algarve coast (Portugal) were related with Cu and Cd concentrations, whereas Mourgaud et al. (2002) demonstrated significant correlations between MT levels and the accumulation of Cd, Cu, Ni and Zn in transplanted mussels from the French Mediterranean coast.

These results indicate that MTs can give a biological meaning to the chemical concentration values, thus providing a suitable monitoring procedure to assess biological availability and early impact of heavy metals on marine organisms.

Micronuclei frequency

MN test has been applied for assessing genotoxic effects of environmental contaminants in mussels in both laboratory (Mersch et al., 1996; Mersh et al., 1997; Venier et al., 1997; Bolognesi et al., 1999; Bolognesi et al., 2004; Siu et al., 2004) and field studies (Brunetti et al., 1988; Bolognesi et al., 1992; Burgeot et al., 1996; Dolcetti and Venier, 2002; Dailianis et al., 2003; Izquierdo et al., 2003; Barsiene et al., 2004; Venier and Zampieron, 2005). Although MN frequency can be conducted in different tissues, we selected gills to apply this test as brachial epithelium represents the primary target for water-borne and tends to have higher MN frequencies than haemocytes (Venier et al., 1997; Bolognesi et al., 1999; Dailianis et al., 2003).

The frequency of MN in gills of the mussels from the Iberian Mediterranean coast varied in a range from 1.9 to 11.4 ‰ (Figure 3). The lowest frequency was found in the reference station of Cadaqués (1.9 ‰), an unpolluted area located in the northeast Spain. The temperature recorded in this station during the survey was 17 ºC. Water temperature is also known to have an influence on MN frequency as reported for mussels (Brunetti et al., 1992). Thus, MN

frequency measured at this reference station was similar to the spontaneous gill MN frequency recorded on *M. galloprovincialis* from unpolluted areas of the Mediterranean Sea within the temperature range of 15 to 20 ºC (Brunetti et al., 1992).

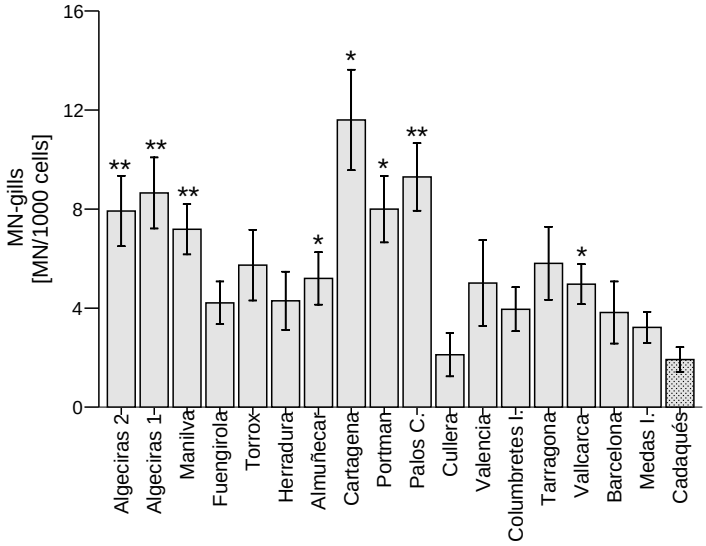

Figure 3. Mean (± S.E.) micronuclei frequency (‰) in gill cells. Levels significantly higher than at the control site (Cadaqués) are indicated: * p <0.05, ** p<0.01 (Mann-Whitney U-test).

The statistical analysis showed significantly higher MN frequencies in Algeciras1-Punta Carnero, Algeciras 2-Gudarranque, Manilva, Almuñecar, Portman, Palos Cape and Vallcarca than in Cadaqués. MN frequency levels in these sampling stations ranged between 5-12 ‰.

The results from the chemical monitoring point out that the mussels collected from Cartagena, Portman, Algeciras 2, Tarragona and Vallcarca presented high concentrations of PAHs, PCBs, DDTs or metals. These chemical pollutants may explain the high MN frequencies found in these sampling stations. The low extent of genotoxic effects observed in Barcelona was clearly in contrast with the high levels of organic contaminants found in mussels from this hot spot. The low MN frequency found at Barcelona might be related to adaptation phenomena: it is known that animals subjected to long-term exposure to genotoxic compounds may reduce their MN frequencies (Majone et al., 1988; Scarpato et al., 1990). Similarly, Bolognesi and Degan (2001) found in mussels from La Spezia harbour (Ligurian coast), an area with a high degree of pollution, a low MN frequency level in contrast with the chemical analysis results.

MN frequencies reported in this work were not always consistent with the pattern of pollution of some sampling stations. Almuñecar and Manilva presented high MN frequency levels, which cannot be explained by the concentration of contaminants found by the chemical monitoring. These results could be partly attributed to the fact that the contaminants studied by the chemical monitoring are a fraction of the total bulk of the toxic agents present in the marine environment. Thus, these results showed the feasibility of MN test on mussel gill cells as a tool to detect the presence of genotoxic pollutants in the marine environment and to complement the chemical monitoring programme as another means of evaluating water quality.

Lysosomal membrane stability

Mean retention times (RTs) are shown in Figure 4. NRR times showed significant differences between specimens collected from different sampling sites. Results showed two homogenous subsets and significant differences were found between Manilva and Portman ($p = 0.002$), Cartagena ($p = 0.007$), Columbretes Islands ($p = 0.007$), and Barcelona ($p = 0.013$) (Figure 4). The highest mean values in NRR times obtained were observed in mussel populations from Manilva

(54.0 ± 26.7 minutes) and Medas Islands (41.10 ± 7.81 minutes) as these areas could be characterized as control sites as far as chemical monitoring is concerned (Unpublished data, IEO). In contrast, mussel populations from Cartagena (18.0 ± 5.0 minutes), Portman (15.00 ± 7.02), Barcelona (19.7 ± 3.6 minutes in 2003) and Columbretes Islands (18.0 ± 4.1 minutes in 2003) showed lower capacity to retain the dye.

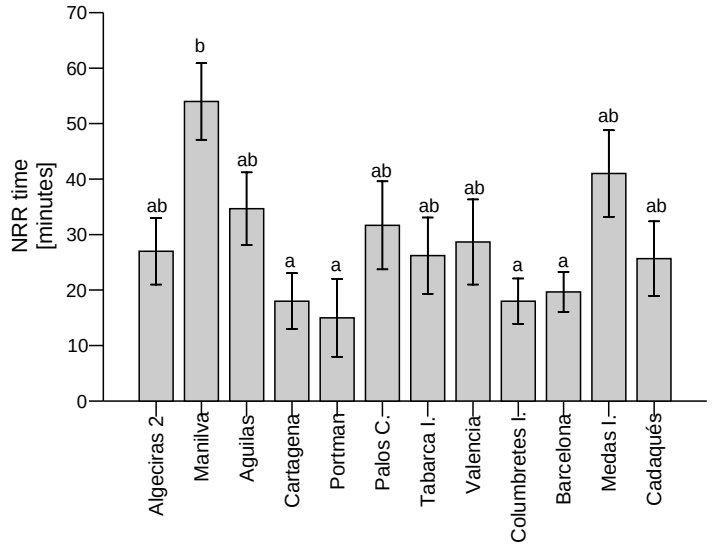

Figure 4. Mean (± S.E.) neutral retention times (minutes) obtained in samples from each station (n = 15). Stations with a common letter subscript were not significant different in a Tukey's test following a 1-way ANOVA.

Portman, Cartagena and Barcelona are highly contaminated sites (urban and industrial activities). In the case of Columbretes Islands, declared as a marine reserve, trace metal levels in mussels are not particularly high, though Cd levels indicate contamination by this metal. Probably these low RTs observed in these samples could be due to pollution derived from the industrial and urban area of Castellón. Similar RT values have been reported in mussels from impacted zones of the Venice Lagoon (Lowe et al., 1995) and the Portuguese coast (Castro et al., 2004). In our study, mean RT values measured at all stations were lower than the minimum optimum established to consider that mussel populations are healthy (WGBEC, 2005). Following these assessment thresholds for NRR assay, mussel populations of all sampling sites selected along the Iberian Mediterranean coast in our study should be considered severely stressed, except Manilva the only station that might be considered stressed but compensating.

CONCLUSIONS

Overall, the biomarker results obtained clearly indicate the existence of biological effects in the Iberian Mediterranean coast due to pollution and provide the baseline levels of these biomarkers (MTs, MN frequency and LMS) in mussels from this sensitive Mediterranean Sea coast. The biomarker responses obtained appeared to be related mainly to the chemical pollutant levels, although the complexity of environmental contaminants can induce varieties of responses in organisms that are not necessarily correlated (Viarengo et al., 2000).

Thus NRR assay, biomarker related with the exposure to organic and inorganic pollutants, showed an important degree of general stress in the mussels collected from Portman and Columbretes Islands. MT levels found in these stations suggest that the concentrations of bioavailable heavy metals are responsible for the biological effects. This latter point appears to be also confirmed by

some of the high levels of cadmium detected in mussels from these locations. These results are in accordance with the significant negative correlation between LMS and MT content reported by Domouhtsidou et al. (2004) in mussels collected from the Eastern Mediterranean Sea. The high MN frequencies and low NRR times found in mussels from Cartagena appear to be more related to the existence of biological significant levels of organic pollutants. This station is located near an important urban and industrial area, and the mussels have high levels of PAHs and PCBs. However, the biomarker responses were not always consistent with the pattern of pollution indicated by the chemical analyses.

Thus, the biomarkers used in this work appear to be useful for application in regular monitoring activities, and reinforce previous studies about the utility of a battery of biomarkers as an early warning signal with a capacity to identify areas where inorganic and organic pollution is relevant.

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Biomonitoring the pollution in Thermaikos Gulf - northern Greece (2001-2005) using cellular biomarkers

by

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ABSTRACT

The pollution levels of Thermaikos Gulf-northern Greece were estimated using four pollution biomarkers ('lysosomal membrane stability in cryosections', 'lysosomal membrane stability-neutral red retention assay', 'metallothionein content' and 'acetyl-cholinesterase activity'). The biomarker values were estimated in ten 6-monthly samplings from 2001 to 2005. Even though there were significant differences in each biomarker between sampling stations in the same sampling period, the overview of the four biomarkers in the ten 6-monthly samplings showed an 'homogenous' state of the pollution in the gulf, since the pollution levels estimated by the four biomarkers were comparable. The results of the last years should be regarded as significant for the levels of pollution in Thermaikos Gulf, since former measurements of our laboratory (period 1996-1998) using lysosomal membrane stability test, had shown pollution much lower levels, as well a clear differentiation of the pollution levels between the eastern and western coasts of Therrmaikos Gulf.

MATERIALS AND METHODS

Mussels (*Mytilus galloprovincialis)* were collected from 4 stations in Thermaikos Gulf and one station in Strymonikos Gulf in ten six-monthly sampling periods from 2001-2005. Mussels were sampled in early June and late October from Aggelochorion (1) cultured population, Peraia (2) natural population, from a natural population located near the outlet tube of the sewage treatment plant of Kalohori (3), as well as from cultured populations in Halastra (4). Mussels were also sampled from a cultured population in Olympiada (5) located in Strymonikos Gulf, which was selected as a reference station (Fig. 1).

Figure 1. Map of the sampling stations in Thermaikos Gulf. Aggelochorion (1), Peraia (2), Outlet tube (3) and Halastra (4). Reference station, Olympiada (6) is placed in Strimonikos Gulf.

Biomarkers used

The biomarker “lysosomal membrane stability” in cryosections (LMS) is based on the cytochemical detection of the lysosomal enzyme N-acetyl-β-hexosaminidase and on the time of acid labilization treatment required to produce lysosomal maximum staining intensity (Fig. 2). The test of “lysosomal membrane stability” is carried out on digestive gland cryosections and minor values are investigative of relatively polluted marine areas, while higher values are investigative of less polluted areas. This procedure was performed according to Moore (1976) and UNEP/RAMOGE (1999).

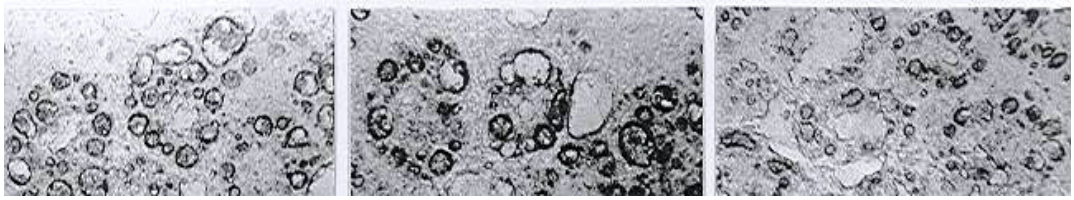

Figure 2. The time of the maximum staining intensity of the lysosomes characterizes the examined area.

The biomarker ‘lysosomal membrane stability-neutral red retention assay’ (NRR) is a rapid and sensitive test for determining the lysosomal membrane stability against environmental toxic factors. The NRR measures the retention of neutral red (a weak base dye) within the lysosomal compartment (Fig. 3). According to the test carried out on haemocytes, lower values correspond to mussels from polluted areas, while higher values to mussels from clean ones. The procedure was performed according to Lowe and Pipe (1994), with slight modifications.

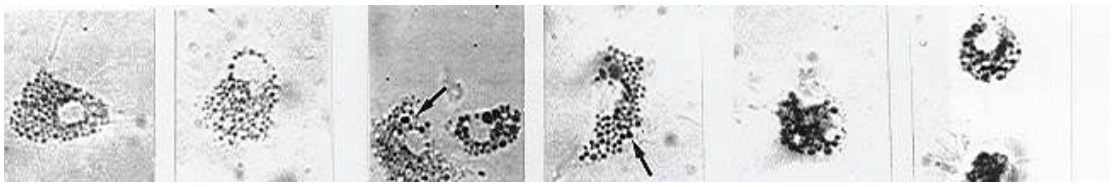

Figure 3. Various stages of the sequestration of the dye neutral red inside the mussel haemocytes (arrows).

Metallothionein (MT) determination in tissues of organisms exposed to heavy metals is regarded as a significant biomarker of environmental stress. The procedure was done according to Viarengo et al. (1997) and UNEP/RAMOGE (1999) and is based on the determination of the sulphyhydryl (-SH) residues content. The metallothionein content was analysed in the digestive gland of mussels *Mytilus galloprovincialis*. High amounts of -SH groups in samples tested represent a good biomarker of heavy metal pollution.

Inhibition of acetyl-cholinesterase (AChE) activity has been demonstrated as a good procedure evaluating the presence of organophosphates and organic pollutants in the marine environment. For the measurement of AChE activity, a modification of the method of Ellman et al. (1961), proposed by Galgani and Bocquene (1988) and UNEP (1993) was used. Acetylthiocholine iodide (21.67 mg/ml) was added as substrate to initiate the enzymatic reaction with the use of DTNB (Ellman et al. 1961). The enzymatic activity of acetylocholinesterase was tested in gills and digestive gland of mussels *Mytilus galloprovincialis*.

Statistical methods used

Data on the LMS, AChE activity and metallothionein content was tested using non-parametric statistics (Mann–Whitney U-test, $p<0.05$), while data on NRR assay, was tested using Duncan's test ($p<0.05$, breakdown and one way ANOVA). The analyses were carried out using the STATISTICA statistical package (STATISTICA, Microsoft Co.).

RESULTS AND DISCUSSION

Evaluation of lysosomal membrane stability in cryosections

The results, as well as their statistical interpretation (Domouhtsidou et al., 2003; Koukouzika and Dimitriadis, 2005) for the ten 6-monthly samplings from 2001-2005 are summarized in Fig. 4. A simplifying representation of the levels of pollution using the values of this biomarker is presented in Fig. 5. According to them, the 'lysosomal membrane stability' values for the Olympiada station were statistically higher (lower levels of pollution) compared to the values of the sampling stations in Thermaikos Gulf. Even though there were differences among sampling stations in Thermaikos Gulf in each sampling period, as an overview the pollution levels showed an 'homogenous' status, since the values of this bomarker at all stations and in all sampling periods were more or less comparable.

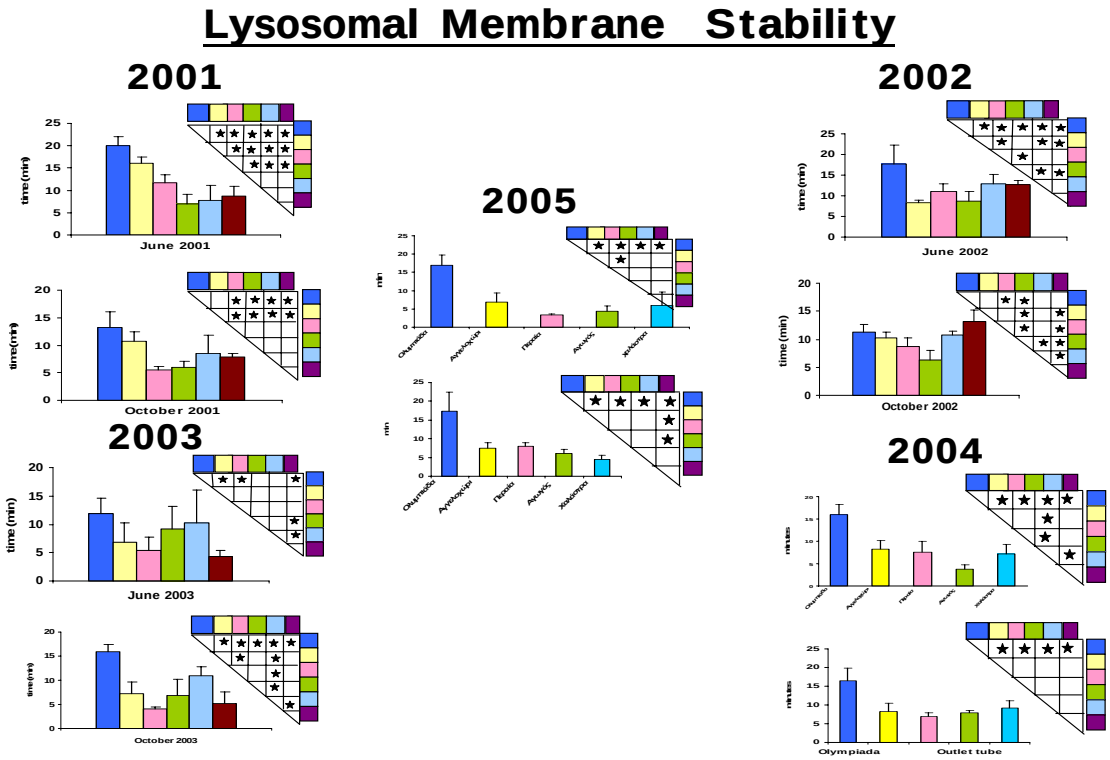

Figure 4: Values of lysosomal membrane stability from the sampling stations in Thermaikos and Strymonikos Gulfs in the 10 six months sampling period from 2001-2005. Statistical signification was based on Mann- Whitney U-test ($p< 0.05$).

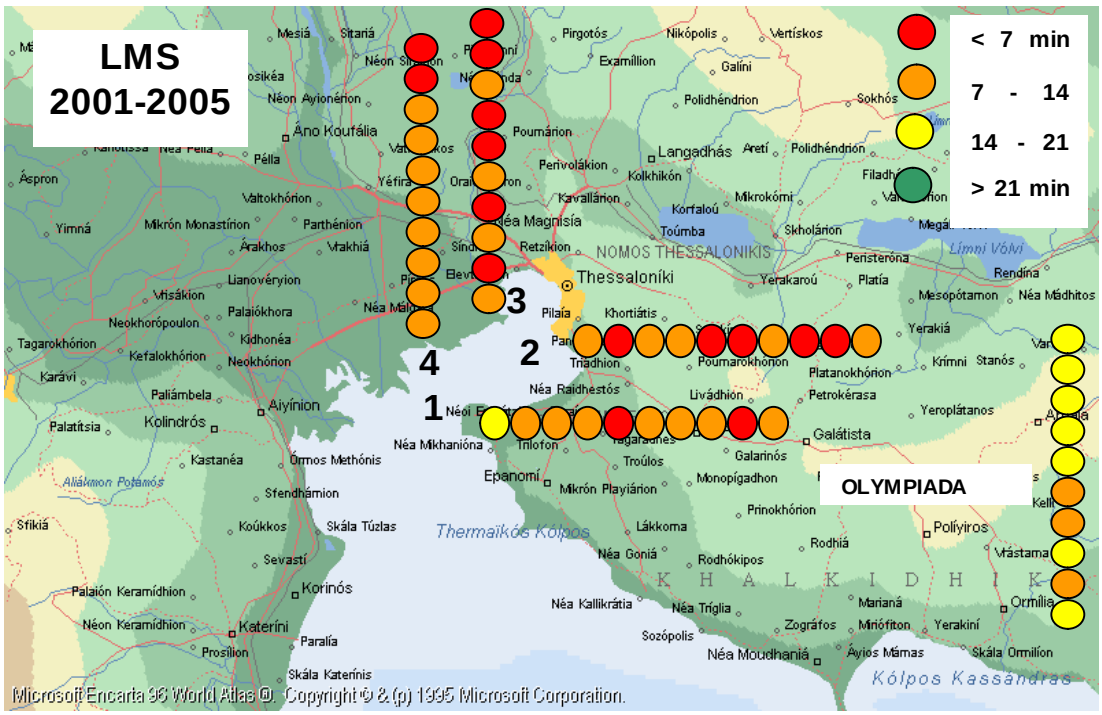

Figure 5. Simplified representation of the levels of pollution using the values of the biomarker 'lysosomal membrane stability'. The cycles from the base to the top or from left to the right represent the ten six-monthly sampling periods from 2001-2005 for Aggelochorion (1), Peraia (2), Outlet tube (3) and Halastra (4). On the lower right, there is the evaluation for reference station (Olympiada), while on the upper right is the scale of values.

Evaluation of 'Lysosomal membrane stability-neutral red retention assay'

According to the results, their statistical interpretation ((Dailianis et al., 2003; Koukouzika and Dimitriadis, 2005), as well as the simplified representation of the levels of pollution using the values of this biomarker (not shown), the "neutral red retention assay" values for the Olympiada station was statistically higher (lower level of pollution), in relation to the values of the sampling stations in Thermaikos Gulf. Similar to the biomarker LMS, the pollution levels using neutral red retention assay show a 'homogenous' status. Even though there were differences of the biomarker between sampling stations in the same sampling period, the overview of this biomarker for the 10 six monthly sampling periods show comparable values. The only station presenting lower level of pollution in general according to this biomarker was the station of Halastra.

Evaluation of 'Metallothionein content' and 'Acetyl-cholinesterase activity'

According to the results, their statistical interpretation (Dailianis et al., 2003; Domouhtsidou et al. 2003), as well as the simplified representation (not shown), the pollution levels estimated by these biomarkers were statistically lower in Olympiada station, compared to the levels in Thermaikos Gulf. Even thought there were differences among sampling stations of Thermaikos Gulf in the same sampling period, the general view of both biomarker in all stations and in all periods was more or less comparable. However, the overall pollution levels evaluated by these biomarkers (affected mainly by heavy metals and pesticides, respectively) were relatively lower compared to those estimated by the former biomarkers used, i.e. LMS and NNR.

Evaluation of the levels of pollution using <u>a combination of all biomarkers</u> used

The evaluation of the levels of pollution using a combination of the values of all biomarkers used by summing them up is still under investigation (Raftopoulou, et al., 2006). However, the preliminary results are shown in the figure below (Fig. 6). According to this, the pollution levels estimated by the combination of the four biomarkers in the 10 six month sampling periods (2001-2005) are, more or less, comparable in the whole gulf, showing in that way an 'homogenous' state of the pollution. The results of the last years should be regarded as significant for the levels of pollution in Thermaikos Gulf, since former measurements of our laboratory for the period 1996-1998 (Domouhtsidou and Dimitriadis, 2001) using lysosomal membrane stability test, had shown pollution levels much lower, as well a clear differentiation of the pollution levels between the eastern and western coasts of Thermaikos Gulf (Fig. 7).

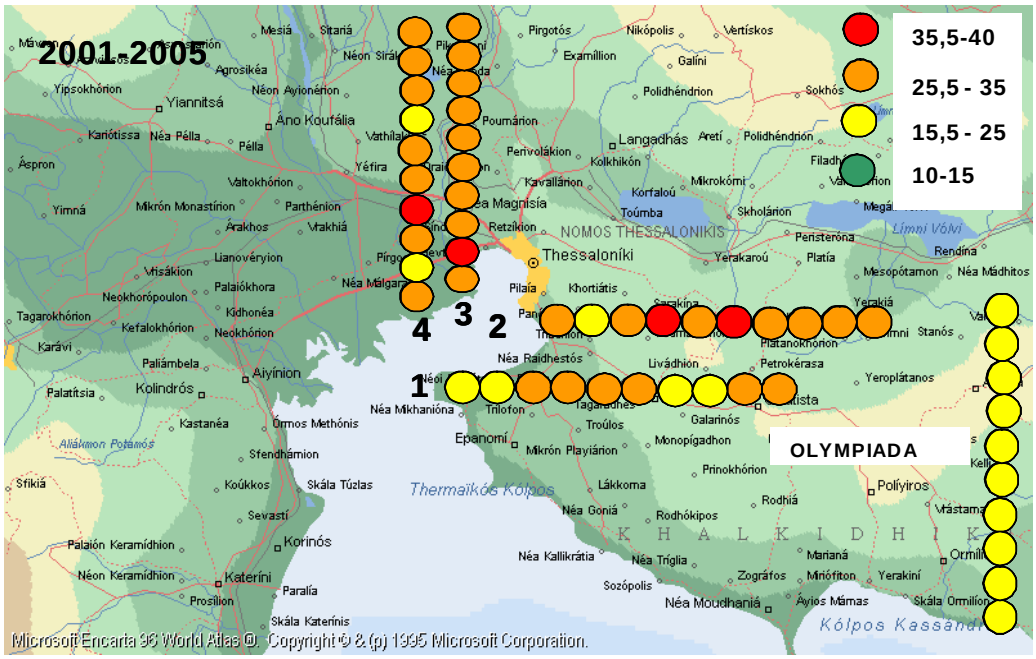

Figure 6. Simplified representation of the levels of pollution using preliminary results of a combination of the values of all biomarkers used. The colored cycles from the base to the top or from left to the right represent the two sampling periods on 2005 for Aggelochorion (1), Peraia (2), Outlet tube (3) and Halastra (4). On the lowest right, there is the evaluation for reference station (Olympiada), while on the upper right is the scale of values.

Comparing the results of physical and chemical contaminants from other studies

In the final report of ΔΕΠΕΘ (Laboratory Network of the Environmental Quality Monitoring of the Hellenic Seas), which refers to results of 2004 and 2005 in Thermaikos Gulf, it is reported that the overall concentration levels of pollutants, mainly DDTs, PCBs and PAHs were found to be not particularly high in the water and in mussels of Thermaikos Gulf.

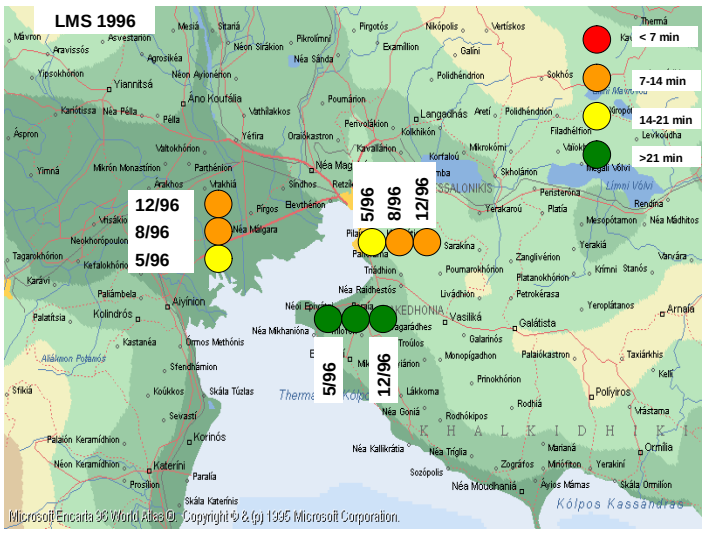

Figure 7. Levels of pollution evaluated by LMS on May, August and December 1996 (Domouhtsidou and Dimitriadis, 2001). Note that Aggelochorion (1) displayed low levels of pollution compared to the results of 2001-2005. Mikro Emvolo (2), Halastra (3). On the upper right is the scale of values.

Moreover, the report of the Hellenic Center for Marine Research (HCMR) includes data from Thermaikos Gulf for the year 2000 on a number of chemical and physical parameters including heavy metals, nitrates, dissolved oxygen etc. In the report, the overall concentration levels of most of the contaminants were below the maximum permitted limits, while relatively high concentrations were recorded for Fe and Mn. It was concluded that there is a need to remove nitrates and phosphates from the wastes of the wastewater treatment plant of the city of Thessaloniki. In addition, in mussel cultures, an oxygen demand was recorded, which was attributed to the organic pollutants in these areas.

Comparing the results of the present report with those above mentioned, it could be supported that the relatively high specific and general stress on mussels reported in the Thermaikos Gulf (Dailianis et al., 2003; Domouhtsidou et al., 2003; Koukouzika and Dimitriadis, 2005), is probably related to the increased levels of nitrates and phosphates recorded in the Gulf, as well as to the low levels of oxygen close to mussels cultures. In any case, the crucial point is the following: according to chemical monitoring, a close estuary system such as Thermaikos Gulf (located near a big city and its industrial zone) polluted by a huge number of chemicals could be characterized as a system that feels the 'environmental quality standards' (since the majority of the pollutants are under the maximum permitted limits); however, its water quality is quite inappropriate and harmful for the physiology of the living organisms and that is showing by the ecotoxicological methods.

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Biomonitoring of the Gulf of Patras (Greece) using caged mussels

by

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ABSTRACT

The Gulf of Patras is located in Northern Peloponnese, Greece. Despite the fact that most of the industries have been transferred to other regions and a sewage treatment plant has been in operation for five years, some pollution sources, like Glafkos River and a local harbour continue polluting the gulf. Five years ago, a long-term field study was initiated in the framework of the UNEP/MAP/MED POL Biomonitoring programme. Specimens of *Mytilus galloprovincialis* were placed in bow nets and immersed at 3-10 m depth in Itea, a clean coastal region (reference area), and in two marine stations along the Gulf of Patras. Heavy metals in the surrounding waters and a battery of biomarkers in the digestive gland and gill cells isolated from caged mussels were assessed. In addition, the polysome content and the efficiency of ribosomes from digestive gland cells at initiating protein synthesis were estimated. Covering a 5-years period, the whole set of data suggests that the degree of pollution in the Gulf of Patras differs between different sites and depends on agricultural, urban and industrial influences. Although, a progressive decrease of pollution is observed in the Gulf of Patras during the last two years, the continuation of the biomonitoring programme seems to be necessary in order to arrive at more concrete conclusions. In addition, the results support the notion that downregulation of the global protein synthesis is an important component of the cellular stress response and may be exploited to monitor biological effects of pollution.

MATERIALS AND METHODS

Mussels and area descriptions

Mussels, (5-7 cm shell length, 7-9 g soft tissue wet wt), never exposed to growth stimulants, were provided by a marine farm. Specimens of these mussels were placed in bow nets and immersed at 3-10 m depth, in a site near Itea region, far from pollution sources (reference area). Another sample of mussels was transplanted by the same system in two coastal stations along the Gulf of Patras. Station 1 (38o 12′ 48" N, 21o 42′ 42" E) was in front of the estuaries of Glafkos River, which crosses several intensive agricultural areas and also receives domestic and industrial effluents. Station 2 was in a less contaminated area, 20 Km east of Patras town, with little organic pollution, but enriched in metals, particularly in Zn and Cr. After exposure for 1 month, the caged mussels were collected and their soft parts were dissected into gills and digestive glands, within 3h.

Physicochemical analysis of seawater, sediments, and mussel digestive glands

Heavy meals in seawater and sediments, except for mercury, were measured by flameless atomic absorption spectrophotometry (AAS), according to Jan & Young (1978). Mercury was determined by the cold vapor AAS method (Freimann & Schmidt, 1984). Metal were also analyzed in a composite sample of digestive glands excised from 10 mussel specimens and digested with nitric acid according to Stein et al (1998). Dissolved oxygen (DO), salinity and biological oxygen demand during 5 days (BOD_5) in seawater were analyzed according to Franson (1995).

Biomarker analysis

Micronucleus frequency was measured in gill cells, as indicated by Bolognesi et al. (2004). Metallothioneins were partially purified from digestive gland extracts and determined as described by Viarengo et al. (1997). Lysosomal membrane stability assay was performed according to Regoli (1992).

Isolation of ribosomes from digestive gland cells and estimation of polysome content

Digestive gland pools (3 g) were homogenized in 9 ml of buffer A (20 mM Tris-HCl pH 7.6, 150 mM NH_4Cl, 10 mM magnesium acetate, 0.5 mM EDTA, 58 µg/ml PMSF, 250 mM sucrose and 6 mM β-mercaptoethanol) at 4°C. The homogenate was adjusted to 0.5% deoxycholate and 200 µM cycloheximide, and then was centrifuged twice at 13,000×g for 20 min. The final supernatant was centrifuged at 120,000×g for 6 h at 4°C. The pellet was resuspended in 10 ml of buffer B (50 mM Tris-HCl pH 7.6, 150 mM NH_4Cl, 10 mM magnesium acetate 200 µM cycloheximide, 6 mM β-mercaptoethanol) and recentrifuged as above. Finally, the pellet was resuspended in 2 ml of buffer B, diluted against the same buffer, and stored at –70°C until analysis. To obtain the sedimentation profile of ribosomal particles, 10 A_{260} units of ribosomal material were loaded on a 15-40% linear sucrose gradient in buffer B, centrifuged at 37,000×g for 6 h at 4°C in a SW60 rotor (Beckman), and analyzed by optical scanning at 260 nm.

Protein-synthesis initiation test

Runoff ribosomes, that are ribosomes stripped of endogenous mRNAs and peptidyl and/or aminoacyl-tRNAs, were prepared by incubating the ribosomal suspension (10 A_{260} units of ribosomes per ml) with 0.5 mM puromycin, at 30°C for 30 min (Ferreras et al., 2004). Crude acetyl[^{3}H]phenylalanyl-tRNA (Ac[^{3}H]Phe-tRNA) charged with 15 pmol of [^{3}H]Phe per A_{260} unit, and partially purified translation factors were prepared from digestive gland extracts, as described previously (Kalpaxis et al., 2003). To test the efficiency of ribosomes to initiate protein synthesis, runoff ribosomes (32 A_{260} units per ml) were incubated at 25°C for specified time intervals, with 13.4 A_{260} units/ml Ac[^{3}H]Phe-tRNA in buffer 50 mM Tris-HCl pH 7.2 containing 50 µM spermine, 2 mM spermidine, 50 mM NH_4Cl, 10 mM $MgCl_2$, 0.4 mM GTP, 320 µg /ml poly(U), 400 µg/ml translation factors and 6 mM β-mercaptoethanol. The ternary ribosomal complex (Ac[^{3}H]Phe-tRNA·poly(U)·ribosome) formed, was purified through adsorption on cellulose nitrate filters. The amount of the ternary complex formed was calculated by measuring the trapped radioactivity on the filters.

RESULTS

Chemical characterization of the mussel sampling sites and bioaccumulation of metals in digestive gland cells

The concentrations of heavy metals in digestive glands of mussels were higher in Stations 1 and 2 than at the reference area. This implies that both stations represent areas polluted by metals. Heavy metal analysis of sediments and seawater from the same regions confirmed that Station 1 was seriously polluted by Cu and Pb, while Station 2 was polluted by Zn and Cr. BOD_5 analysis and the somewhat lower values of pH and DO in seawater of Station 1 hind an impact of organic contamination. Nevertheless, aromatic hydrocarbons (PAHs) or organochlorine pesticides (OCPs) were never found in high concentrations. The degree of bioaccumulation varied among the tested metals, being higher for Cd, Cu, and Zn. In addition bio-accumulation varied seasonally, reaching the lowest values in autumn (Fig. 1).

Standard-biomarker determinations

Metallothionein content in digestive glands was less in samples from the reference area than that found in samples from Stations 1 and 2. Also, metallothionein content was found lower in mussels from Station 2 than that measured in specimens from Station 1, except for the first year (Fig. 2). Generally, fluctuations in metallothioneins were related to fluctuations in heavy metal content of mussel digestive glands. This was verified by statistical analysis on data for each of the sampling campaign, regardless of the sampling site of mussels.

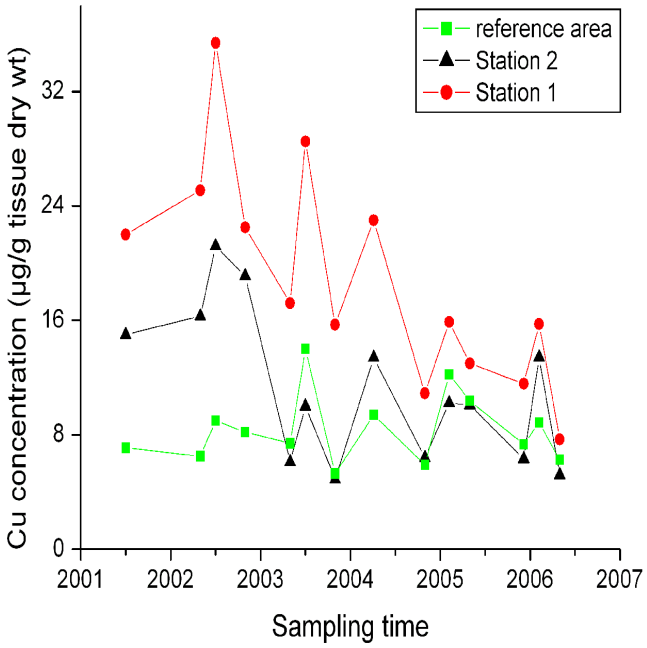

Fig.1. Cu concentration in mussel digestive glands from the reference area (Itea) and two stations in the Gulf of Patras.

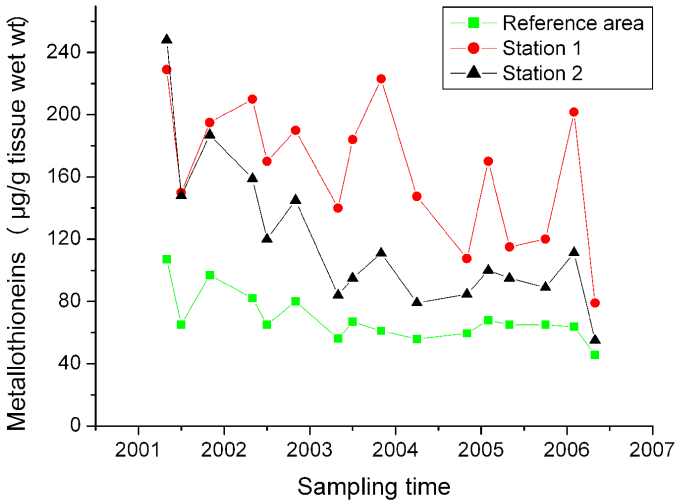

Fig. 2. Metallothionein content in mussel digestive glands from the reference area (Itea) and two stations in the Gulf of Patras.

Spatial and seasonal variability in the stability of lysosomal membranes in digestive glands of caged mussels is shown in Fig. 3. LP values in samples from Stations 1 were always lower than those measured in samples from Station 2 or the reference area. Moreover, LP exhibited seasonal variability, achieving low values in winter and autumn, except for samples collected in Station 1 during the autumn sampling campaign, in 2004. Such variability may be related to the reproductive cycle of mussels. This biological function beginning in October, results in severe lysosomal perturbations leading to unstable lysosomal membranes. Despite seasonal variability, with some exceptions, LP values were significantly correlated to metallothionein content and cytosolic metal concentrations (negative correlation).

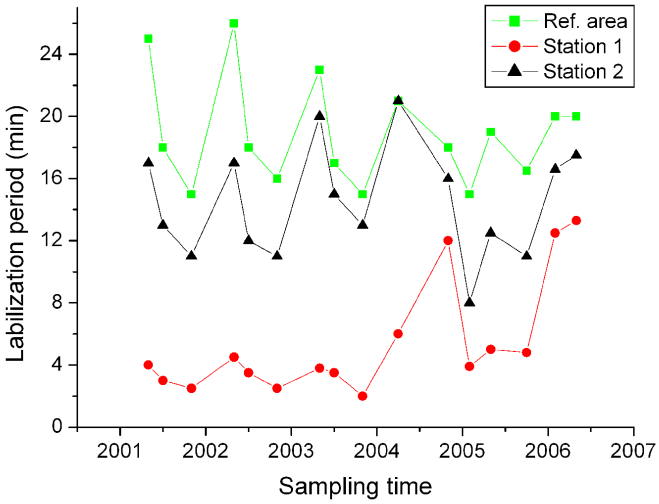

Fig. 3. LP values in mussel digestive glands from the reference area (Itea) and two stations in the Gulf of Patras. Labilization period (LP) is the average pre-incubation time of cryotome sections of digestive glands at pH 4.5 and 37^{o}C that is required to artificially destabilize the lysosomal membranes.

Higher values of micronucleus frequency were recorded in gill cells of mussels transplanted to Station 1, compared to those measured in samples from Station 2 or the reference area, regardless of the sampling time (Fig. 4). Generally, micronucleus frequency was lower in autumn than in other sampling seasons. Nevertheless, some exceptions of this rule are obvious (Fig. 4), and may be related to non-canonical climatic or metabolic changes modulating the bio-availability of pollutants and/or the efficiency of the cellular defense mechanisms.

Downregulation of global protein synthesis by pollutants

When exposed to environmental stresses, organisms downregulate protein synthesis, by storing inactive ribosomes, which recover when conditions improve.

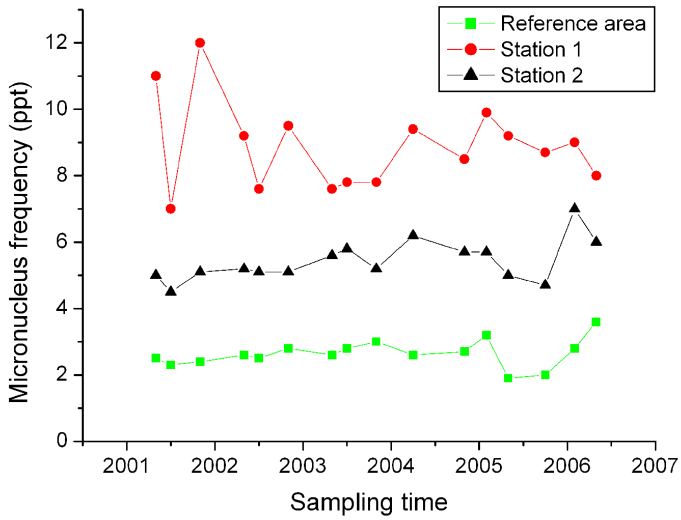

Fig.4. Micronucleus frequency in mussel gills from the reference area (Itea) and two stations in the Gulf of Patras.

Therefore, an effective way to reveal responses to pollution stress is to measure the subpopulation of aggregated ribosomes, which serve as an indicator of the translation efficiency status. Following this approach, ribosomes were isolated from the post mitochondrial supernatant of digestive gland extracts and analyzed by sucrose gradient centrifugation. Representative sedimentation profiles of ribosomal particles are shown in Fig.5 (Pytharopoulou et al., 2006). As illustrated in this figure, the isolated ribosomal material contains polysomes as well as 80S monomers and ribosomal subunits. Compared to profiles obtained from samples collected either from Station 2 or from the reference area, the polysome peak in extracts from Station 1 shifts towards a lower density, suggesting a disaggregation of polysomes to monosomes and ribosomal subunits. This was valid, regardless of the sampling campaign. In addition, seasonal variations in polysome profile were observed for each sampling site. Namely, the polysome content was lower in winter and summer than in either spring or autumn (Table 1).

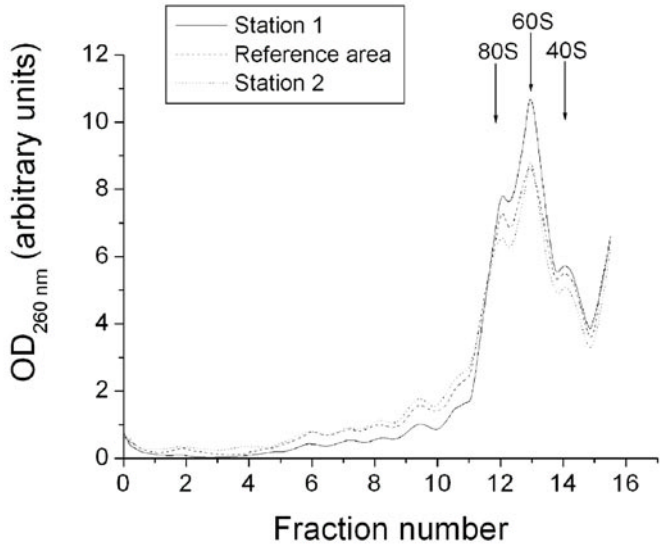

Fig. 5. Sucrose gradient analysis of ribosomal material isolated from digestive gland cells of caged mussels collected from a reference area (Itea) and two stations in the Gulf of Patras during the winter sampling, in 2005. The gradients were calibrated with 80S monosomes and ribosomal subunits prepared from yeast cells (From Pytharopoulou et al., 2006).

Table 1. Seasonal variations in the polysome proportion (%) in ribosomal material isolated from digestive gland cells of caged mussels from a reference area (Itea) and two stations in the Gulf of Patras.

Sampling Campaign*	Sampling station		
	Station 1	Station 2	Ref. area
2001			
SP	14.4 ± 0.7	20.4 ± 1.0	29.8 ± 1.5
SU	13.8 ± 0.7	17.5 ± 0.5	23.8 ± 0.9
AU	13.5 ± 0.7	21.1 ± 1.0	27.3 ± 1.2
2002			
SP	19.5 ± 0.9	27.8 ± 1.2	30.5 ± 1.5
SU	14.2 ± 0.7	16.1 ± 1.0	18.4 ± 1.1
AU	20.9 ± 1.0	29.5 ± 1.2	32.8 ± 1.3
2003			
SP	23.4 ± 0.9	34.0 ± 1.7	31.2 ± 1.1
SU	8.9 ± 0.7	15.8 ± 0.9	12.8 ± 0.6
AU	30.0 ± 1.0	37.9 ±1.8	38.0 ±1.9
2004			
SP	21.7 ± 1.1	38.0 ± 1.5	31.6 ± 1.5
AU	32.3 ± 1.2	41.3 ± 1.1	32.6 ± 1.2
2005			
WI	18.6 ± 1.2	31.3 ± 2.0	28.9 ± 1.0
SP	26.3 ± 1.2	33.6 ± 0.9	31.7 ± 1.3
AU	30.2 ± 1.9	36.1 ± 2.0	33.8 ± 1.8
2006			
WI	21.4 ± 1.1	29.7 ± 1.2	32.0 ± 1.3
SP	30.2 ± 1.0	36.2 ± 1.2	33.6 ± 1.1

* WI, SP, SU and AU, winter, spring, summer and autumn sampling, respectively

Changes in the level and composition of the environmental pollution seem to be the main reason of such variations. Nevertheless, other exogenous and endogenous factors must be regarded with caution. For instance, hypoxia and low temperature shift marine organisms to reduced translational rate (Larade & Storey, 2002; Fraser et al., 2002). On the other hand, increased temperature, which elevates in summer by 7^{o}C, may be detrimental to cells protein-synthesizing under conditions of food limitation (Kalpaxis et al., 2004). Moreover, degenerative processes occurring during the spawning season may also activate anabolic pathways, and subsequently translation rate. The latter hypothesis can also explain the fact that polysome content and LP values are not significantly correlated in autumn. On the contrary, polysome content was always found negatively correlated with micronucleus frequency, metallothionein content and certain metals (Cu and Hg).

Analysis of ribosomal capacity to initiate protein synthesis revealed marked deviations among weakly contaminated and highly contaminated samples (Fig. 6, see also Kalpaxis et al. 2003, 2004). Therefore, it was reasonable to suggest that pollution stress may downregulate global protein synthesis via induction of translational checkpoints at the level of the initiation step.

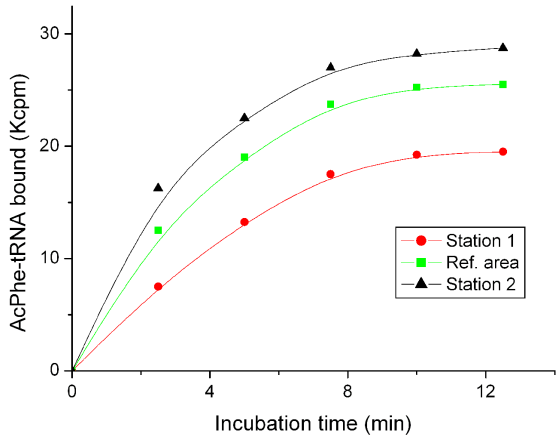

Fig. 6. Time course of Ac[^{3}H]Phe-tRNA binding to *M. galloprovincialis* poly(U)-programmed ribosomes. The values of radioactivity have been corrected for the nonspecific binding downregulate global protein synthesis via induction of translational checkpoints at the level of the initiation step.

ACKNOWLEDGEMENTS

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Chemical bioaccumulation and its biological effects on caged mussels along French Mediterranean coasts

by

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ABSTRACT

Chemical contaminant monitoring in the French Mediterranean is based on two national networks. Routine chemical monitoring has been conducted on native or farmed mussels (Mytilus galloprovincialis) by the national RNO network since 1978. This "passive biomonitoring" approach assesses the trends of chemical contaminants bioaccumulated in mussel tissues collected along the Mediterranean coasts. A more recent "active biomonitoring" approach was implemented on caged mussels deployed in the median geographical area and a standard protocol was developed and validated by Ifremer in 1996 (Andral et al., 2004) in order to reduce variability of the results due to various trophic conditions. Since 2000 the bioindicator network (RINBIO) deployed 100 stations along the French Mediterranean coast (1,800 km) in sea and in lagoon every three years, using man-made cages containing mussels (Mytilus galloprovincialis). Heavy metals, persistent organic compounds and radionuclides were analysed in mussel tissues. The field methodology and data management has been thought in order to limit interferences with the effects of trophic level on the bioaccumulation.

INTRODUCTION

Several European and national projects have supported the development of biomonitoring in the Mediterranean Sea. The first programme (BEEP - Biological Effects of Environmental Pollution in marine coastal ecosystem), launched in 2005, studied several biological markers of response to chemical stresses in marine organisms dispersed along the coasts of the Baltic Sea, the North Atlantic Sea and the Mediterranean Sea. Specific campaigns along the Greek, French, Spanish and Italian Mediterranean coasts were conducted to validate the use of selected core biomarkers at specific sites for both routine assessment of chemical contamination and in the aim of improving national and international monitoring programmes. Data collected from the majority of biomarkers analysed in mussels (Mytilus galloprovincialis) revealed a strong station / campaign interaction and high natural variability (Beliaeff and Bocquené, 2004). A series of practical biomarkers / endpoint recommendations were drawn up (Garrigues et al., 2005) for the application of core biomarkers to biomonitoring (lysosomal stability, acetycholinesterase, metallothionein, 7-ethoxyresorufin-O-deethylase, mincronuclei frequency, catalase and glutathione-S-transferase). Secondly, a French national programme backed by the RNO and RINBIO networks studied seasonal variations in six mussel biomarkers at two sites in the French Mediterranean Sea. Physiological indicators, environmental parameters and chemical contamination levels were compared at two sites (La Fourcade and Carteau). Background levels were determined for six biomarkers (acetycholinesterase, DNA adducts, benzo(a)pyrene hydroxylase, heat shock proteins, metallothionein and multixenobitic resistance) at the Carteau native site and the La Fourcade transplanted site (Tab1).

		DNA adducts (Number/10^9 DNTPs)		AChE (nmol/min/mg prot)		BPH (pmol/min/mg prot)		HSP70 (mg/g prot)		MT (mg/g prot)		MXR (arbitary unit)	
		C	F	C	F	C	F	C	F	C	F	C	F
Cohort 1	apr-99	18.6+/-9.1	37.7+/-3.2	54.4+/-7.4	41.7+/-7.7	12.1+/-10.2	12.5+/-7.6	40.4+/-11.2	42.6+/-6.8	0.022+/-0.007	0.024+/-0.011	3.5+/-0.8	ND
	may-99	33+/-3.7	40.8+/-14.6	37.5+/-8.3	50.5+/-6.2	8.3+/-5.7	2.6+/-1.3	48.1+/-9.3	44.2+/-6.8	0.018+/-0.006	0.019+/-0.009	0.1+/-0.1	2.6+/-0.8
	jun-99	9.5+/-5.3	12.6+/-4.1	41.2+/-8.5	53.8+/-6.3	1.4+/-0.2	1.7+/-0.2	34.2+/-9.3	32.8+/-6.8	0.013+/-0.004	0.018+/-0.002	0.7+/-0.6	2.5+/-1.2
	jul-99	19+/-16.9	5+/-2.6	39.1+/-4.7	39.1+/-7.7	5.4+/-3	3.2+/-1.3	28.3+/-4.6	40.8+/-3.5	0.01+/-0.003	0.016+/-0.002	4.3+/-1.2	0.8+/-0.6
	aug-99	24.3+/-1.7	9.3+/-5.3	43.5+/-9	38.7+/-5.6	16.9+/-12.8	31.5+/-12.4	38.4+/-8.6	37.3+/-5.4	0.011+/-0.004	0.022+/-0.006	6.6+/-0.9	8.4+/-2
	sept-99	27.9+/-3.3	23+/-9.2	27.6+/-6.8	28.1+/-7.3	29.2+/-21	21.8+/-8.6	23.4+/-1.3	30.6+/-4.3	0.01+/-0.004	0.01+/-0.004	5.8+/-1.6	4.7+/-1
	oct-99	18.2+/-15.7	ND	34.9+/-4.8	43.5+/-2.7	35.4+/-25.7	43.1+/-20.9	24.9+/-4.5	ND	0.008+/-0.003	ND	1+/-0.1	ND
	dec-99	10.5+/-12	32+/-10.1	42+/-6.9	48.1+/-4.4	48.6+/-19.7	33.7+/-8.5	27.2+/-9.5	32.3+/-3.4	0.007+/-0.002	0.017+/-0.007	4.8+/-2.5	3.1+/-2.6
	jan-00	3+/-2.6	ND	47.6+/-16.6	ND	46.6+/-22.4	ND	27.1+/-6.9	ND	0.013+/-0.007	ND	9.8+/-5.2	ND
	feb-00	44.6+/-12	48.5+/-44.4	44.1+/-8.7	50.8+/-14.3	23+/-18.4	55.2+/-21.8	32.8+/-4.1	29.8+/-4.9	0.013+/-0.003	0.011+/-0.003	9.1+/-3.1	5.4+/-4.2
	mar-00	8.8+/-7.2	12.9+/-0.5	44.2+/-8.2	35+/-5.5	12.2+/-5.8	18.7+/-10.4	17+/-0.8	33.6+/-5.9	0.009+/-0.004	0.02+/-0.007	2.7+/-3.1	5.8+/-1.6
Cohort 2	apr-00	17.3+/-2.4	18+/-8.4	52.6+/-8.9	56.7+/-8.1	5.6+/-4.2	0.8+/-0.3	24.1+/-4.8	30.6+/-4.8	0.011+/-0.005	0.014+/-0.005	6.4+/-2.6	2.9+/-3.6
	jun-00	18.1+/-8.1	22.9+/-26.6	42.4+/-8.9	43.5+/-11.2	2.5+/-1.4	13.1+/-8	30.8+/-6.1	34+/-4.4	0.012+/-0.006	0.016+/-0.007	3.2+/-1.9	8.8+/-3.1
	jul-00	44.7+/-23.9	9.8+/-4.8	44.2+/-20.1	37.6+/-10.1	24.5+/-2.8	28.5+/-11	24.7+/-2.7	ND	0.01+/-0.004	ND	ND	10.7+/-2.2
	aug-00	54.1+/-0.8	17.2+/-13.4	37.1+/-5.8	36.4+/-6.2	21.5+/-1.3	28.9+/-10.4	21.9+/-5.6	39+/-6.6	0.054+/-0.024	0.016+/-0.006	12.1+/-3.6	6.3+/-2
	sept-00	6.1+/-6.5	38.3+/-7.7	38.7+/-9	51.9+/-17.6	25.9+/-27.8	84.3+/-24.3	35.9+/-3	36.6+/-7.7	0.015+/-0.004	0.016+/-0.004	9.1+/-1.9	0.1+/-0.3
	oct-00	ND	17.5+/-0.1	36.7+/-7.2	53+/-8.5	38.2+/-12.9	30.8+/-23.4	21.9+/-5.9	34.3+/-4.7	0.055+/-0.015	0.012+/-0.005	2+/-1.9	0.6+/-0.2
	nov-00	ND	15+/-15.2	ND	54.9+/-8.8	ND	79.1+/-13.7	ND	38.3+/-6.5	ND	0.019+/-0.004	ND	0.7+/-0.8
	dec-00	29.5+/-10.4	12.1+/-4.4	34.2+/-11.5	57.6+/-16.8	78.6+/-7	14.9+/-7.2	ND	37.1+/-5.6	ND	0.02+/-0.008	0.4+/-0.4	0.1+/-0.2
	jan-01	17.1+/-0.7	9+/-6.2	35+/-9.9	58.5+/-11.8	33.1+/-14.4	11.9+/-16.2	23.3+/-4.3	24.8+/-6.8	0.065+/-0.018	0.045+/-0.017	0.1+/-0.4	7.5+/-1.8
	feb-01	7.6+/-2.9	ND	38+/-8.4	ND	71.1+/-23.1	ND	33.3+/-5.1	ND	0.052+/-0.012	ND	0.1+/-0.2	ND
	mar-01	ND	24.6+/-10.4	33+/-6.8	34.1+/-5.2	23.4+/-5.4	59.3+/-9.7	24.4+/-5.6	32+/-11.8	0.054+/-0.015	0.05+/-0.012	14.9+/-1.4	ND
	Mean level	21.7	21.4	40.4	45.7	26.8	28.8	29.1	35.0	0.023	0.02	4.8	4.2
	CV (%)	65.6	58.4	15.7	19.8	78.2	85.2	26.0	14.0	86.3	52.2	88.8	79.3

Table 1: Biomarkers values in *Mytilus galloprovincialis* collected at Carteau (C) and La Fourcade (F) (Bodin *et al*., 2004)

Evaluation of the seasonal variations of biomarkers showed comparable base levels for the two sites. The coefficients of variation for each biomarker, except AChE and BPH, were slightly higher at Carteau, the most contaminated area with PAH and PCB. Findings from these two pilot sites demonstrated the suitability of transplanted mussels for use as biomarkers and for establishing physiological endpoints for chemical exposure. Lastly, the MYTILOS and MYTIMED European projects (backed by the INTERREG IIIB MEDOCC programme) were initiated with the aim of developing studies on caged mussels (on the basis of RINBIO protocol), improving current knowledge on inputs and fate, and reducing chemical contamination in the Mediterranean Sea. The MYTILOS programme (2004-2006) was implemented throughout the Western Mediterranean (120 mussel cage stations) in France, Italy, Spain, Morocco, Algeria and Tunisia (Fig 1).

CONCLUSIONS

Chemical contaminant monitoring in the French Mediterranean focuses on the active monitoring of caged mussels and the passive monitoring of native or farmed mussels. An integrated programme incorporating both chemical and biological approaches is necessary, but French biomonitoring is still in the experimental phase. A clearly-structured approach must now be developed within MEDPOL for efficient integrated chemical and biological monitoring along the Mediterranean coasts. Assessment criteria must also be harmonised with those of Northern European organizations and the OSPAR Conventions. Harmonisation should encompass biomarker selection, specific pilot sites, standard operating protocols and data management, as well as common inter-calibration exercises, training courses and databases.

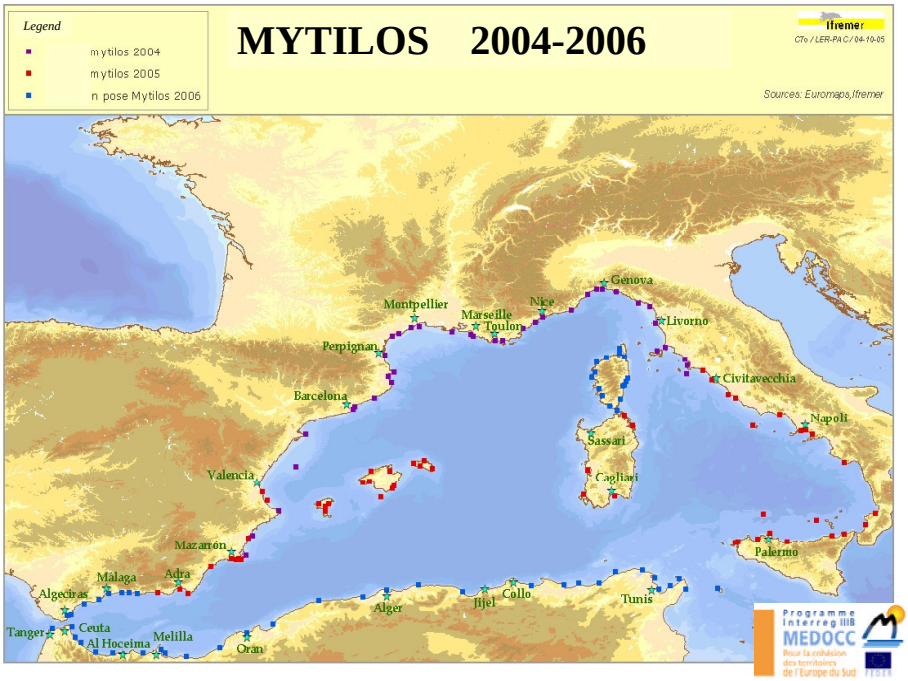

Fig 1 : Mytilos campaign using man-made cages containing mussels (Mytilus galloprovincialis).

The bioaccumulation level of CB 153 in mussel tissues demonstrated a high variation levels between Italy France and Spain (Fig 2). With the support of MAP/MEDPOL, the MYTIMED programme (2007-2008) will be implemented along Eastern Mediterranean coastal waters (about 120 stations).

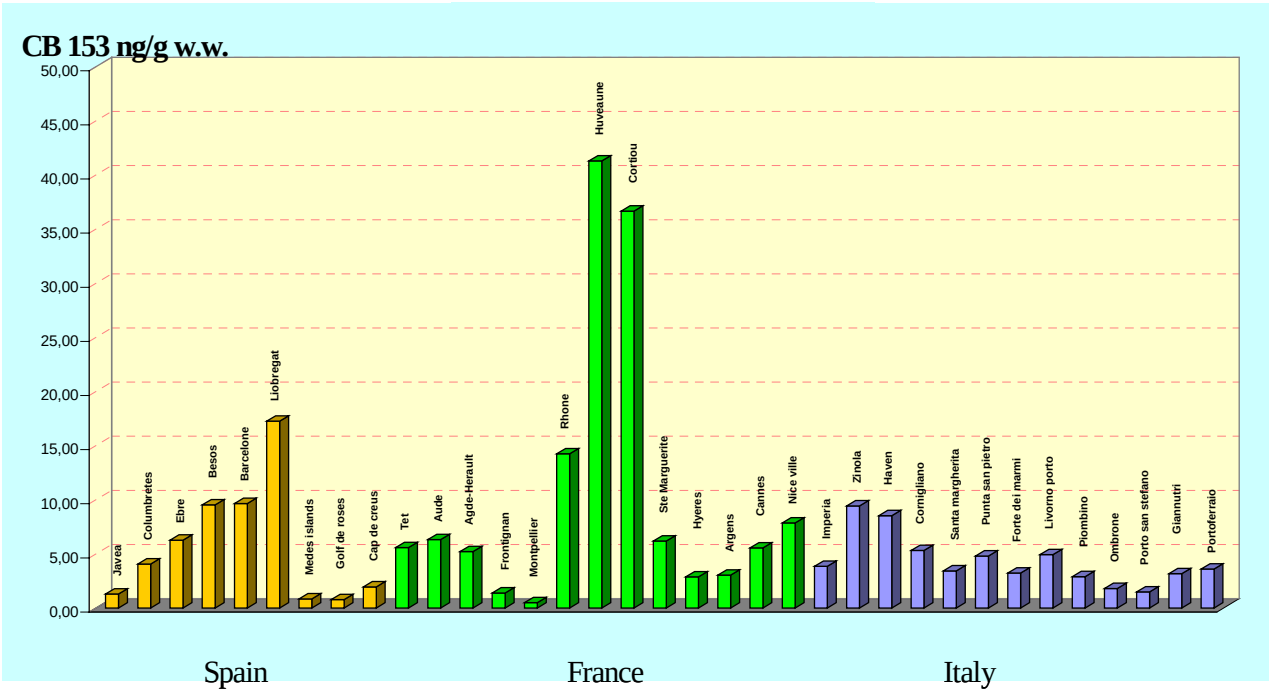

Fig 2: Chemical concentration of the CB 153 congener bioaccumulated in caged mussels along the Spanish, French and Italian Mediterranean coasts

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Monitoring the Tunisian coastline using biomarkers on *Ruditapes decussates*

by

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The present report is a summary of the work undertaken aiming at the assessment of the quality of the marine environment along the Tunisian coasts using biochemical and cellular markers. Clams, *Ruditapes decussatus* were collected during four seasons on seven different sites from the Tunisian coasts. Oxidative stress was evaluated in the digestive gland using catalase activity (Cat), neutral lipids, lipofuscine and malonedialdehyde (MDA) accumulation. Glutathion-S-Transferase activity (GST) is related to the conjugation of organic compounds and was evaluated in both gills and digestive glands. Acetylcholinesterase activity (AChE) was evaluated as biomarker of exposure to organophosphorous and carbamate pesticides. Total metallothionein protein was evaluated in the clam's digestive gland as biomarker of exposure to heavy metals. Furthermore, levels of the oxidative DNA damage 7, 8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) and catalase (CAT) activity were measured in the digestive gland and gills.

The specific sampling sites were chosen because of their proximity to urban, industrial and agricultural areas. Faroua (site1) and Menzel Jemil (site 2) are located in the north of Tunisia in the Bizerta lagoon (BL) which is a Mediterranean lagoon covering nearly 15 Km^2. The lagoon is subject to urban and agricultural pollution. Wastes of agricultural origin may reach the lagoon as a result of leaching from inland activities devoted to the cultivation of cereals and thus the lagoon is subject to eutrophication during the summer (Dellali et al. 2001). Rass Dimass (site 3), is located in the middle of the Tunisian coastal area, this site is highly subject to urban pollution, especially because of the presence of used water depuration station. The other 4 sites belong to the south region and are represented by Luza (site 4) which is 50 km from Sfax, Aouebed (site 5) 20km farther and Gargour (site 6) and Mahres (site 7) as shown in the Figure 1. Sfax is the most important industrial pole in Tunisia, causing important pollution problems in the region. Control clams were purchased from a local mussel farm and acclimated in aquaria containing re-circulating clean seawater (1L/animal).

The application of statistical analysis shows significant differences between sites and seasons compared to control samples. Faroua (sites1) and Menzel Jemile (site2) seem to be the less polluted in respect to the other sites for all seasons. Gargour (site 6) shows the highest contamination level.

Among the different sites, clams sampled at Menzel Jemilee and Mahres (see Fig. 1 for spot locations) showed the highest heavy metal accumulation rate (Fig. 2). In fact, clams from the latter two sites showed the highest content of Cd, Cu and Zn with values of 0.19, 2.13, and 23.58 µg/ g wet weight (ww) respectively in the clams digestive glands from Menzel Jemile, and 0.24, 2.42 and 32.47µg/ g ww in the clams from Mahres. Cd concentration was about two fold lower in sites Chaara and Gargour with respective values of 0.11 and 0.09 µg/g ww. Conversely, no Cd accumulation was registered in Rass Dimes, Luza, and Aouebed. the latter two sites displayed also the lowest copper content (0.29 and 0.49 µg/g ww respectively), while Ras Dimes and Chaara showed copper loads comparable to Menzel Jemile and Mahres (respectively around 1.5 and over 2 µg/g ww). In general, zinc amounts were comparable in all sites, except Mahres where a significant higher content was found.

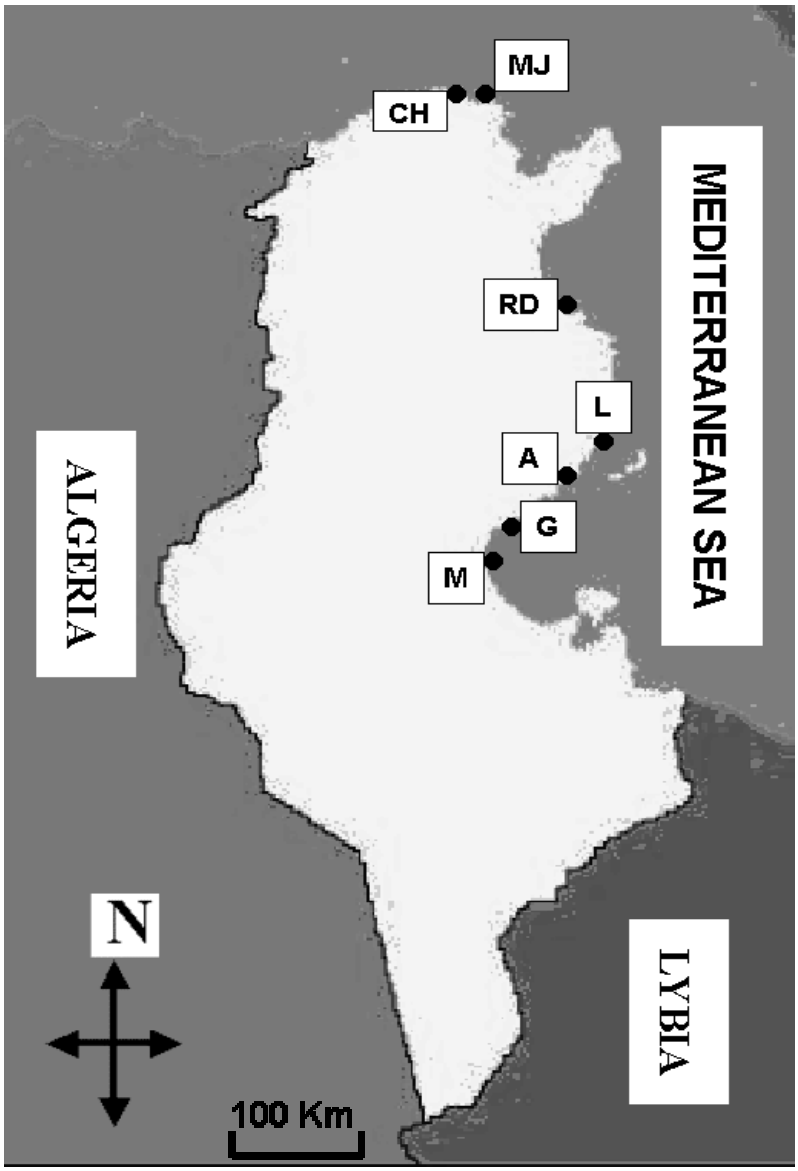

Fig.1. Location of sampling sites along Tunisian coastal areas. Chaara (Ch), Menzel Jemile (MJ), Rass Dimass (RD), Luza (L), Aouebed (A), Gargour (G) and Mahres (M).

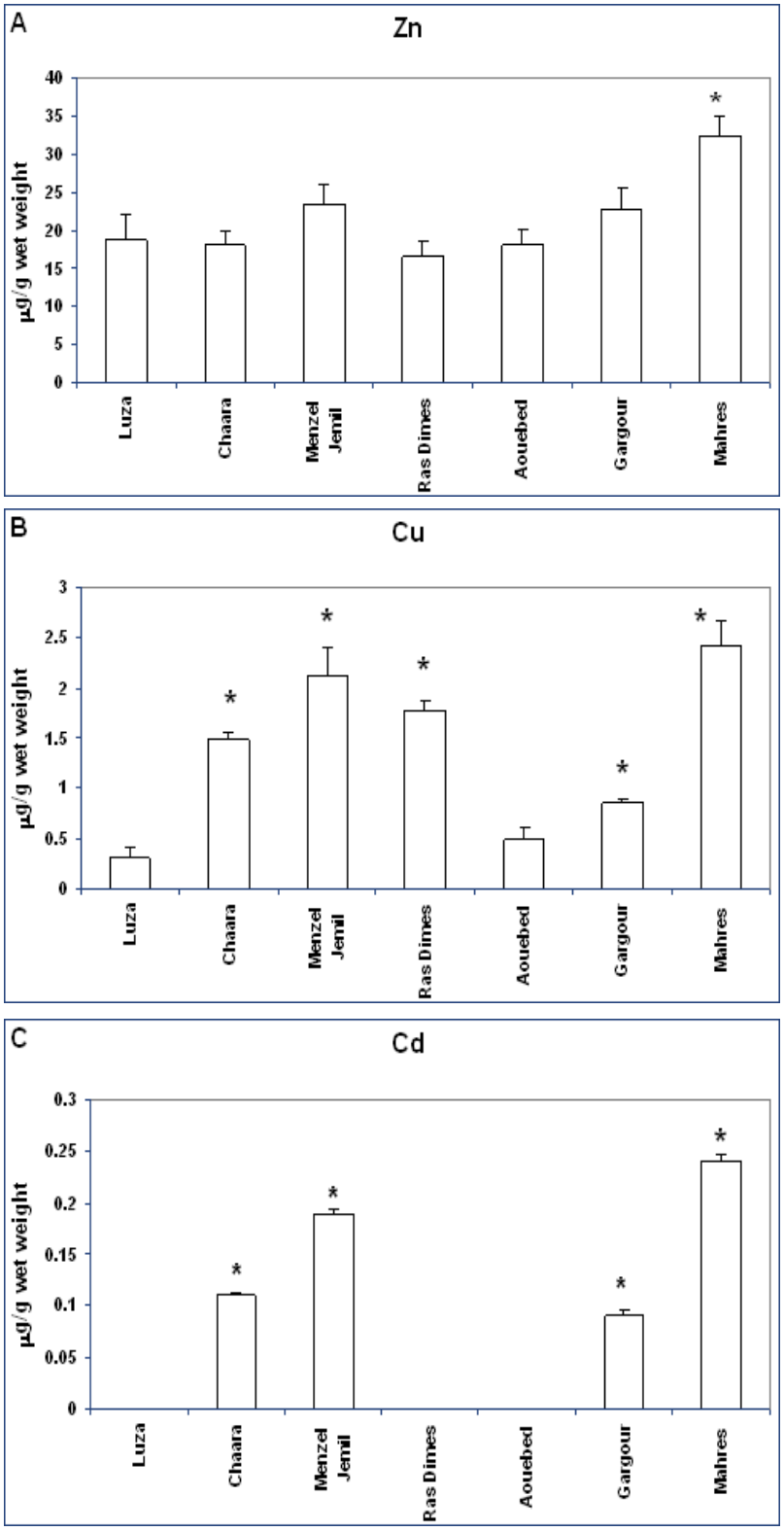

Figure 2. Heavy metals contents (µg/g wet weight) in the digestive gland of *M. galloprovincialis* from the different sites after 45 days caging experiment. Shown are, Zn concentration (A), Cu concentration (B) and Cd concentration (C). Analyses were performed by means of atomic absorption spectrophotometry. * Significantly different, Holm-Sidak ANOVA Multiple Comparison test versus Luza (n= 5; $p<0.05$). Luza was used as control site because of the lowest values for heavy metals.

As Luza showed the lowest heavy metals amounts, it was chosen as the reference site for further biomarker analysis (Banni et al., 2007).

A discriminant analysis applied to the set of biomarkers shows a significant difference between several sites. The more effective biomarkers in inter-site discrimination are AChE and neutral lipid

accumulation measured respectively in gills and digestive gland. MDA and GST activities in digestive gland contribute less frequently in site discriminations (Banni et al 2005).

The use of a biomarker index facilitates the comparison between sites and sampling periods.

Index calculation was performed as described by Narbonne et al. (2001). Biomarkers data were analysed by carrying out a one-way ANOVA and Tukey test. For each parameter at each site the mean (average) was calculated. A mean confidence interval (CI) for each parameter is determined at the desired significance level (traditionally 0.05) for each site and season. The response factor (RF) is the ratio between the higher and the lower mean, the response range (RR) is the arithmetic difference between the higher and the lower mean. A discriminatory factor is calculated as DF = RR+CI/CI. This factor serves to determine the theoretical number of significant differences among the sites being compared. This DF is converted in discriminatory level (DL), integral number from analysis of significant differences between means. By using this scale, each biomarker response must be ranked ‘‘1’ or ‘‘2’’ when there are two levels from ‘‘1’’ to ‘‘5’’ when there are five CI places between lower and higher mean. In order to standardise the biomarker response a biomarker pollution index (BPI) is attributed at each result according to their rank position. For each site an MPI is calculated as the sum of five individual biomarkers related to drug metabolising enzymes, oxidative stress and cholinesterase activity.

Most polluted sites reflected by a higher MPI are sites 6 and 7. Thus, a higher pollution level (5) may be attributed to these sites corresponding to “highly contaminated environment”. The results confirm the pollution state of Sfax city coasted area, due essentially to the presence of continuous discharge of heavy metals and also of organic compounds from local industrial activities as described by Smaoui-Damak *et al* (2004). Moreover, other studies performed on sites neighbouring Sfax city showed an increase of total metallotioneins protein levels in the tissue of clam Ruditapes decussatus (Hamza-Chaffai et al, 2003; Banni et al, 2003).

Different profiles were observed for 8-oxodG levels in the two tissues analyzed, between the different sites. Digestive glands of clams from sites Menzel Jemile, Aouabed, Gargour and Mahress presented levels of 8-oxodG significantly higher than control clams, with the animals from site Gargour presenting the highest values. In gills, significant differences were observed between 8-oxodG values from control animals and those from sites Chaara, Luzza and Gargour, presenting higher values (Jebali et al 2007).

The quality of marine coastal environments can be assessed by means of several methods. Various early warning biological methods have been assembled for detecting, quantifying and identifying spatial and temporal changes in the quality of marine coastal environments. However, no single method can satisfy all these objectives.

Over the past decade, molecular, biochemical and cellular markers have been extensively used in pollution monitoring of aquatic environments. Biochemical markers have been selected among early molecular events occurring in the toxicological mechanisms of main contaminants. Biomarkers are useful descriptors of field situations that may allow the identification of chemical stressors and their potential ecological risk. They give additional information that cannot be obtained from chemical analysis of pollutant concentrations alone, and they may integrate effects of mixtures of chemicals over long exposure periods.

ACKNOWLEDGEMENTS

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Heavy metals and metallothionein levels in mussel samples from the Albanian sea coast

by

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ABSTRACT

The work was performed in the framework of the MED-POL III (MAP/UNEP) programme during the years 2001 – 2005 in two “hot spot” areas of the Albanian coast, located in Durres and Vlora Bays. Butrinti Lagoon is used as a background station.

The objective of this study is the determination of heavy metals and metallothionein (MT) levels in mussels to provide evidence that MTs are involved in responses to heavy metals pollution. The concentration of heavy metals in homogeneous mass of different size of mussels, mussel’s tissue, as well as digestive gland of mussels collected in Durres, Vlora and Butrinti stations are analysed by AAS and ICP. Some comparisons of the levels of heavy metals in each system were carried out. The highest concentrations of heavy metals were found in the digestive gland of the mussels.

Digestive gland has been selected as the most appropriate tissue for monitoring of metallothionein (MT) level because of their highest induction capacity and extraction efficiency.

A spectrophotomeric method is optimised to assess the concentration of MTs in the digestive gland of marine organisms. The procedure takes into account precautions to obtain a complete metallothionein precipitation and to avoid the oxidation of sulphydryl groups (SH), the contamination by soluble low molecular weight thiols and enzymatic protein degradation that can occur during sample preparation in our laboratory conditions. MT levels reflect better than total HM contents the pollution impact of HM in marine environments.

Keywords: metallothionein, heavy metals, mussel, mussel tissue, digestive gland, biomarker of exposure

INTRODUCTION

Mussels are generally used as indicator organisms for marine pollution control owing to their geographical wide spreading as well as to their large capacity for accumulation and storage of trace metals, like heavy metals (HM), so they could be considered as a biomarker of exposure to heavy metal pollution, (Duka, 2005).

Metallothionein (MT) was discovered in 1957 when Margoshes and Vallee identified in equine kidney a cadmium-binding protein responsible for the natural accumulation of cadmium in this tissue. MTs are still the only biological compounds known to naturally contain this metal, (Viarengo *et al.,* 1997). MTs show particular biochemical features; they are of low molecular weight (about 6000-8000 Da), soluble, sulphydryl-rich proteins (about 30% cysteine content mammal MTs), with a peculiar aminoacid (characteristic distribution of cysteinyl residues such as: Cys-X-Cys, Cys-Cys, Cys-XY-Cys, where X and Y are aminoacids different from cysteine). They are thought to play roles in both the intracellular fixation of the essential trace elements zinc and copper and in controlling the concentration of the free ions of these elements regulating their flow to their cellular destination, and in neutralising the harmful influences of exposure to toxic elements such as cadmium and mercury and in the protection from a variety of stress conditions (Viarengo *et al.,* 1999).

MT are metal binding proteins whose neosynthesis represents a specific response of the organisms to pollution by heavy metals such as Cu, Zn, Cd and Hg, (Talbot and Magee, 1978). MT levels are utilized as an early indicator of the biological effects of heavy metals which represent an important source of pollution in the coastal areas of industrialized zones, (Viarengo *et al.,* 1999; Roesijadi, 1996), and it is recommended in MED POL III Programme as sensitive biomarker of marine environment contamination by heavy metals, (Manual on the Biomarkers UNEP/MAP, Athens 1999).

Determination of MTs presents a challenge for every chemical and biochemical laboratory, because various methods as UV spectrophotometry, polarography, AAS, capillary zone electrophoresis, and others are referred in the literature (Dabrio and Rodriguez*,* 2000; Lecoeur *et al.,* 2004; Minami *et al.,* 2002). A modified variant of Viarengo's spectrophotometric method is used in this study (Viarengo *et al., 1997;* Duka, 2005).

MATERIALS AND METHODS

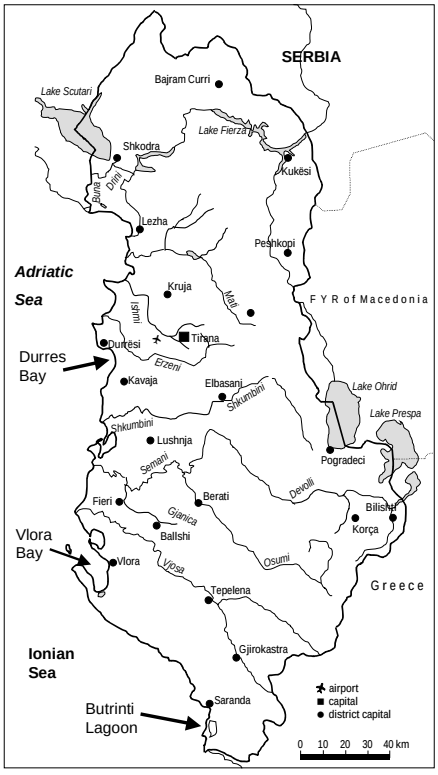

Fig.1. Map of Albania; Sampling sites

1. Sampling area

Durrës, with more than 150,000 inhabitants, is the main harbour of the country, located in the central-western part of Albania at the Adriatic Sea coast. At the beach of Porto Romano, about 6.5 km north of the city centre, is located a former chemical plant, extending on an overall surface of about 50 ha. This plant produced, until 1990, sodium dichromate, pesticides, such as Lindane (gamma-HCH), and other chemicals. All production processes have been idle since that time and the plant's buildings have been totally destroyed. East of the site a drainage system leads the drainage water from the plain, but also the sewage from Durrës city, through a main channel into the Adriatic Sea by means of the pumping station in Porto Romano. Fishing in the Adriatic Sea is practiced near the discharge point of this channel. At present, this area represents a "hot spot", comprising the remains of the Lindane and dichromate factory, a dumpsite nearby and a chemical waste storage site. The Cape of Bishti Palles and Lalzi Bay located 2 km north of the site are considered to be one of the areas, which could be used for the development of a tourist industry in Central Albania.

Vlora, with a population of about 120,000 habitants is found in the Southwest part of Albania; it is located in the gulf having the same name, Vlora Bay and presents the natural boundary between the Adriatic Sea and Ionian Seas. Four kilometres north of the city, at the beach, it's the site of a former chemical complex including a chlorine-alkali plant. During its operation (1967-1992) liquid wastes (about 500 m^3/hr) containing 1.1 mg Hg/L were discharged directly into the sea. Soda – PVC plant lie down in an area of about 20 ha; another area of 25 ha, positioned between the Plant and the coast, was used as a technological dump's disposal. The plant was closed in 1992, and its buildings have been completely destroyed. Mercury used in Electrolysis Plant and PVC Plant was spread around on the territory of the plant. From former investigations, drops of metallic mercury in the area of the electrolysis plant and in all of its drainage canals were observed. Mercury content levels > 10,000 mg/kg (6^{th} European Framework Programme (2004-2007) in the soil samples are

found exceeding more than 1,000 times typical EU thresholds. In July 2002, a mission of UNEP/MAP (GEF Project GF/ME/6030-00-08) had identified this area as a “hot spot”.

Butrinti Lagoon is situated in Saranda district, opposite the island of Corfu and close to the Greek-Albanian border. The area is largely a wetland site with saline lakes and dissected by two rivers, the Bistrica to the north and Pavllo to the south. To the east, Mile Mountain (824 m) separates the wetland complex from the interior of Albania. The area has an outstanding landscape value with a high variety of natural, semi-natural and artificial habitats. This site was used as a reference area.

2. Sample preparation

Mussels (*M. galloprovincialis*) collected from Durres and Vlora were grown in wild natural conditions, whereas mussels from Butrinti came from a farm. Preparations of the composite samples for determination of HMs were carried out following the UNEP procedure, (UNEP(OCA)/MED WG.128/2, 1997). Four to five pools of samples each composed of 20 mussel specimens were mixed. Composite analytical samples were prepared for each station, homogenized and kept in a refrigerator at -20 ^{0}C until chemical analysis.
For the determination of HMs in soft tissues and digestive glands, the mussels were dissected, damp dried to minimise differences in water content, homogenized and frozen at -20 ^{0}C. The same procedure was followed for the preparation of the gland samples for the determination of MTs.

2.1 Total metal analysis

-Reagents
Nitric acid (69.5%) was obtained from Ridel de Haen, H_2O_2 30% from Fluka and standard solutions for Cd, Cr, Cu, Fe, Mn, Hg, Ni, Pb, and Zn were prepared from 1000 mg/L (2% HNO_3) standards of Merck.

- Analytical procedure and apparatus
1. Samples for chemical analysis by AAS were digested with nitric acid and H_2O_2 into Teflon vessels for 2 h at 240^0 C, and then processed for analysis. An Atomic Absorption Spectrometer Varian SpectrAA 10/20 is used: flame technique for determination of Cu, Ni, Mn, Fe and Zn, graphite furnace for Pb, Cd and Cr and cold-vapour system for Hg determinations. Instrumental parameters were optimised using validation procedures and manual instructions. The accuracy was tested by analysing SRM-2976 (mussel tissue) from NIST.

2. Metal concentration in digestive gland samples were analysed also using an Inductively Coupled Plasma Mass Spectrometer HP4500 (Agilent, Waldbronn, Germany). A portion of 500±1 mg of sample was digested with 5 ml HNO_3, using a microwave oven (UltraCLAVE 2^{TM} EMLS, Leutkirch, Germany) and then diluted to 25 mL with distilled water. The accuracy of this method was tested by analysing a certified reference material DORM-2 (dogfish muscle) obtained from the National Research Council of Canada.

2.2 Metallothionein analysis

- Reagents
Sucrose, Trizma hydrochloride, Leupeptine, Phenylmetylsulfonyl fluoride (PMSF), Ellman’s reagent, 5,5’-dithio-bis (2-nitrobenzoic acid), Glutathione (reduced form) and Ribonucleic acid (RNA) were purchased from SIGMA Chemicals. Ethanol absolute was obtained by Riede-deHaen and β-mercaptoethanol was obtained by Fluka.

- Determination of metallothionein content
MT concentrations in digestive gland cells were determined according to the method published by Viarengo et al. (1997). Some modifications were applied based on our laboratory conditions: first

separation was performed using lower speed of centrifugation, *10 000 x g for 30 min at 0-4^{0}C* (instead of 3*0 000 x g for 10 min*), and measurements of final absorbance were carried out after a constant time of 2 min (due to instability of the absorbance) (Duka, 2005). An UV-VIS Spectrophotometer Shimadzu 2401 PC is used for absorbance measurements. A refrigerated centrifuge type Z323 from Hermle is used for all separations.

2.3 Identification of metals binding metallothioneins

Digestive gland samples were homogenized with 4 ml solution which contains 1 mM dithiothreitol and 10 µM PMSF. The suspension was sonicated for 60 seconds and then centrifuged at 5000 rpm for 120 minutes at 4^{0}C (Duka, 2005). The clear supernatant was used for size-exclusion chromatography (SEC) measurements. The high-performance liquid chromatography system was a Hewlett-Packard (Waldbronn, Germany) equipped with a quaternary pump, a vacuum degasser column oven, and an autosampler with a variable 100 mm^3 injection loop. For data evaluation the chromatographic software G1824C Version C.01.00 was used. The column eluent first entered the UV detection cell and then was split into two directions: the main part of the eluent (approx. 1mL/min) was directed to the fraction collector, the minor part (approx. 0.2 mL/min) was transferred to the spray chamber of the ICP-MS. The specific element determinations were performed on an Agilent 7500C ICP-MS.

3. Results and Discussion

3.1 Total Heavy Metal levels in mussels

Concentrations of heavy metals in mussel samples from Durres and Vlora stations found during the years 2001 to 2005 are presented in Table 1 and Figure 2 (data obtained in the framework of MED POL III Programme for Albania, (UNEP, MED POL – Phase III 2002).

Although the compatibility of the data present some shortcomings because of the variability in time of sampling and in mussel parameters (length and weight) during these years, the following can be noted on the results obtained:

Table 1. Concentrations of heavy metals in mussel samples from Durres and Vlora “hot spots” (during years 2001- 2005)

Sampl. station	*Year (month)*	*Concentration, µg/kg DW*								
		Cd	*Pb*	*Hg*	*Cu*	*Zn*	*Cr*	*Ni*	*Mn*	*Fe*
C 1.2 (Durres)	2001 (7)	721	1388	152	11515	177515	3970			808667
	2003 (10)	269	1640	219	8360	80500	3930	4480	181300	309200
	2004 (10)	366	3856	195	10044	59800	4332	2194	22890	438800
	2005 (12)	624	5662	83.1	28908	217840	8994		53550	1579380
C 2.2 (Vlora)	2001 (7)	288	4031	242	10366	121675	12484			1214188
	2003 (10)	771	2380	421	6740	244600	1630	3940	139300	293900
	2004 (8)	285	2618	418	4612	64500	1438	6180	12338	191400
	2005 (12)	424	5694	381	13308	198640	9718		46168	1336900

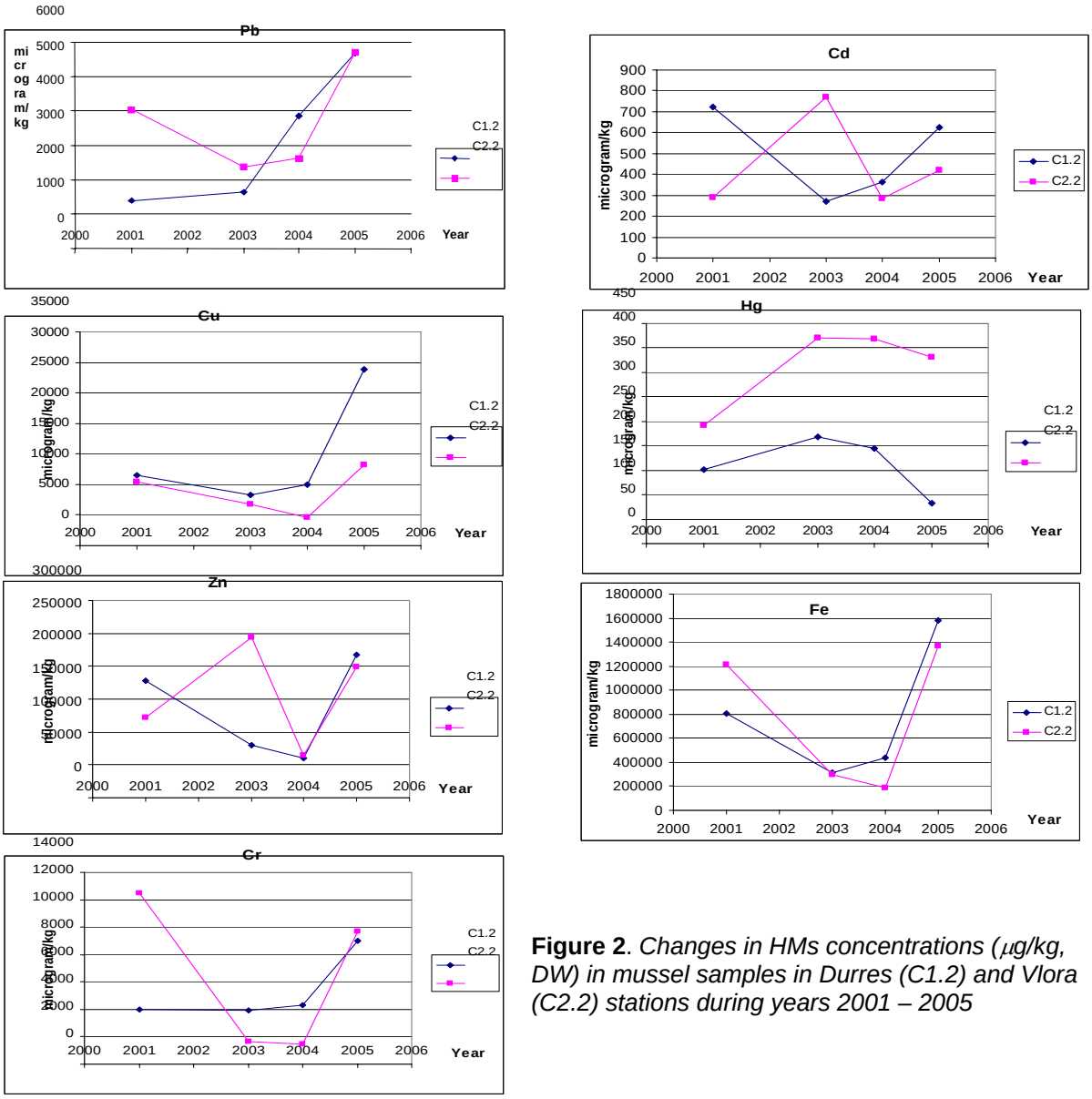

Figure 2. *Changes in HMs concentrations (μg/kg, DW) in mussel samples in Durres (C1.2) and Vlora (C2.2) stations during years 2001 – 2005*

(a) The levels of most heavy metals are higher in 2005 than in previous years. It is related mostly with bioaccumulation process of heavy metals, when the concentrations of HM are increased with the size of mussels.

(b) Levels of Cd, Cu, Fe and Mn found in the station C1.2 (Durres) are higher than those found in the station C2.2 (Vlora). It is related mainly with the geology of the area. The geological formation of Durres Bay consists mainly of silicate sandy mixed with clay, and that of Vlora Bay consists mainly of Ca+Mg carbonates (more than 90%). The same findings result also in the previous monitoring programmes (Cullaj *et al.,* 2000; Celo *et a*l., 1999; Babi *et al.,* 1998).

(c) Mercury levels in samples of Vlora Bay are more than four times higher than those of Durres Bay. The same conclusion resulted also in the previous years, caused from the impact of the former discharges from a PVC plant (Lazo and Cullaj, 2002; Cullaj *et al.,* 2004; Lazo, 2003).

(d) Use of the wild mussel samples for trend monitoring in our programme has an important shortcoming resulting from the natural variability between years. To overcome this problem, caged mussel sampling, as well as the mussels of the same class, with similar dimensions and weight, should be used.

The analyses are carried out in mussel samples of the years 2003 and 2004. Results are presented in Table 2 and Figure 3.

Table 2. HM levels in mussel tissue and digestive gland from different stations of Albania (mg/kg DW)

HM	*Vlora*		*Durres*		*Butrinti*	
	Digestive gland	*Tissue*	*Digestive gland*	*Tissue*	*Digestive gland*	*Tissue*
Cu	14.60	4.30	8.85	6.37	7.43	5.43
Zn	163	180	81.84	81.83	38.47	44.09
Fe	666.1	197	1077	262.2	327	160.7
Ni	47.57	9.5	71.65	25.2	10.16	22.13
Cr	6.72	15.3	14.86	19.67	-	-
Cd	0.518	0.506	1.417	0.166	1.25	1.35
Pb	4.05	2.24	3.02	1.80	0.60	0.58

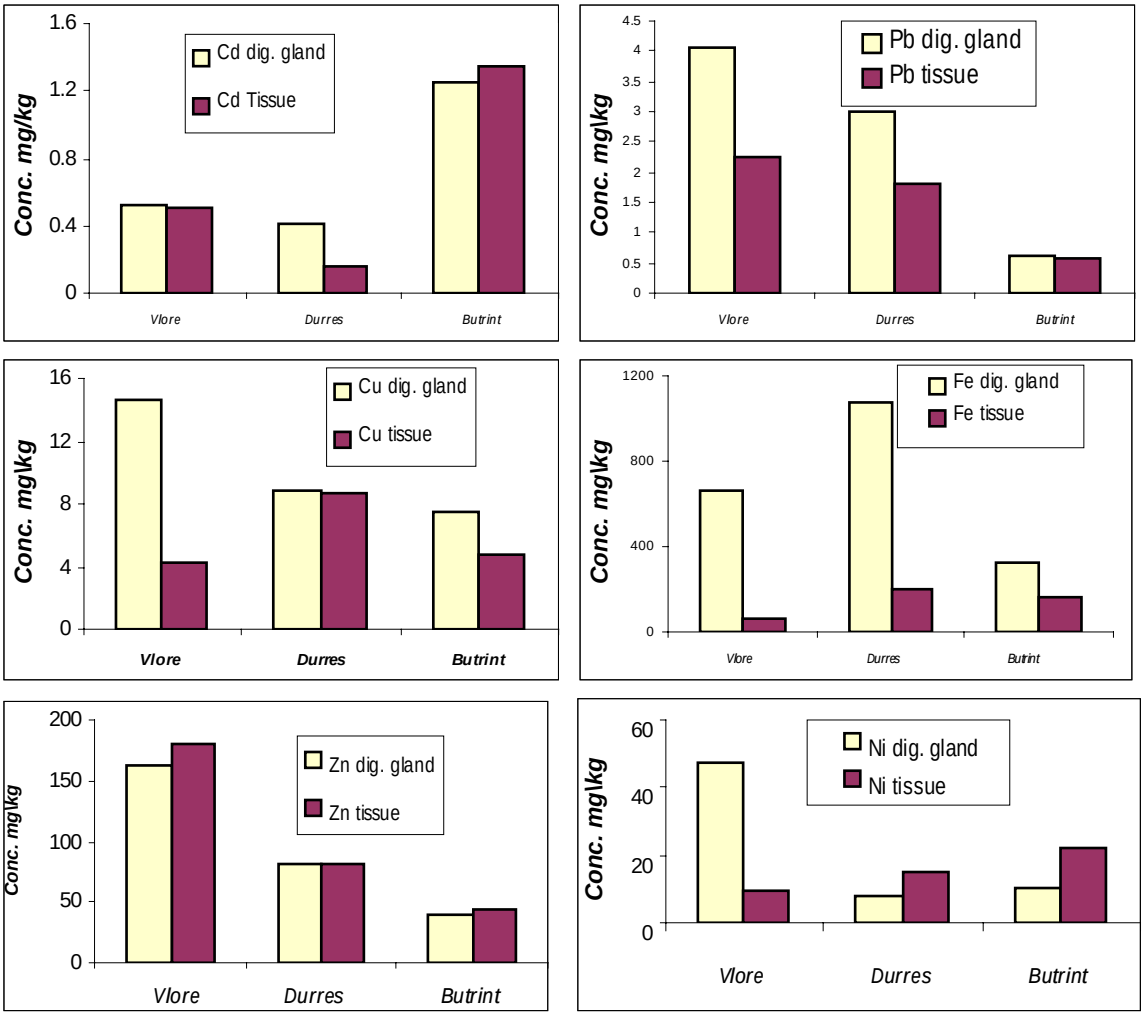

Figure 3. HM in digestive gland and tissue of mussel samples taken from three different stations (in mg/kg DW)

Because the digestive gland is a good bioaccumulator of HMs, the concentration of HMs in digestive gland is generally higher than in respective soft tissue of mussels. Enrichment rate of HMs in digestive gland relative the tissue is very different for various metals and various

sampling areas. Moreover, for zinc and chromium (not presented in Figure 2) we didn't find any bioaccumulation of these metals in gland relative to tissue. This indicate that: (i) MT have not the same affinity to different HMs, due the different affinity of thiolic groups -SH; (ii) levels of HMs in

water milieu, but also the size and weight of mussels have a strong influence on bioaccumulation of HMs in digestive gland of mussels.

(iii) From the results found, it is derived that the enrichment factor of HMs in the digestive gland to the tissue is higher in Vlora samples than in Durres samples. Lower enrichment factor (except Cd) is found for the samples taken from lagoon Butrinti.

3.3. Metallothionein spectrophotometric analysis

3.3.1 Calibration procedure

MT concentration in the sample is quantified by evaluating the SH-residues content by a spectrophotometric method, using Ellmans reagent. The reaction produced stoichiometric amounts of thionitrobenzoat, a yellowish compound with maximum absorbance at 412 nm. We use reduced glutathione as a reference standard to construct the calibration curve.

The chemical composition of standard calibration solutions is shown in Table 3, and calibration curve is shown in Figure 4. We found very good linear relationship (r = 0.998) between absorbance in 412 nm measured after 2 minutes and concentration of glutathione (reduced form).

*Table 3**. Composition of calibration solutions***

Calibration standard	*Conc. of MT nmol/ml*	*Conc. of MT (μg/ml)*	*GSH μl*	*NaCl 0.25M μl*	*HCl 1N + EDTA 4N, μl*	*D.T.N.B. ml*	*Final volume ml*
Blank	0	0	-	150	150	4.2	4.5
Standard 1	14.4	4.44	20	130	150	4.2	4.5
Standard 2	28.8	8.88	40	110	150	4.2	4.5
Standard 3	43.2	13.32	60	90	150	4.2	4.5

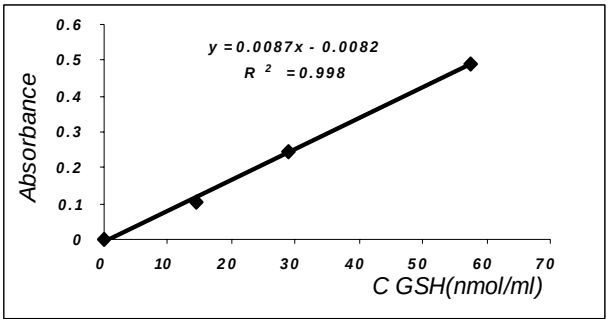

Figure 4. *Standard calibration curve obtained by spectrophotometric evaluation of equimolar –SH concentration of GSH from rabbit liver.*

As it is shown in Figure 4, this procedure allowed the determination of nmolar levels of MT. To quantify exactly the absolute value of metallothionein content in mollusc tissues, a homologous metallothionein standard, not yet commercially available, should be used. However, due to the low cost of the standard GSH (in respect to metallothionein), this compound have to be preferred for routine analyses in biomonitoring programmes where variations of metallothionein levels in different samples, rather than absolute amount determinations, are pursued.

3.3.2 Optimising the time of photometric measurements

During our measurements we found that the absorbance was unstable with time. The changes of the absorbance at 412 nm for a period 0 to 1000 seconds after adding the last reagent (DTNB) for two standard solutions are given graphically in Figure 4.

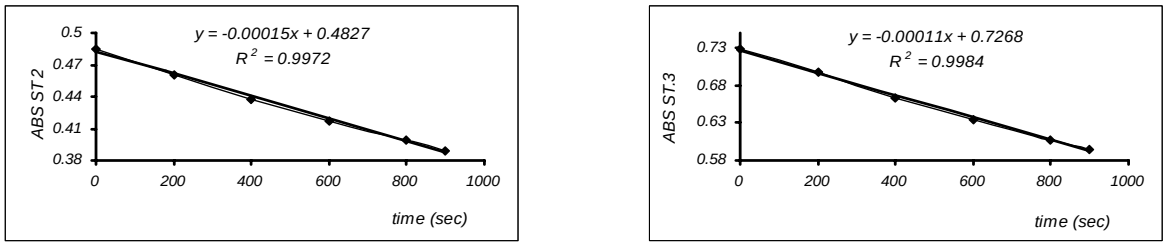

Figure 4. Change of absorbance at 412 nm with time of measurements for standard 30.0 nmol/ml and 60 nmol/ml GSH

As it is shown in Figure 4, the absorbance of the final product decreased nearly linearly with time, so the absorbance of standards and samples should be measured as soon as possible, and at the same time. As it is shown in Figure 4, the rate of change of absorbance value does not depend on the concentration. The measurements done within 2 minutes do not affect the results (the variance of absorbance is only 2%), so the time of measurements is chosen within this interval.

3.3.3 MT in digestive gland of mussel's

The analyses are performed in mussel samples taken in the years 2003 and 2004. The results are presented in Figure 5 (Duka and Cullaj, 2006).

The levels of most MTs in 2004 result are than in previous years. It is related mostly to the bioaccumulation process of heavy metals (see Figure 2). MT concentrations in mussels of Durres and Vlora stations are higher than of Butrinti Lagoon, used as background station. Our results of MT levels are comparable with the reported results for the region: the levels of Vlora and Durres samples are comparable with those of Patras Gulf, Greece (148-150 μg/g FW) (Kalpaxis *et al., 2004),* but lower than those reported for three Italian seacoast sites (140-202 μg/g FW) (Viarengo and Canezi, 1999). The levels of Butrinti samples are lower than those of other stations.

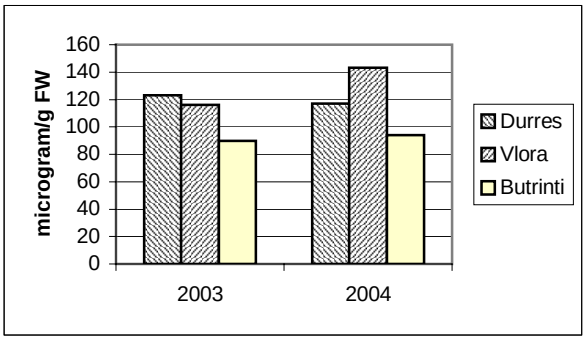

Figure 5. MT in the digestive gland of mussels (mg/kg, Fresh Weight) for years 2003 and 2004 in three Albanian stations

3.4 Identification of metal-binding metallothioneins

This analysis has been performed at the Institute of Analytical Chemistry at the Karl-Franzens-University, Graz (October 2005). In order to find the HMs that are bound to metallothionein, we used a separation procedure by size-exclusion chromatography, determining the bound metals in the effluent of the column by using ICP-MS as a element-selective detector.

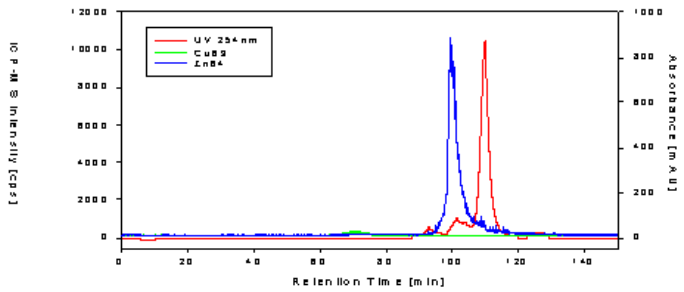

Figure 6. Chromatogram of a mussel sample from Butrinti lagoon, digestive gland. Chromatographic conditions: Column: Superdex 75 pg; 16/60, Mobile phase: 100 mM NH_4HCO_3; 1mM DTT, Flow rate 1.2 ml/min.

MT shows typical absorbance spectra due to the particular interaction of different cations in metal thiolate clusters. These metallotetrathiolate clusters provide the protein with a highly stable structure that renders it heat stable. In the chromatogram obtained by SEC-ICP-MS (Fig. 6) we have identified quantitatively metal binding protein in digestive gland. It is clearly shown in these chromatograms that soluble metals, Zn and Cu are associated with metallothionein fraction.

CONCLUSIONS

1) The content of heavy metals in mussel samples strongly depend on the level of contaminants in the water system. Mercury levels in samples from Vlora Bay are more than four times higher than those from Durres Bay due to the impact of former discharges from a PVC plant. At the same time, however, levels of Cd, Cu, Fe and Mn found in the station Durres are higher than those found in the station Vlora. This is related mainly with the geology of the area, but also with the impact of anthropogenic sources.

2) The content of heavy metals in mussel samples varies from one year to another. It is believed that this results from the natural variability of size and weight of mussels grown in natural wild conditions. To overcome this problem, the caged mussel sampling, as well as mussels of the same maturity, with similar dimensions and weight, should be used.

3) The concentration of HMs in the digestive gland is generally higher than in the respective soft tissue of mussels, but enrichment rate is very different for various metals and various sampling areas. This indicates that: (i) MTs do not have the same affinity to different HMs; (ii) the concentration levels of HMs in the water, but also the size and weight of mussels have a strong influence on the bioaccumulation of HMs in the digestive gland. The enrichment factor of HMs in the digestive gland to the tissue is higher in Vlora samples than in Durres samples. Lower enrichment factor (except Cd) is found for the samples taken from lagoon Butrinti. Our results show that the digestive gland is a better bioindicator of HM than the mussel's tissue.

4) The spectrophotometric method for the determination of MT as modified on the basis of our laboratory conditions gives relatively good results; calibration curves are reproducible and show a good linearity.

The spectrophotometric method allows simple, reproducible and relatively low cost detection of low concentrations (nmol) of metallothionein in biological samples and therefore it can be used as a tool for MT quantification in biomonitoring programmes. MTs seem particularly involved in responses to heavy metals like Zn and Cu, and they should therefore be considered a biomarker of exposure to heavy metal pollution. High levels of MT would generally indicate the presence of a metal pollution stress in the animals at the sampling location.

5) MT concentrations in mussels of Durres and Vlora stations are higher than those of Butrinti Lagoon, used as background station. Our results of MT levels are comparable to those reported for Patras Gulf, Greece and Italian seacoast sites.

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Biomonitoring programme in the Slovenian coastal sea during MED POL Phase III (1999-2005)

by

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ABSTRACT

According to the Agreement between the United Nations Environment Programme – Mediterranean Action Plan (UNEP-MAP) and the Government of Slovenia, the National Monitoring Programme was established and implemented in Slovenia in 1999, in order to fulfil the demands of the Barcelona Convention and national legislation. The National Monitoring Programme is part of the Programme for the Assessment and Control of Pollution in the Mediterranean Region (MED POL) carried out within UNEP-MAP. This programme also involves monitoring the contaminant levels in biota and sediments, including trend monitoring and biomonitoring (Turk et al., 2000).

INTRODUCTION

The Slovenian coastline is located in the southeastern part of the Gulf of Trieste in the northern Adriatic. Pollution hot spots and sensitive areas were identified along the Slovenian coast according to the recent data and UNEP/WHO recommendations (Turk & Potočnik, 2001). The gulf is affected by land-based pollution sources like sewage discharge, urban runoff and drainage of agricultural areas, as well as nautical tourism and intensive maritime traffic to the ports of Trieste, Monfalcone and Koper. As a consequence, increased concentrations of hydrocarbons were detected in the upper sediment layer in the marinas and municipal ports (Bajt, 2000). The principal industries in coastal regions in the past were metal manufacturing, chemical production and food industry. Main pollution inputs to the Bay of Koper include primarily and secondarily treated urban and industrial wastewater from the town of Koper and inland agglomerations along the river Rižana and Badaševica. The Dragonja River carries agricultural discharges into the inner part of the Bay of Piran. Moreover, tourism, aquaculture activities and a wastewater treatment plant also contribute to the pollution of the Bay of Piran. No major pollution source was detected in the Bay of Strunjan (Turk & Potočnik, 2001). Few studies on surface sediments in the Gulf of Trieste, particularly in its industrialized area, were devoted to the detection and characterization of the sources of persistent organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs) (Bajt, 2000). The PAHs content in the sediments of the Gulf of Trieste was estimated to be between 30 and 600 ng g^{-1} dry weight, rapidly decreasing with increased distance from the shore (Notar et al., 2001). Contamination of sediments with PAHs should be declared as low to moderate. The Soča River continuously washed away cinnabar deposits into the Gulf of Trieste, which is substantially polluted by mercury, thus making it one of the most polluted areas in the Mediterranean. However, since 1995, acquisition of the cinnabar deposits has been abandoned, and mercury pollution in the gulf decreased (Horvat et al., 1999, Faganeli et al., 2003). The estimated gross flux of heavy metals from wastewater treatment plants and industry at the Slovenian coast was 3492 kg in the year 2000 (Turk & Potočnik, 2001).

MATERIALS AND METHODS

The biomonitoring programme was established in 1999, and two biomarkers of exposure were utilised: genotoxicity and metallothionein content, as recommended by UNEP (UNEP/RAMOGE, 1999). The sentinel organism was the blue mussel (*Mytilus galloprovincialis*) and was collected at permanent sampling stations in the Bay of Koper, Bay of Piran and Bay of Strunjan, the last as a reference station (Figure 1.). Mussels were collected twice per year (March and September) starting in September 1999. The station in the Bay of Koper is located in the vicinity of the marina and Port of Koper. Blue mussels were collected from their natural beds in the Bay of Koper and

from shellfish farms at the stations in the Bay of Strunjan and at the Bay of Piran. Related environmental parameters such as temperature of seawater, salinity and oxygen saturation were measured at 2 m depth in the proximity of the sampling stations. Genotoxic effects were measured in haemolymph using the alkaline filter elution method (UNEP/RAMOGE, 1999) and metallothionein concentrations in hepatopancreas (Viarengo et al., 1997). The biomonitoring process also included chemical analyses of mercury, cadmium as well as PAHs in the mussel tissue. The results of biomonitoring are regularly published in the yearly reports in the series of reports, National Monitoring Programme of Slovenia, from 1999 onwards.

Figure 1. Sampling Stations for the Biomonitoring Programme in Slovene Coastal Waters

The effects of genotoxic agents in the seawater were evaluated in mussel haemolymph and expressed as SSF (strand scission factor). In years 2001 and 2002, calculated SSF values were positive (spans from 0.0215 to 0.0228). From 2003 onwards, SSF values were negative in all sampling stations during late winter sampling. Moreover, the station in the Bay of Koper also showed permanent negative SSF, based on samples taken in the late summer period. Meanwhile, sampling stations in the Bay of Strunjan and the Bay of Piran had revealed negative SSF values in the late winter and positive SSF in the late summer period. During the monitoring period SSF values did not indicate sites of bad DNA integrity in the mussels.

Metallothionein content was evaluated in the hepatopancreas of the blue mussels at the same sampling stations. Generally, metallothionein concentrations in the samples were slightly lower in late summer compared to late winter. No significant differences were found between sampling stations or between years. The level of metallothioneins spans from 53 to 163 µg/g wet tissue during late winter sampling and from 46 to 138µg/g wet tissue in late summer. The amount of mercury and cadmium in the mussel tissue spans from 70 ng/g d. w. to 240 ng/g d. w. and from 680 ng/g d. w. to 1270 ng/g d. w., respectively (Turk et al., 2005). In conclusion, the evaluated content of metallothioneins in mussels indicates that there was no site highly polluted with metals that induce metallothioneins in Slovenian coastal waters.

In order to employ a battery of biomarkers the activity of hepatic ethoxyresorufin-O-deethylase **(**EROD) was measured in black gobies (*Gobius niger*) collected from seven sampling sites along the Slovenian coast during spring and winter. EROD activity was generally in good correlation with increased total PAHs content in sediments and sediment toxicity, and was significantly higher during the non-spawning period in winter than during the spawning period in spring. Sediments from the same sites were tested for their toxicity and for total content of polycyclic aromatic hydrocarbons (PAHs) (Ramšak et al., 2004).

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Monitoring of pollution biological effects using the mussels Mytilus galloprovincialis in the Algerian west coast

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ABSTRACT

The absence of urban and/or industrial sewages treatment stations and the industrial development in the Algerian western area, resulted in the coastal marine environment becoming the main receptor of several forms of pollution with a consequent contamination of this biotope. This work concerns the impact assessment of this coastal pollution by using a suite of recommended marine biomarkers including lysosomal membrane stability in living cells by the Neutral Red Retention Time (NRRT) method and the evaluation of micronucleus (MN) frequency in the mussels *Mytilus galloprovincialis*, the most used sentinel organism in Mediterranean marine environment biomonitoring programmes sampled from the large Oran polluted harbour (OH) (Oran bay, Algerian west coast) and a reference non-urban area of Maârouf (Mrf) (marine mussel farm, extreme north of Algerian west coast) between July 2005 and April 2006. The difference of the annual physical parameters variations between OH and the Mrf site corresponds to the influence of the domestic and industrial wastewater released by the city of Oran without any treatment. The mussels' biological data (condition index, protein content) recorded of both sites were related to their natural reproductive cycle. This implied that intrinsic variation between sites due to a different mussel development phases was minimal. The higher NRRT recorded in the granular haemocytes during the autumn and spring in the Mrf mussels compared with those of the OH are rather dependent on the quality of the biotope and the levels of chemical contamination but also on the generic factors of stress. Moreover, the variation of MN frequency, overall reflecting non-significant seasonal and spatial genotoxic effect of the two sampling sites' contamination requires further investigations related to the biotic and abiotic variations.

Key words: Biomarkers, Algerian west coast, Mytilus galloprovincialis, lysosomal membrane stability, micronucleus, Oran harbour, Maârouf

INTRODUCTION

The concept of biomonitoring belongs to the indicators of the environment global change. This predictive approach of the biological effects of the contaminants is currently used through many scientific programmes in various Mediterranean countries with an aim of promoting a common and integrated strategy of using marine biomarkers in recommended species sentinels (UNEP, 1993; 1997; Viarengo *et al*., 1997; UNEP/RAMOGE, 1999; Cajaraville *et al*., 2000; Viarengo *et al*., 2000a; Viarengo *et al*., 2000b; ICES, 2004). Biomarkers are early warning biological tools able to detect pre-pathological alterations or disturbances as responses to environment pollutants at cellular and organism level such as the mussels *Mytilus* spp (Moore, 1985; Amiard *et al*., 1986; Viarengo *et al*., 1990; Lionetto *et al*., 2003; Regoli *et al*., 2004; Gravato *et al*., 2005).

The increase in the human population (the Algerian littoral zone concentrates more than 40% of the population), the absence of urban and/or industrial sewage treatment stations and the industrial development in the Algerian western zone, made the coastal marine environment the main receptor of several forms of pollution. The assessment of marine pollution developed by our research laboratory this last decade on various pollutants bioaccumulated by some marine species (mussels, sea urchins, crustaceans, fishes and cetaceans) from several sites of the Algerian western coast revealed high concentrations of heavy metals (Hg, Cd, Pb, Zn, Cu, Mn, Ni, Mg), organochlorinated compounds (PCB and chlorinated pesticides) and polyaromatic hydrocarbons (chrysene, phenanthrene) (Taleb, 1997; Taleb *et al*., 1997; Taleb & Boutiba, 1999; Boutiba *et al*.,

2003).

In accordance with the actual national priorities of the environmental policy (National Actions Plan for the Environment and Sustainable Development, 2002, PNAE-DD; Algerian Ministry of the Environment; http://www.ambalgott.com/download/algerie-paned.pdf), our laboratory recently developed the first regional marine biomonitoring project entitled: "Use of the biomarkers for the assessment of marine pollution impacts in western Algerian coastal area" in which some recommended marine biomarkers (UNEP, 1997; UNEP/RAMOGE, 1999) were introduced in Algeria for the determination of lysosomal membrane stability in living cells by the Neutral Red Retention Time (NRRT) method (general stress) and the evaluation of micronucleus (MN) frequency (genotoxic effects) in the mussels *Mytilus galloprovincialis*, the most used sentinel organism in Mediterranean marine environment biomonitoring programmes.

MATERIALS AND METHODS

Sampling sites

Mussels (*M. galloprovincialis*) were collected from the large Oran harbour (OH) where 90 million m^3 of wastewaters are released annually by the Oran metropolis and many industrial units without any treatment. High levels of heavy metals, polyaromatic hydrocarbons and bacterial density were recorded in the tissues of *M. galloprovincialis* at this site (Boutiba *et al*., 2003). The second sampling site was a marine mussel farm located in the non-urban area of Maârouf (Mrf) at the extreme north west of Algeria and at approximately 200 km away from of the first site (Fig. 1).

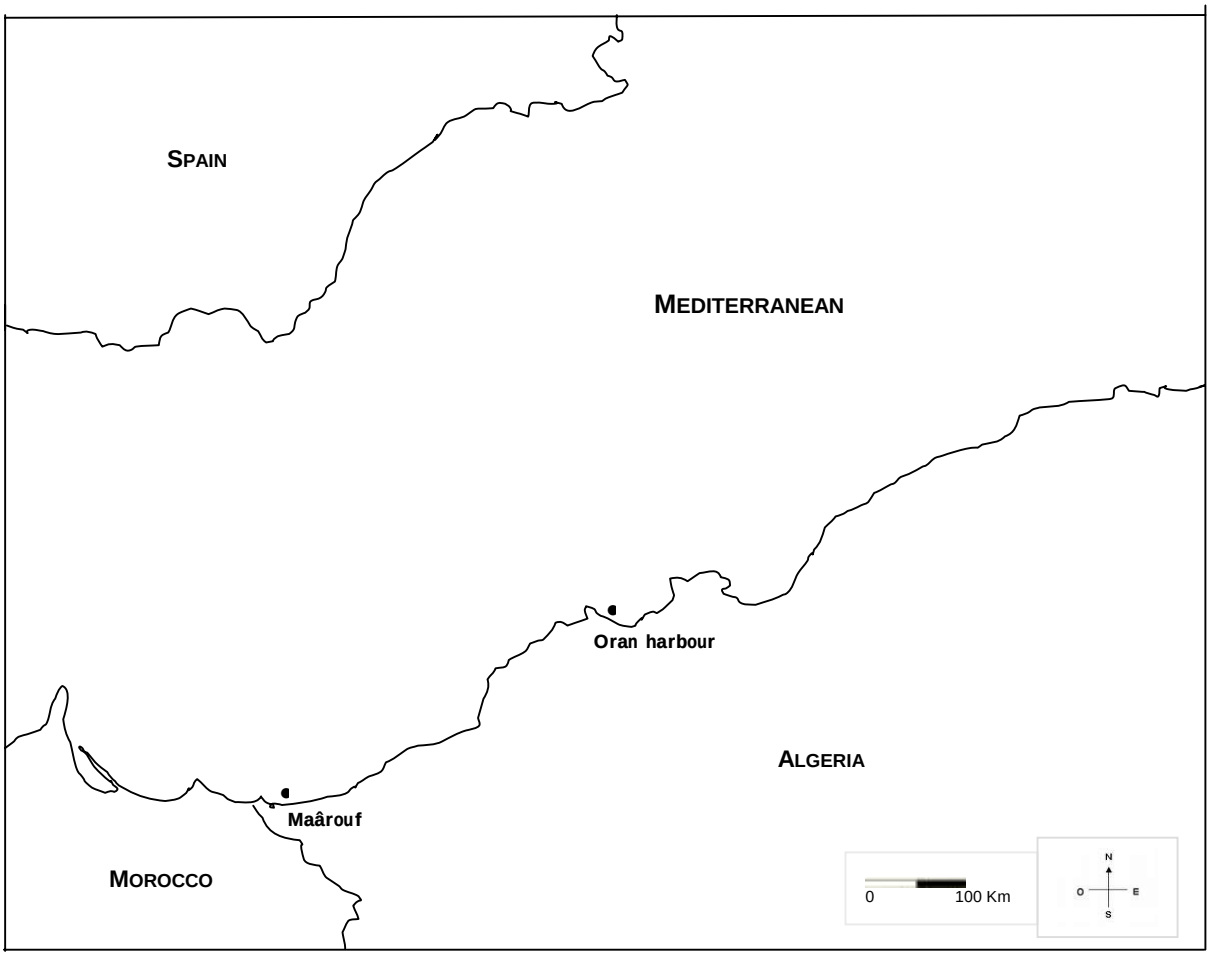

Fig. 1: Sampling sites located along the Algerian west coast.

To assess the seasonal pattern of naturally (physical and biological) induced stress response, water dissolved oxygen, pH, temperature and salinity were monitored *in situ* and in the laboratory the food supply (chlorophyll *a*) (Lorenzen, 1967), protein contents (Bradford, 1976) in some organs and mussels condition index calculated as follows: CI = tissue wt (g)/shell length (mm) x 100. The sampling sites were geo-referenced with Garmin GPS 12 (Table 1).

Table 1: Data of Oran harbour (OH) and Maârouf (Mrf) sampling sites

Sampling site	Sampling date	Position	Temp. (°C)	Salinity (‰)	Turbidity (NTU)	Dissolved O_2 (mg/l)	Chl *a* (mg/m^3)
(OH)	July 2005	N35°42'663" ; W 000°39'320"	27.3	34.5	4.5	3.6	2.60
	October 2005		22.4	36.5	2.5	3.9	0.51
	January 2006		13.0	37.5	1.7	4.4	0.57
	April 2006		24.0	37.0	1.5	6.8	1.88
(Mrf)	November 2005	N35°04'249"; W 002°03'790"	20.7	39.5	1.5	6.3	0.62
	March 2006		21.7	38.5	1.0	6.2	1.05

Mussels collection and handling

Mussels comprised both male and female and were collected randomly each season from mussel stocks and shipped in coolers with ice packs to the laboratory where they were maintained in flow through raceway systems of seawater at ambient temperature and salinity for at least 2 days prior to experimental use. Depuration of mussels facilitates the removal of any residual sediment in the soft tissues or body cavity.

Evaluation of NRRT in haemolymph

The analytical method was performed according to Lowe *et al.* (1992) and Lowe *et al.* (1995) and proposed by UNEP/RAMOGE (1999), with slight modifications.

Haemolymph was withdrawn from the posterior adductor muscle of ten mussels in physiological saline so as to obtain a 1:1 v/v of cell/physiological saline suspension. The suspension, obtained from each mussel, was spread on Poly-L-Lysine (1/10) prepared slides and transferred to light proof humidity chamber for 15 min to allow the cells to attach. After incubation, 40 µl NR working solution were dropped onto each slide. At the end of 15 min, the slides were quickly examined at x400 magnification and images are digitalized using computer-enhanced automatic image analysis. The system included a charged couple device (CCD) Sony colour camera mounted on a Zeiss light microscope. Image software (Pinnacle Studio, v. 8) electronically captured the microscopic images displayed on a television screen (Sony Trinitron) and stored them on a personal computer. The time after the NR probe application, where there was evidence of dye loss from the lysosomes to the cytosol in at least 50% of the examined cells, belonging to the granular haemocytes, represented the NRRT for the mussel.

MN in the haemolymph and gill tissue

The determination of micronuclei frequency was applied according to the procedure proposed by UNEP/RAMOGE (1999). Then, haemolymph was withdrawn from the posterior adductor muscle of ten mussels in physiological saline so as to obtain a 1:1 v/v of cell/physiological saline suspension. Suspensions were spread on slides, transferred to a lightproof humidity chamber, and allowed to attach. Cells were then fixed in methanol:acetic acid (3:1), stained with 3% Giemsa, and mounted in Eukitt. Gill cells were isolated by enzymatic digestion with a solution of Dispase I (Neutral protease, Boehringer Mannheim, Germany). The cellular suspension obtained by filtration was centrifuged, and aliquots of the re-suspended pellet were fixed in methanol: acetic acid (3:1) overnight, spread on slides, stained with 3% Giemsa, and mounted in Eukitt. The stained slides were analysed under the same Zeiss light microscope at a final magnification of 1000x under oil immersion. The scoring of slides comprised the examination of more than 1000 agranular haemocytes and epithelial-like gill cells. The criteria used for the identification of micronuclei are referred in UNEP/RAMOGE (1999).

Statistical analysis

Statistical analysis of data on condition index, protein content, NRR of the haemocytes and the MN frequency of the haemocytes and the gill cells were based on Duncan's test for multiple comparison and Student *t*-test between pairs of mean values using Microsoft STATISTICA (v 6.0) statistical software. The significance level for all statistical tests was set at $p < 0.05$.

RESULTS AND DISCUSSION

1. Physical parameters and chlorophyll a

Shell length, tissue weight and condition index are of mussels from OH are summarized in Table 1. They reflect the influence of the domestic and industrial wastewater released by the city of Oran without any treatment, mainly in July marked by the lowest salinity and dissolved oxygen and by the higher temperature and turbidity. However, in (Mrf) station, all the physical parameters remained relatively constant during the two months of sampling except for the Chl *a* corresponding to the seasonal bloom of phytoplankton.

2. Biological parameters

The length of collected mussels did not vary markedly over the sampling, lengths for natural mussels population from (OH) ranged from 50 to 82 mm and 55 to 84 mm in cultural mussels population of (Mrf) site (Table 2). However, there was an increase in somatic weight during July and April compared to the other months (October and January) for the mussels of (OH) and between November and March for those of (Mrf) (Table 2). The condition index reflects significantly this seasonal change of the somatic weight from period (Table 2).

Table 2: Shell length (mm), tissue weight complex (g) and condition index of mussels, *Mytilus galloprovincialis* (value are mean± SD; *n* = 10 per month, per site) sampled from Oran harbour (OH) and Maârouf (Mrf) station.

		Shell length (mm)	**Somatic weight (g)**	**Condition index**
(OH)	July 2005	66.7 ± 6	7.16 ± 2.17	26.38 ± 7.06
	October 2005	64.9 ± 9.8	6.33 ± 3.2	24.20 ± 10.88
	January 2006	69.8 ± 5.4	6.24 ± 1.76	13.76 ± 6.79
	April 2006	68.2 ± 5.5	7.3 ± 2.34	19.95 ± 7.52
(Mrf)	November 2005	65.2 ± 6.2	4.06 ± 1.16	8.63 ± 2.20
	March 2006	74.8 ± 6	8.58 ± 1.9	22.41 ± 7.89

The protein level in the digestive gland, haemolymph, gills and mantle/gonad complex of (OH) mussels showed significant variation throughout the sampling periods except between April and October in the digestive gland and between April and January in the haemolymph.

The protein content of (OH) mussels peaked in July (summer) in the digestive gland and the haemolymph and in April (spring) in the gills and the mantle/gonad complex (Fig. 2). For the (Mrf) mussels cultured population, proteins concentration was statistically higher in March (spring) compared with November in all the tissues (Fig. 2). This annual seasonal variation in the protein concentration of both sites was related to their natural reproductive cycle. Protein concentrations usually decrease just after the period of spawning and increase in the resting stage.

The annual seasonal variation of the protein content that reaches, in general, the highest level during April and July, reflects the periods of rest (development) and spawning. The protein level is thus a general indicator that reflects the resting-spawning cycle (Lee, 1986; Mohan & Kalyani, 1989); indeed, the proteins are accumulated and stored for the period of rest in preparation of the phase of spawning, and will decrease after the spawning, which allows us according to the biological data recorded for the (OH) mussels to suppose that the spawning periods are October and January corresponding to the autumn and the winter.

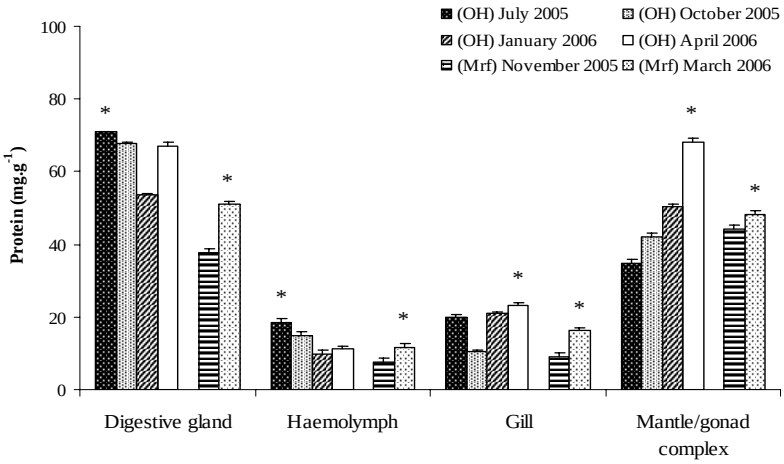

Fig. 2: Seasonal variation of the protein content ($mg.g^{-1}$) in tissues of Oran harbour (OH) and Maârouf (Mrf) *M. galloprovincialis* (mean±SD, *n* = 5). Statistical signification is based on Duncan's test for multiple comparison in different seasons at (OH) and Student *t*-test at (Mrf). * indicates statistical signification at p<0.05

3. Evaluation of NRRT

The NRR times recorded in the granular haemocytes of (OH) and (Mrf) mussels showed that the seasonal variation of this biomarker (Fig. 3) did not follow a clear pattern. The labilisation time of the haemocytes lysosomal membrane of the (OH) mussels was significantly less low during July compared to October and January, whereas for the other periods of sampling, no significant difference was noted in this site and even between the NRRT of the two sampling periods of the (Mrf) mussels.
During the autumn and spring, the NRRT were statistically higher in the (Mrf) mussels compared with those of the (OH) (Fig. 3).

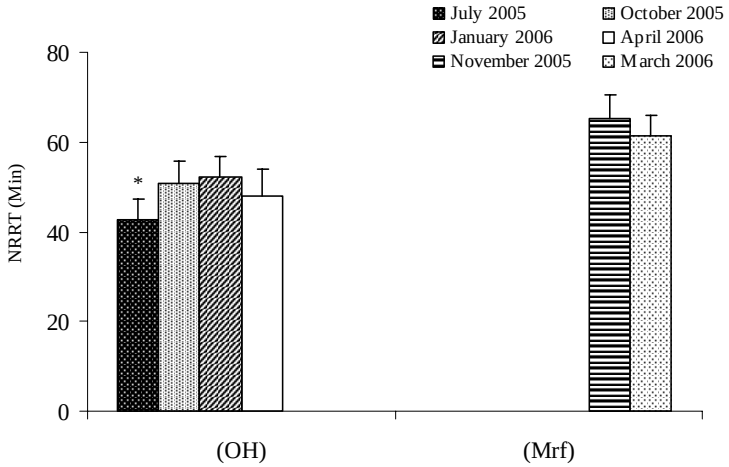

Fig. 3: Neutral red retention times (min) in *M. galloprovincialis* of Oran harbour (OH) and Maârouf (Mrf) site expressed as mean±SD from ten mussels. Statistical signification is based on Duncan's test for multiple comparison and Student *t*-test between pairs of mean values, * indicates statistical signification at p<0.05

The results of the evaluation of the NRRT, indicating the general stress in relation to the seasonal and environmental conditions in mussels collected from an aquaculture farm (Harding *et al.,* 2004), reflect a seasonal variation model of the membrane lysosomal destabilization, correlated with the reproductive cycle of the species and consequently with its condition index. Nevertheless, we noticed in our (OH) samples that for the two periods of spawning (October and January), NRR times are significantly higher compared to those noted during July that represents a period of post-spawning with the highest condition index (Table 2).

The NRRT variations in (OH) mussels compared to those of (Mrf) showed that the lysosomal membrane stability is rather dependent on the quality of the biotope and the levels of chemical contamination. Data of Boutiba *et al.* (2003) reflect significantly the chemical stress caused in particular by heavy metals in *M*. *galloprovincialis* of Oran harbour. The potentially toxic impact of this general stress on the mussel populations of the Oran bay was reported following the low temporal values of the lysosomal membrane stability (Taleb *et al.,* 2003).

It was mentioned, for this purpose a reduction of the lysosomal membrane stability associated with the exposure of the mussels (*M. galloprovincialis*), clams (*Slaps philippinarum*) and oysters (*Crassostrea gigas, Crassostrea virginica*) to the pollutants such as heavy metals and polyaromatic hydrocarbons (Moore, 1991; Lowe *et al.,* 1995; Ringwood *et al.*, 1998; Low & Fossato, 2000; Viarengo *et al.,* 2000c; Woo-Geon & Sang-Man, 2005).
The level of mussels' contamination is proportional to the degree of pollution of the surrounding waters, in particular by the polyaromatic hydrocarbons (Nott & Moore, 1987), which tend to bioaccumulate in some organelles such as lysosomes inducing a continual destabilization of the lysosomal membrane (Woo-Geon & Sang-Man, 2005).

The results of many investigations indicated that the physicochemical factors of the biotope, such as the variations in temperature, the hypoxia and the hyposalinity (Moore *et al.*, 1979; Bayne *et al.*, 1981; Hauton *et al.,* 1998) can influence the integrity of the haemocytes lysosomes. Other studies affirmed that the increase in the water temperature during the summer cause a reduction in the stability of the lysosomal membrane (Tremblay *et al.*, 1998), which could explain also the significantly lowest NRRT in July in the (OH) mussels and where we noted 27.3°C of a water temperature and a low oxygen saturation (Table 1).

Times of the lysosomal membrane destabilization ranging between 40-56.25 min for (OH) mussels and between 56.25-75 min for (Mrf) specimens are comparable with those noted in the same species of the Greek northern coasts (Dailianis *et al.,* 2003) not exceeding 50 min for populations exposed to the anthropogenic pollution and the 65 min at a cultured population (Koukouzika & Dimitriadis, 2005). It is, thus possible to admit, respectively the level of the stress exposure of the mussels populations used in our study and consequently the quality of their biotope.

4. Determination of the MN frequency

The results of the MN test applied in the haemolymph and gill cells of (OH) *M. galloprovincialis*, indicated annual frequency varying respectively from 0.99 to 3.3‰ and 1 to 4.75‰ with a peak of genotoxicity during January, whereas at (Mrf) mussels, MN was ranging between 0.99 and 1.67‰ in the haemocytes and 0.76 to 1‰ in gill cells (Fig. 4). Nevertheless, the Duncan's test did not reveal any significant difference of the annual seasonal variation of the MN frequency in these tissues of (OH) mussels. The same report is noted for the (Mrf) mussels between the two periods of sampling.

In addition, the comparison of the MN induction noted at the (OH) mussels and these of (Mrf) during the autumn and spring reveal significant difference only in the haemocytes.

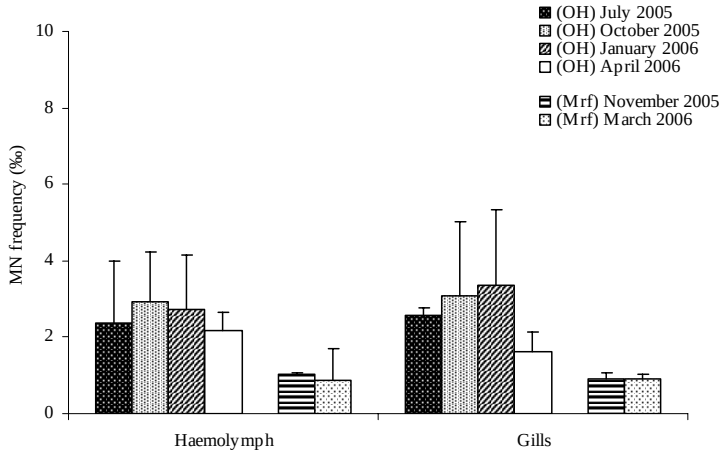

Fig. 4: MN frequencies (‰) in the gills and the haemolymph of (OH) and (Mrf) mussels expressed as mean values±S.D. from five mussels. Statistical signification is based on Duncan's test for multiple comparison and Student *t*-test between pairs of mean values, * indicates statistical signification at p<0.05

The experimental results indicated higher MN frequency in the gill cells compared to the haemocytes in the (OH) mussels. The same result was reported by Bolognesi *et al.* (1999), Baršienė *et al.* (2003) and Dailianis *et al.* (2003). Nevertheless, the agranulocyte haemocytes and the gill cells remain suitable for the genotoxic evaluation for *M. galloprovincialis* (Venier *et al.*, 1997).

The appearance of MN is obvious after exposure of the mussels to chemical contaminants such as heavy metals and organic pollutants (Mersh & Beauvais, 1997; Venier *et al.*, 1997; Bolognesi *et al.*, 1999). However, MN frequency found in the present study showed a significant variation within the same experimental mussel groups (Fig. 4). This internal individual variability of the (OH) *M. galloprovincialis* has also been reported in the same species (Koukouzika & Dimitriadis, 2005) and other bivalves (Wrisberg *et al.*, 1992; Mersh & Beauvais, 1997). Moreover, the variation of MN frequency, overall reflect non-significant seasonal and spatial genotoxic effect of the two sampling sites contamination, knowing that other factors can have a great influence in the formation of the MN such as high death rates cell (Brunetti *et al.*, 1988) and deteriorations on the cell division (Wrisberg *et al.*, 1992). It was even mentioned that the MN induction in gill cells of *M. galloprovincialis* can decrease following a long exposure to a polluted environment (Scarpato *et al.*, 1990).
Thus, the evaluation of genotoxic stress using the MN test seems to require further investigations related to the biotic variations (age, sex, development cycle), and environmental parameters (Dixon *et al.*, 2002; Koukouzika & Dimitriadis, 2005) for its routinely use in biomonitoring programmes of marine pollution.

CONCLUSION

The damage caused to the lysosomal membrane and ADN appears to be universal markers to evaluate the effects of stress on the marine organisms in bivalves. Indeed, results of the NRRT indicating responses to the environmental stress showed that the variation of the destabilization of the lysosomal membrane of the *M. galloprovincialis* haemocytes is dependent on the level of pollution. However, additional research of the MN test is needed to reflect significantly the genotoxic effect of marine water quality.
The results of this study highlighting the environment stress through recommended biomarkers (UNEP, 1997; Ringwood *et al.,* 1999; UNEP/RAMOGE, 1999) will contribute to fill the gaps in terms of ecotoxicology data of the Algerian coastal marine pollution.

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EROD in *Chelon labrosus* and *Siganus rivulatus* from the Syrian coast: field and laboratory studies

by

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ABSTRACT

This paper presents some results of EROD activities in two Syrian fish species: *Chelon labrosus and Siganus rivulatus* from two field stations (Wadi Kandil and Almina). The first station is regarded as a clean, reference station, and the second one is polluted.

We made some laboratory tests and observed EROD differences in relation to temperature and pH changes, and in the presence of aromatic compounds complexes at three different concentrations. The study was undertaken during the year 2004 as part of a Ph.D. thesis, and continued during the year 2006 for the Mediterranean project.

INTRODUCTION

Many oxidation processes are accompanied with stress (proteins, enzymatic, peptides, vitamins), and they can be formed in cell organs to decrease stress (Melancon, 1995). We determined induced enzyme in many ways (Anderson *et al.,* 1988a,b; Larson, 1987). We can use EROD and P-450 as biomarker (Huggett *et al.*, 1992).

We followed Hodson *et al.* (1991) in measuring EROD activity, which presents the ability of using enzymatic complex cytP-450 in fish liver as a biomarker response to organic toxins in the aquatic environment.

Some morphological data of the fish, such as body weight, liver weight, age and sex were recorded. In addition, some environmental data such as temperature, salinity, conductivity, turbidity, oxygen and primary production were measured. Liquid nitrogen was used to prevent any changes in the enzymatic activity P-450 (Forline & Anderson, 1985).

Fish were identified according to Whitehead *et al.* (1986); the benthic species *Chelon labrosus* and the pelagic S*iganus rivulatus* were selected.

In the laboratory tests, the fish were injected under the skin with standard liquid of aromatic compound under various degrees of temperature, pH, using three different concentrations of standard complex that may give an idea about the lethal doses.

MATERIALS AND METHODS

For this study we chose two regions (see Fig. 1):

a) Wadi Kandil region WK.: it's sited to the north of Ras Albasite near Omm Altior area (35˚ 42,` 35˚ 45`). It is extended from Ras Alkanzir to Borg Eslam north of Lattakia city. The depth of the water column ranged from 30-50 m; it is an open station that is exposed to marine currents all the year round, and a recipient of Wadi Kandil river discharges.

b) Almina Altigary MT: We sampled the places where the sewer is discharging near Alsaid and Alnozha ports. This station (35˚ 30 and 35˚ 34) spreads over 3km. The depth of the water column ranged from 5-15 m.

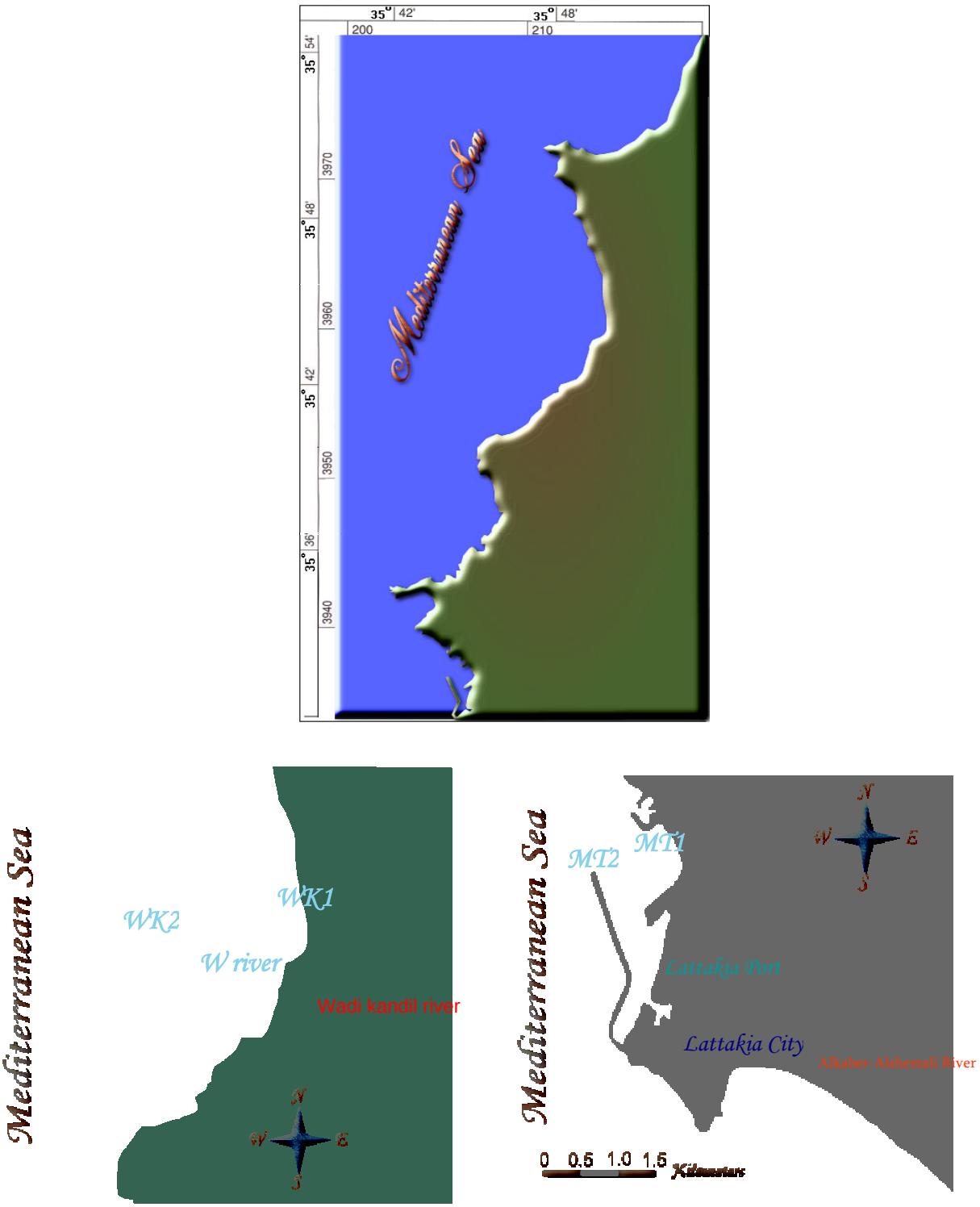


Fig. 1. The Syrian coast and the locations of the two sampling stations

Liver samples were kept with liquid nitrogen for EROD analysis. Another procedure is followed for temperatures 1-4º C. We determined protein concentration according to Bradford (1976) and

Lowery et al. (1957), where Bovine Serum Albumin was used as in Galgani and Bocquene (1988). Standard rizorufine was prepared as described in Klotz et al. (1984). (See Fig.2)

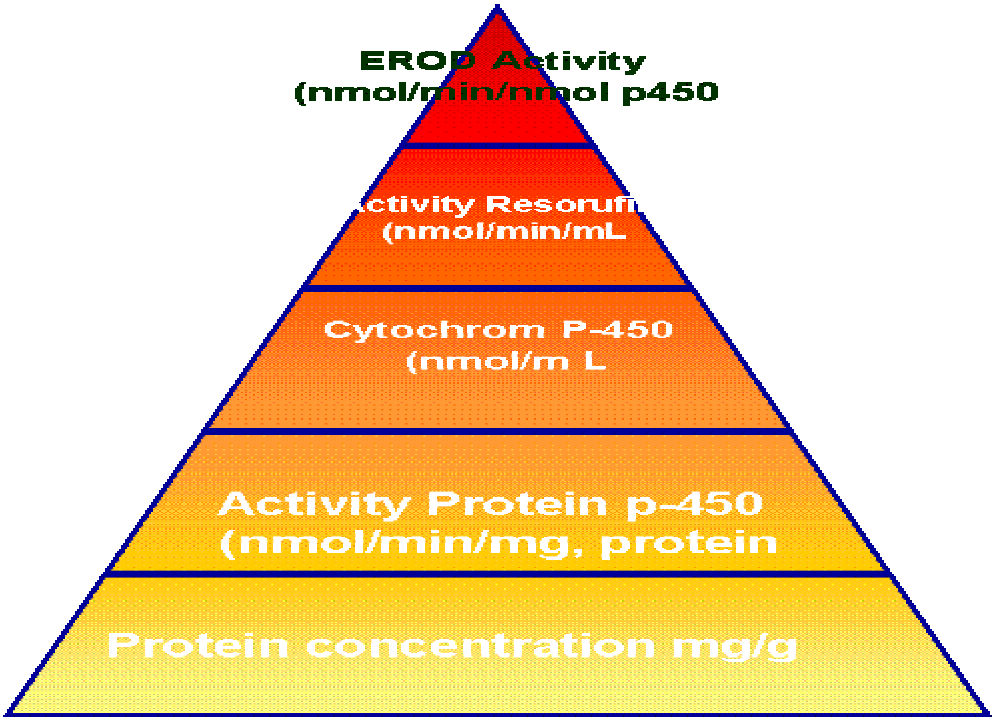


Fig 2: The analysis of EROD activity

RESULTS AND DISCUSSION

a) **EROD activity in female and male C*helon labrosus* of Wadi Kandil**

EROD activity ranged from 9.35-163.51 nmol/min/nmol P-450. 37% from EROD activity samples were more than 50 nmol/min/nmol P-450, and 50% were less than 20 nmol/min/nmol P-450 (Fig. 3).

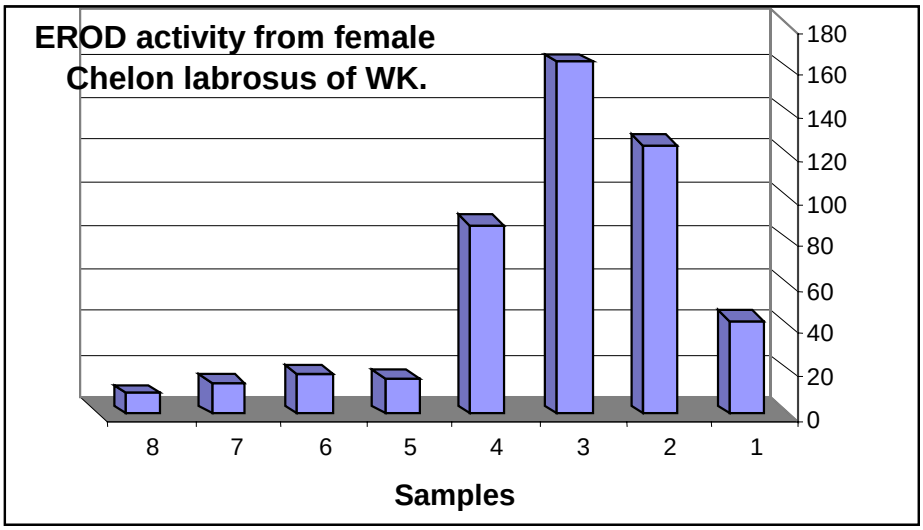


Fig. 3: EROD activity in female C*helon labrosus* of Wadi Kandil

b) **EROD activity in male C*helon labrosus* of Wadi Kandil**

Differences between 95% of samples are so small ranging from 0.04-1.02 nmol/min/nmol P-450 (Fig. 4)

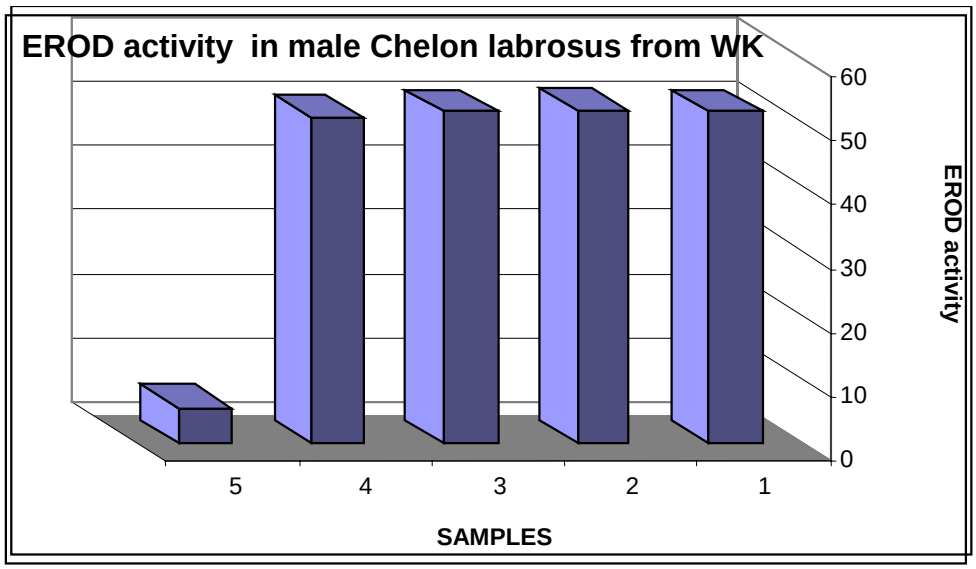


Fig. 4: EROD activity in male C*helon labrosus* of Wadi Kandil

c) **EROD activity in female Chelon labrosus in Almina Altigary**

It ranges between 1.508-87.113 nmol/min/nmol P-450. About 33% of the samples ranged from 36.17-59.57 nmol/min/nmol P-450, and 22% of the samples differed between 74.86-87.11 nmol/min/nmol P-450 (Fig. 5).

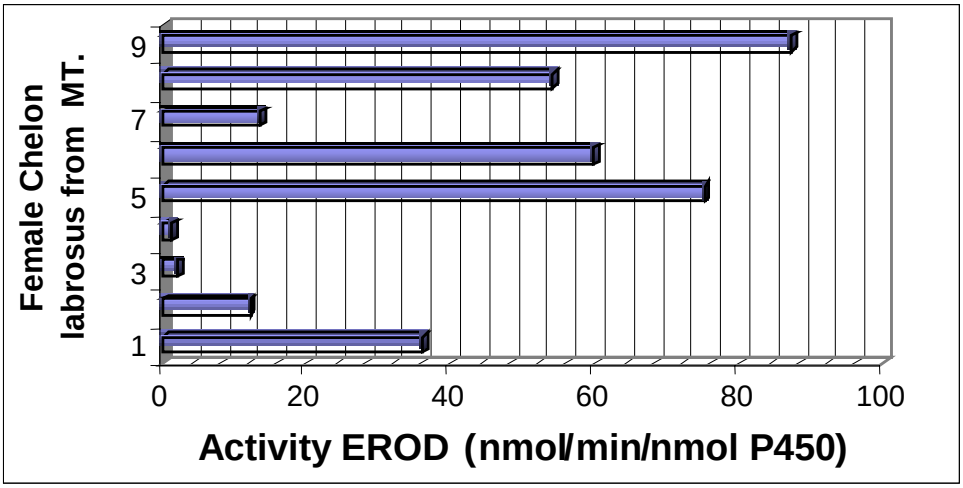


Fig. 5 : EROD ACTIVITY IN FEMALE *CHELON LABROSUS* in Almina Altigary

d) **EROD activity in male Chelon labrosus in Almina Altigary.** It ranges between 4.34-542.41 nmol/min/nmol P-450; only about 8.7% ranged between 318.95-385.53 nmol/min/nmol P-450, and 21% ranged between 122.56-164.98 nmol/min/nmol P-450 (Fig. 6).

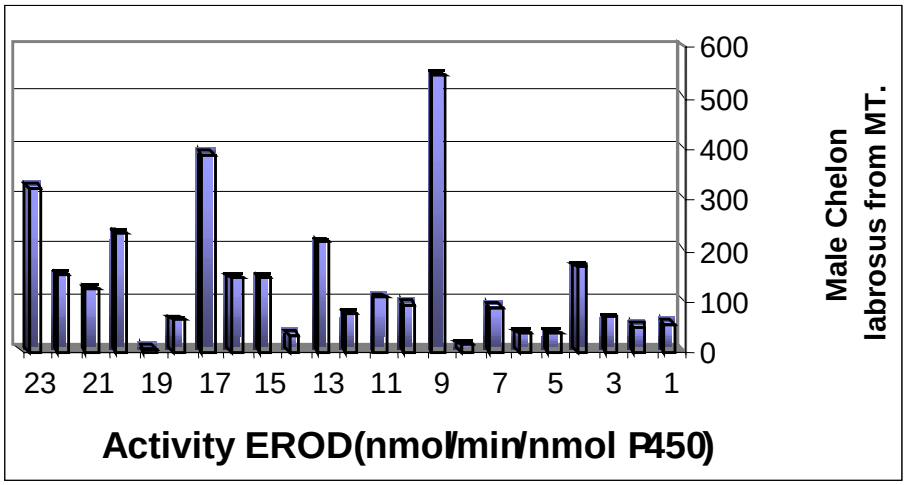


Fig. 6:EROD activity in male *Chelon labrosus* in Almina Altigary

e) **EROD activity in male Siganus rivulatus (Wadi Kandil)**

During 2004 two samples were analysed and the results were 36.892 and 23.8746 nmol/min/nmol P-450.

f) **EROD in male Siganus rivulatus (Almina Altigary)**
During 2004 the results of the four analyzed samples were *33.648, 55.466,65.7356 , 135.684 nmol/min/nmol P-450.*

g) **EROD activity in male C*helon labrosus* (2006)**
EROD activity during September, 2006 doesn't reach 90 *nmol/min/nmol P-450*, whereas it doesn't reach 0.03 *nmol/min/nmol P-450* during May, 2006 in Almina Altigary (Fig. 7).
In Wadi Kandil, it doesn't reach 0.009 *nmol/min/nmol P-450 during* September *2006.*

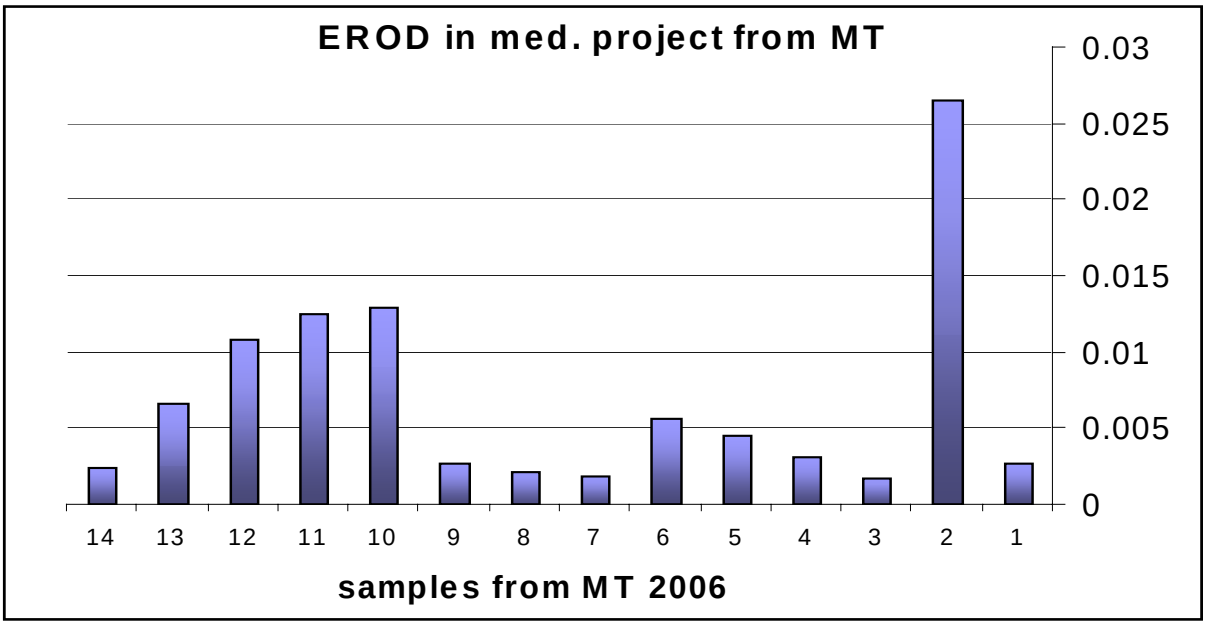


Fig. 7 :EROD activity in male C*helon labrosus* in Almina Altigary (May 2006).

h) EROD activity in male C*helon labrosus in* Almina Altigary (September 2006) (Results of Mediterranean project) (Fig. 8)

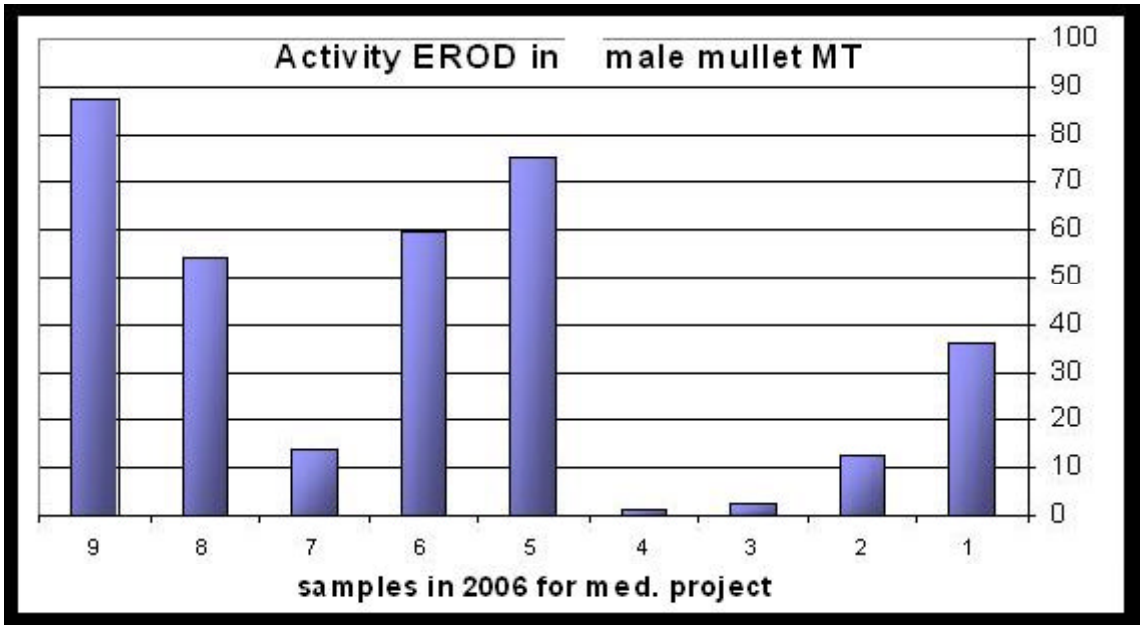


Fig 8:EROD activity in male C*helon labrosus* in Almina Altigary (September 2006)

i) EROD activity in the males of *Chelon labrosus of Wadi Kandil* September 2006 (we made this analysis for Mediterranean project). (Fig. 9)

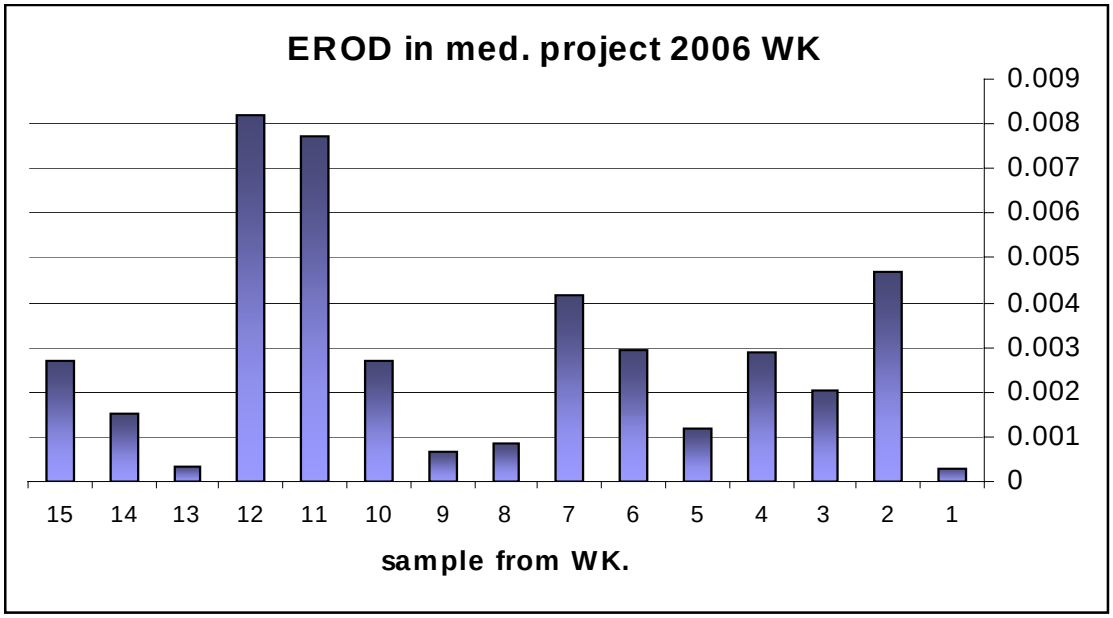


Fig 9. EROD activity in male C*helon labrosus* in Wadi Kandil (September 2006) .

There was a significant relationship of EROD activity with each of total and standard length of fish (69-70%), and with weight (75-75%). In addition, we observed many negative relationships between P-450 and length (-54%, -86%) between weight of liver and total weight. Other relationships were observed between Cytop450 and liver weight, reaching to (70)%. These relationships indicate that EROD activities increase with age and with pollutant concentrations.

LABORATORY STUDIES

We injected the test fish with 10 µL of standard mixture compound (concentration C); diluted 10 times solution (concentration B), and diluted 20 times (concentration A) (see Table 1 for concentrations). The aim of our study was to see how aromatic compounds bio-accumulate in liver under different environmental conditions (Fig 10).

We chose three degrees of temperature (18,20 and 30 ºC), and three levels of pH (6.85,7.5 and 8.2). The fishes were acclimatized for 1, 3 and 5 hours in natural marine water (natural pH). *Chelon labrosus* were injected under the skin of the abdomen.

We chose the fish that are similar in morphology (length, weight) and health while individual fish was marked by tags for differentiation.

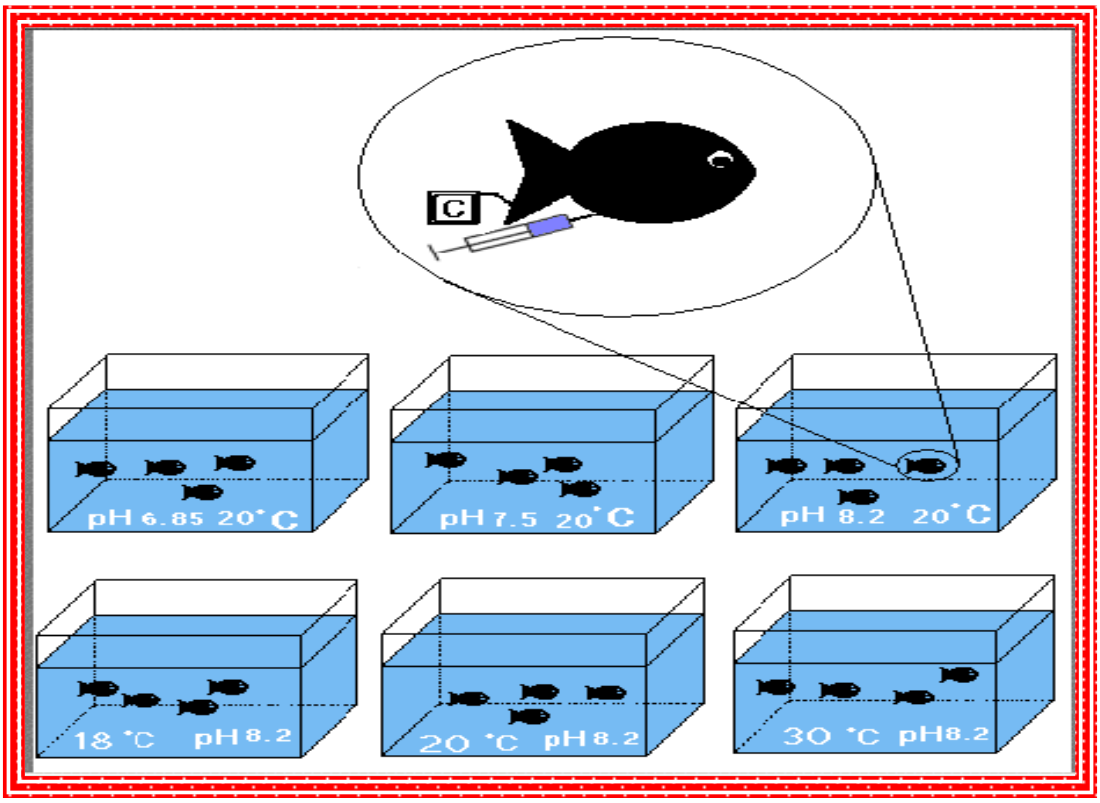


Fig. 10: the aquarium with different degree of temperature,
p H, and concentrations (A,B,C).

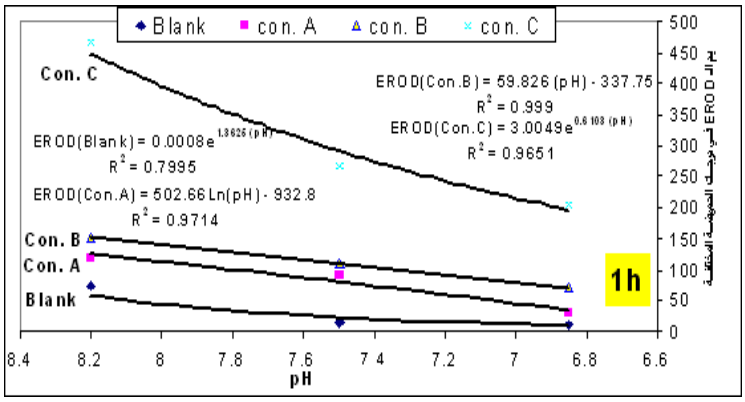
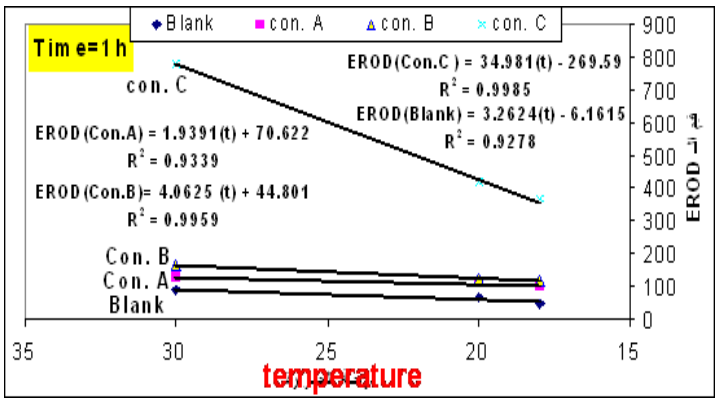
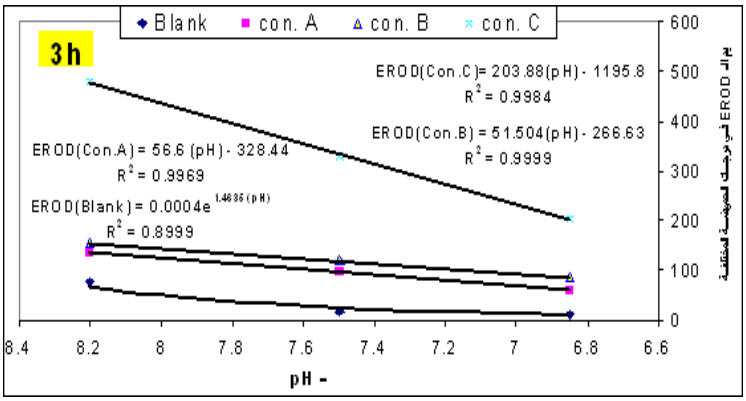
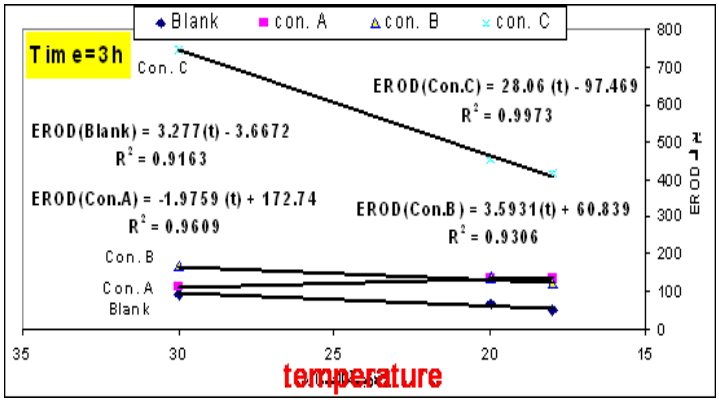
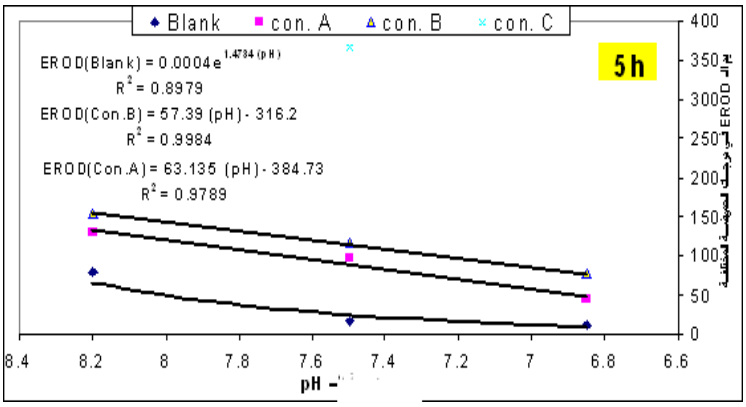
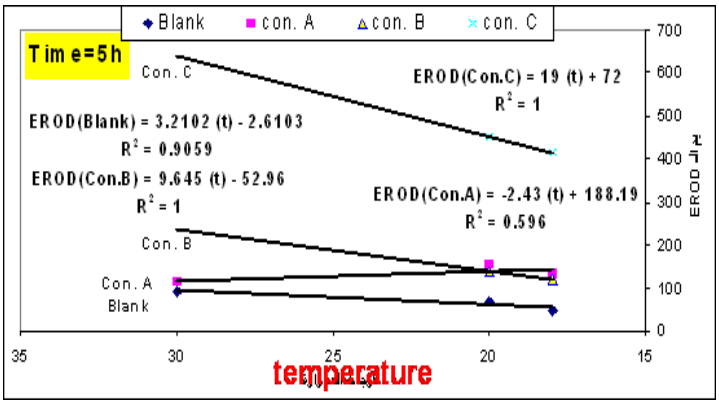
Table 1: Concentrations of aromatic compounds in the standards used

المركبات	Concentration C (ng/µL)	Concentration B (ng/µL)	Concentration A (ng/µL)
C10	15	1.5	0.75
Naphthalene	31.61	3.161	1.5805
C12	15.4	1.54	0.77
1Methyl naphthalene	19.02	1.902	0.951
1 Ethyl naphthalene	16.71	1.671	0.8355
C-14	15.2	1.52	0.76
Acenaphythylene	34.05	3.405	1.7025
Acenaphythene	24.52	2.452	1.226
2,3,6,Trimethyl naphthalene	17.38	1.738	0.869
Fluorene	38	3.8	1.9
C-16	15	1.5	0.75
C-17	15.2	1.52	0.76
Pristane	15	1.5	0.75
Phenanthrene	36.67	3.667	1.8335
Anthracene	33.34	3.334	1.667
n-Octadecene	15.4	1.54	0.77
C-18	15	1.5	0.75
Phytane	12.8	1.28	0.64
2Methyl Phenanthrene	11.38	1.138	0.569
1Methyl naphthalene	2.59	0.259	0.1295
3.6Dimethyl phenanthrene	16.76	1.676	0.838
C-20	15	1.5	0.75
Fluoranthene	20.86	2.086	1.043
Pyrene	30.35	3.035	1.5175
C-21	15	1.5	0.75
C-22	14.95	1.495	0.7475
1Methyl Pyrene	12.86	1.286	0.643
C-24	15	1.5	0.75
Chrysene	28.04	2.804	1.402
C-26	15	1.5	0.75
Squalane	15.4	1.54	0.77
C-28	15	1.5	0.75
Perylene	17.9	1.79	0.895
C-30	15	1.5	0.75
C-32	30	3	1.5
C-34	30	3	1.5

From the plots below it can be seen that:

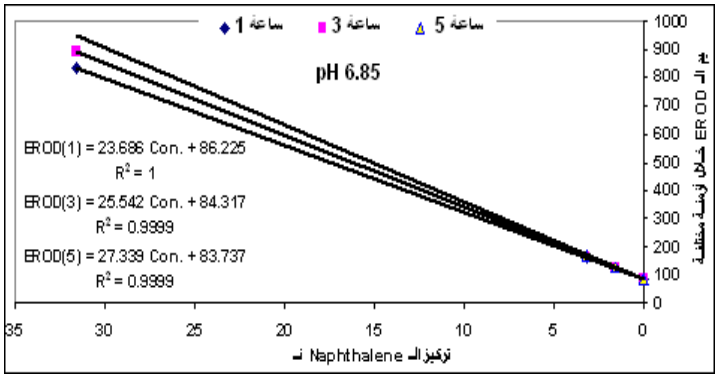
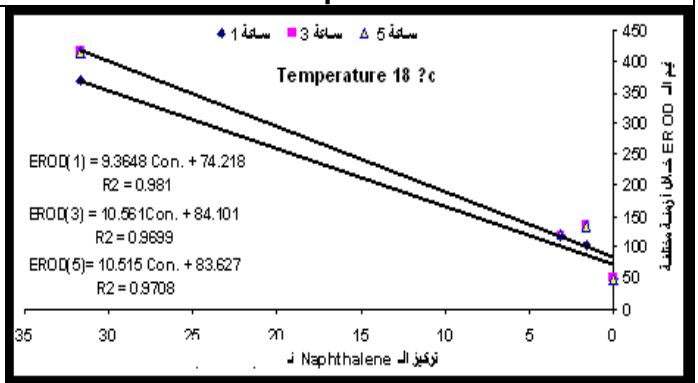
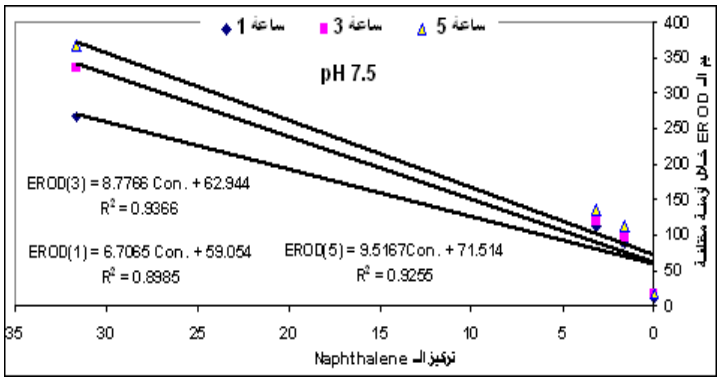
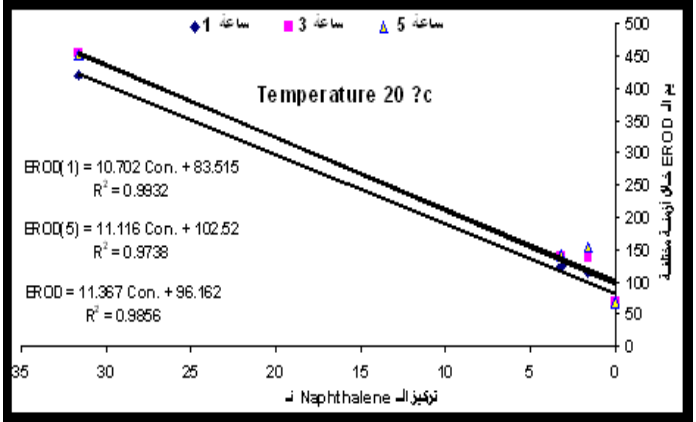
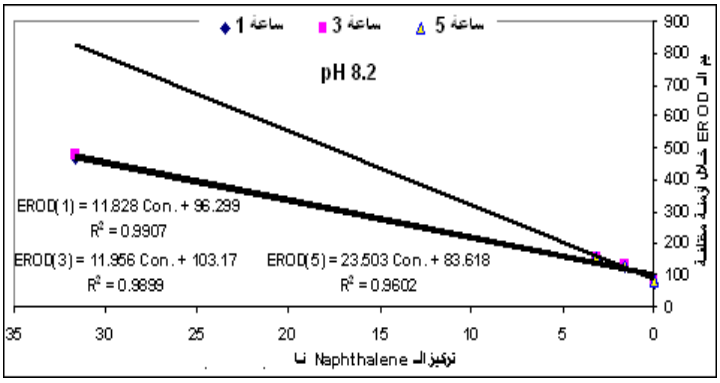
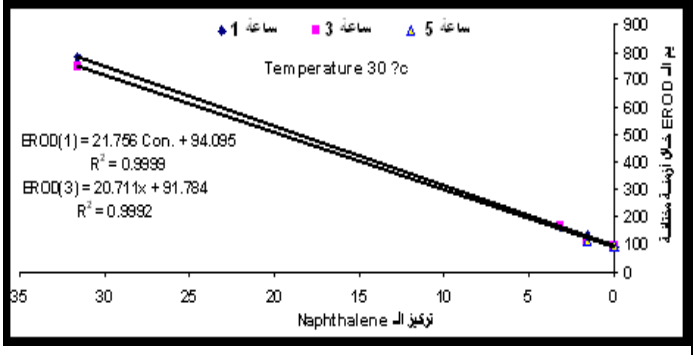
a) The effect of temperature is not obvious in samples that were injected with low concentrations, whereas the effects were very obvious in samples that were injected with high concentrations.

b) The form of our equation varies with pH and increases with time under high concentration.

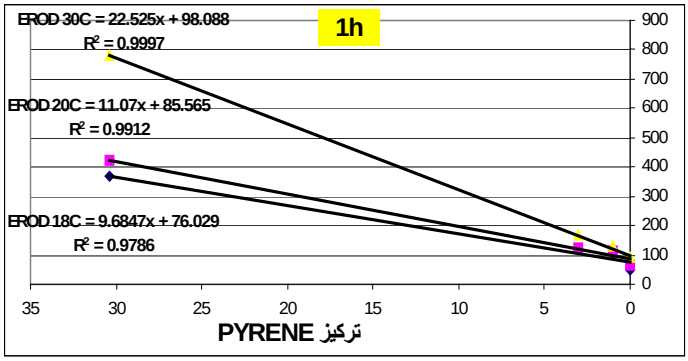
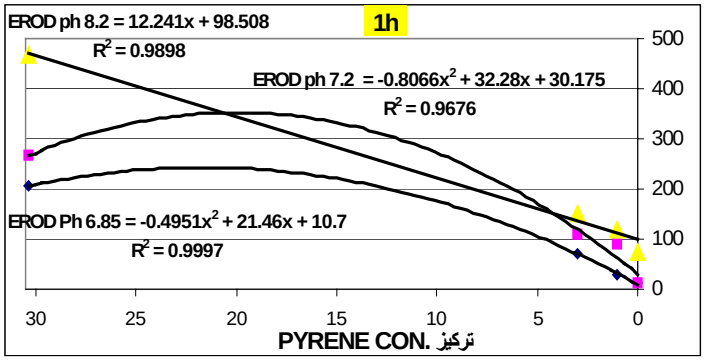
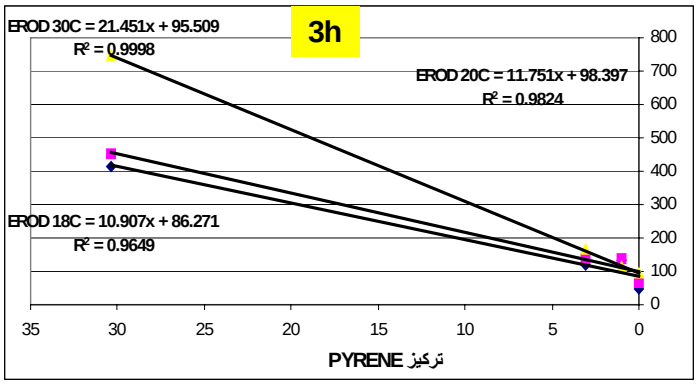
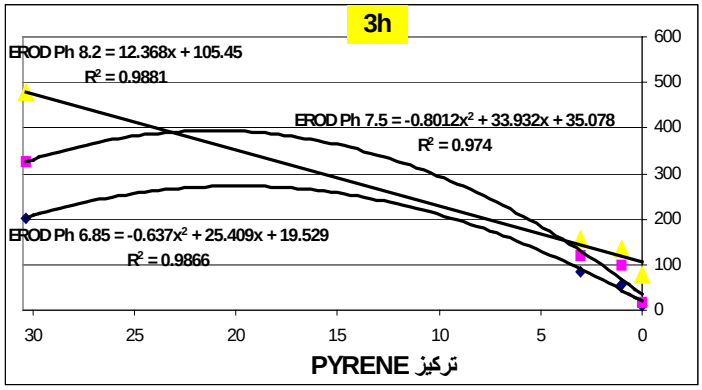
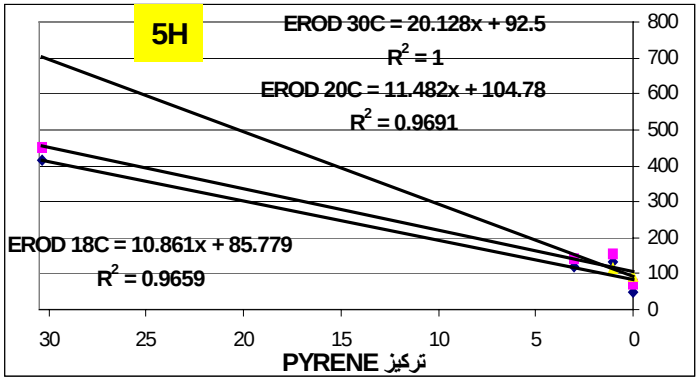
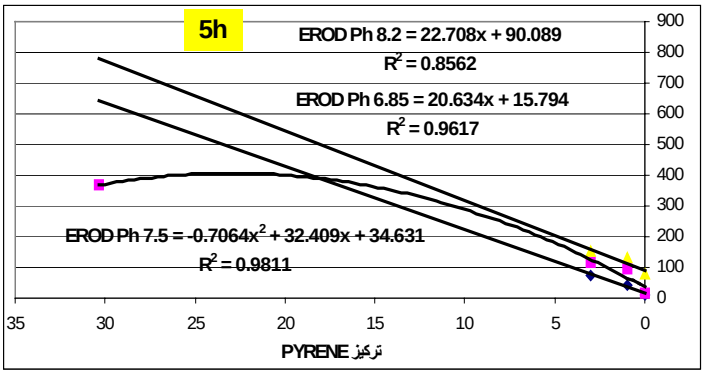
EROD activity changed with pH after 1,3,5 hour and with three concentration	EROD activity changed with temperature of water after 1,3,5 hour and with three concentration
	
	
	

c) *EROD activity changed with temperature, with concentration of Naphthalene (A, B, C and the Blank) and with time.
d) *The concentration of aromatic compound increases with EROD, as median after 3 hour of injection.
e) *EROD activity reached its maximum after 5 to 6 hours
From the plots below it can be seen the effect of temperature and the concentration of pollutants.

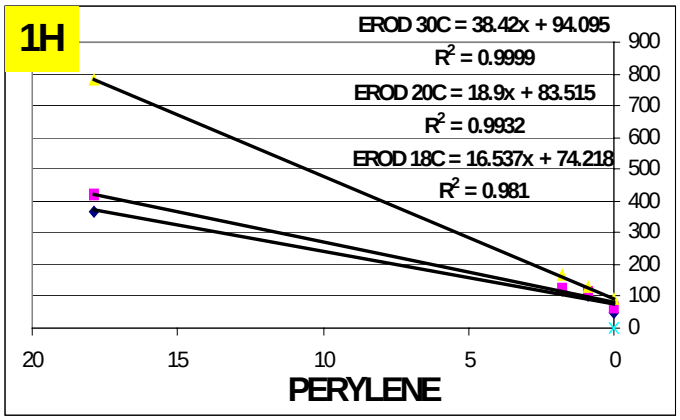
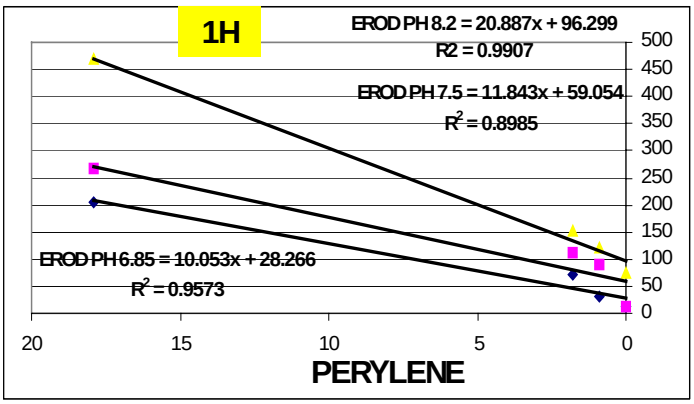
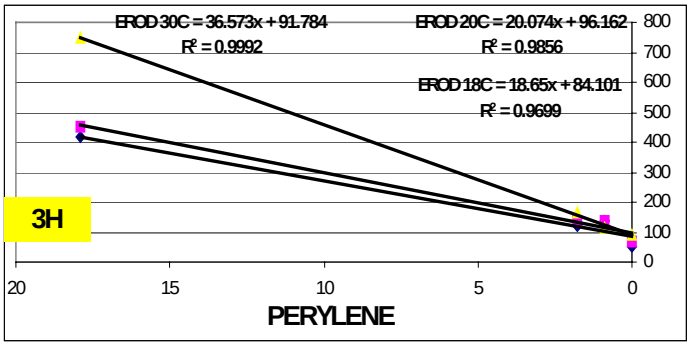
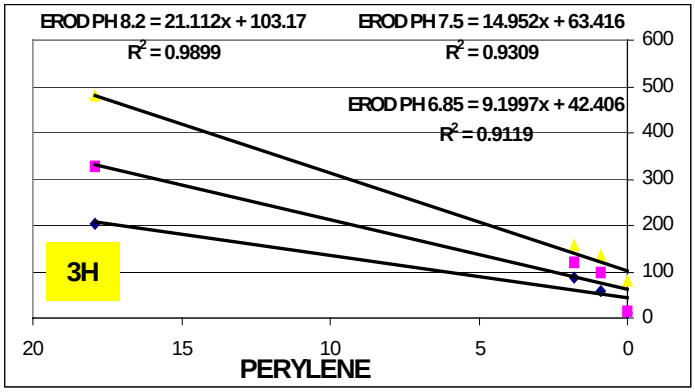
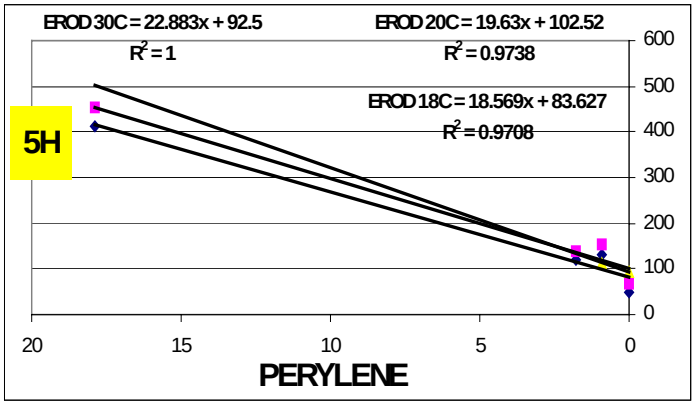
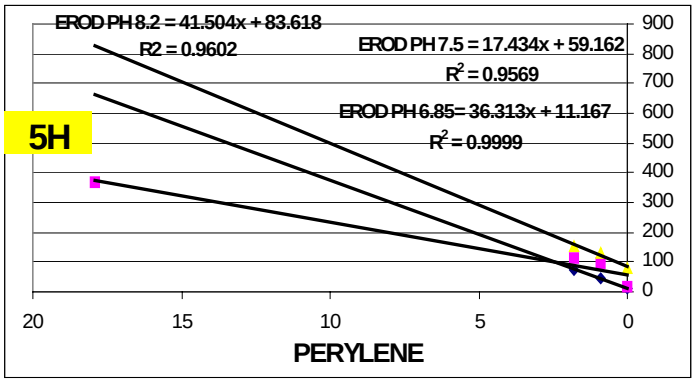
- *the results are similar at pH 6.85 and 7.5 with the changes of naphthalene concentrations and EROD after 1, 3 and 5 hours, whereas in pH=8.2, the effect was noticed clearly after 1 hour.
- * there is a similarity in the results after 1, 3 and 5 hours with different temperatures.

EROD activity with changed pH ,time, and naphthalene concentration	EROD activity with changed temperature, time and naphthalene concentration
	
	
	

- *EROD activity increased at 18 & 20º C with changes of concentration of pyrene, whereas it decreased at 30º C.
- *In general, we can say that EROD activity increases continuously at pH=6.85, whereas it decreases continuously at pH= 7.5 with changing time.

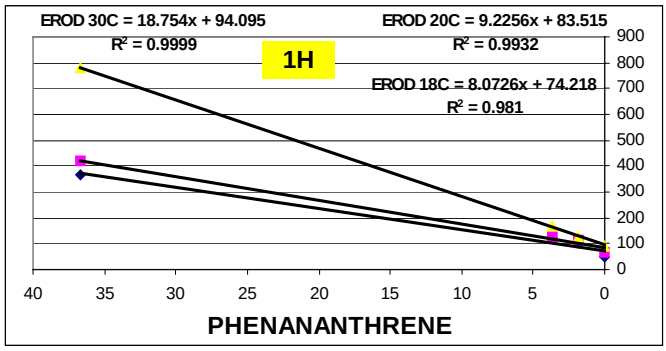
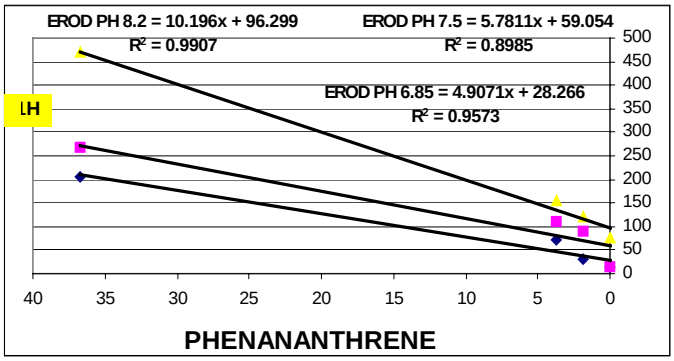
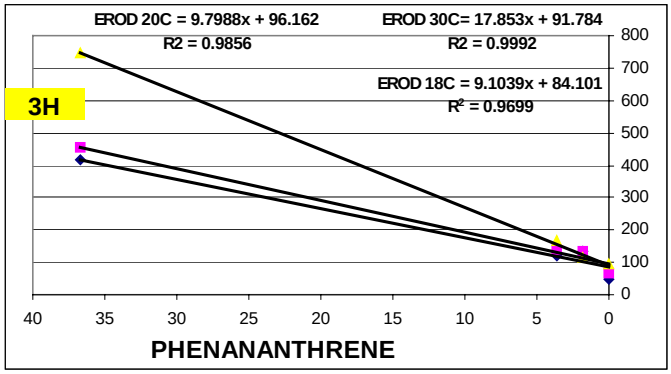
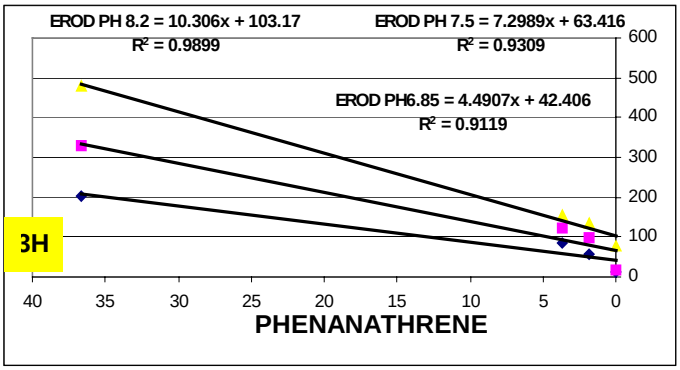
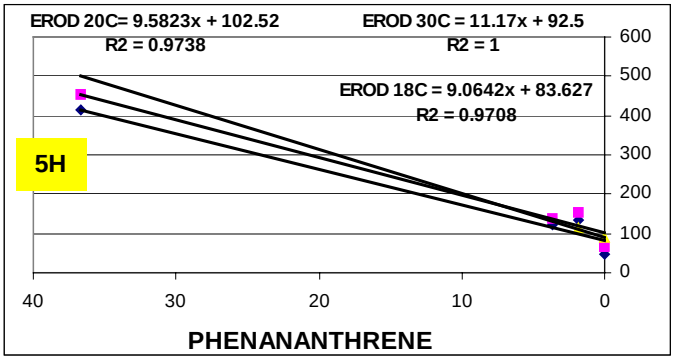
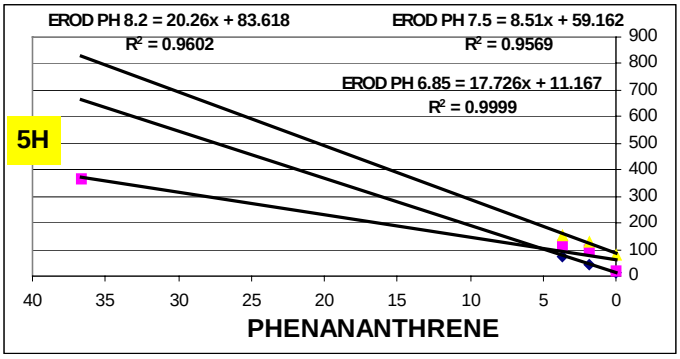
EROD activity with changed temperature, time and pyrene concentration	EROD activity with changed pH ,time, and pyrene concentration
	
	
	

- * EROD activity continuously increases with the concentration of perylene at 18 and 20º C, whereas it decreases at 30º C.
- * At pH 7.5 and 8.2 there is a continuous increase but it is very different in pH = 6.85 with time.

EROD activity with changed temperature, time and perylene concentration	EROD activity with changed pH ,time, and perylene concentration
	
	
	

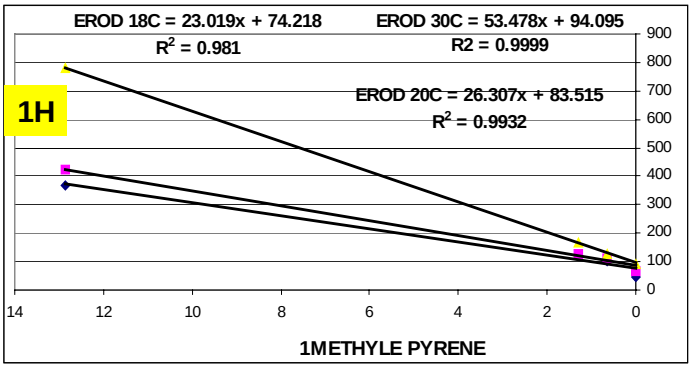
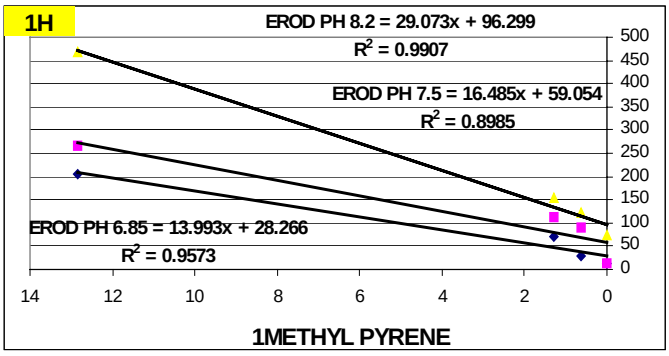
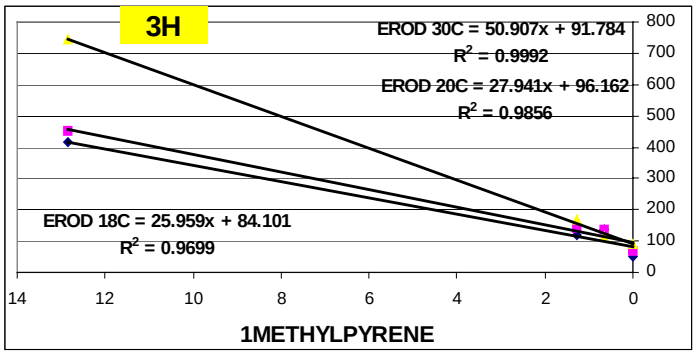
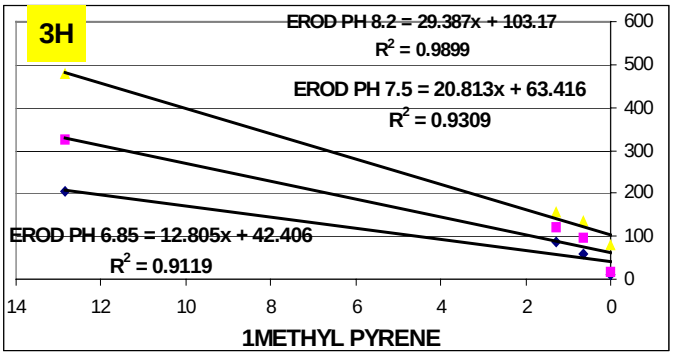
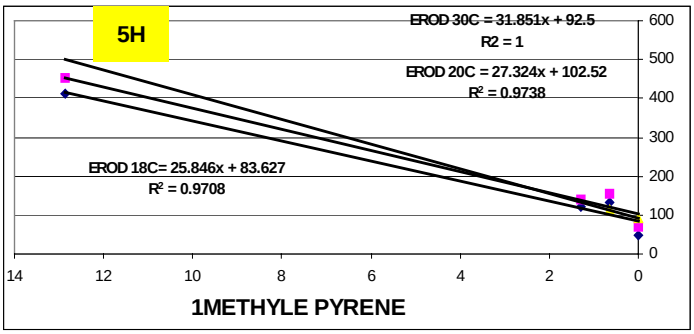
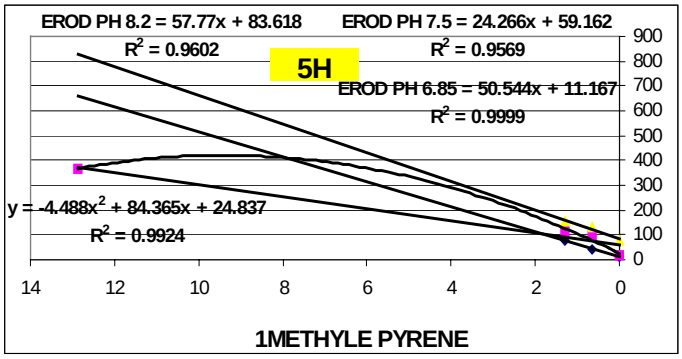
*EROD activity increased for different concentrations of phenanthrene with pH in1 and 3 hours, but the effect was stable after 5 hours.

* EROD activity increased with temperature after 1, 3 and 5 hours.

EROD activity with changed temperature, time and phenananthrene concentration	EROD activity with changed pH ,time, and phenananthrene concentration
	
	
	

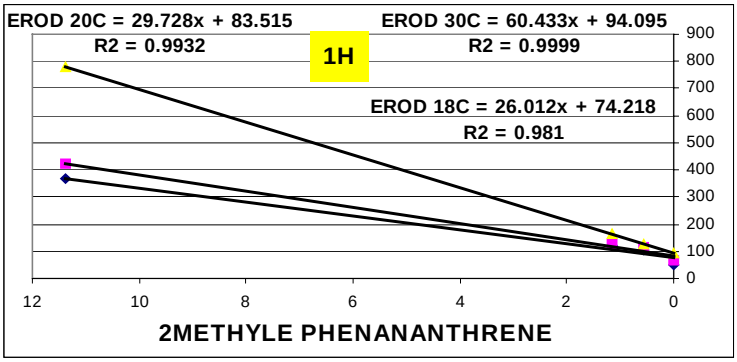
- EROD activity varied with changing the concentration of 1 methylpyrene at 18-20˚C. There was a continuous increase after 1 and 3 hours, a decrease after 5 hours, and a continuous decrease at 30C˚.

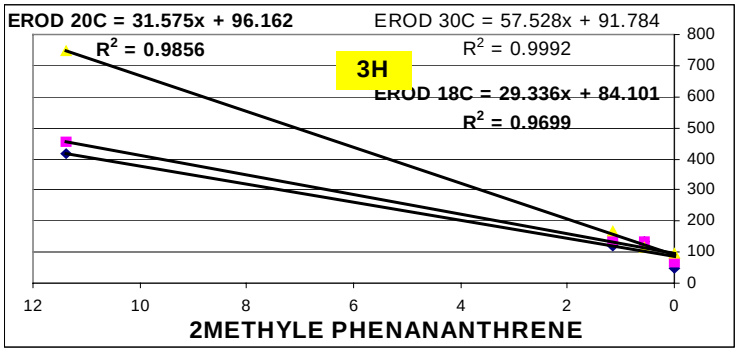
*There was a continuous increase at pH of 7.5 & 8.2 but it varied with time under pH of 6.85.

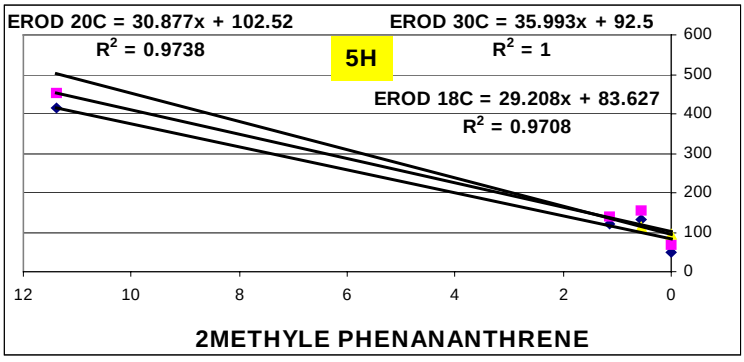
EROD activity with changed temperature, time and 1methylpyrene concentration	EROD activity with changed pH ,time, and 1methylpyrene concentration
	
	
	

- *EROD activity changed with different concentrations of 2methylphenanthrene in two different degrees of temperatures 18 &20º C whereas it increased with time, and decreased at 30º C.
- *At pH 7.5 and 8.2 it continuously increased, whereas it varied with time at pH 6.85.

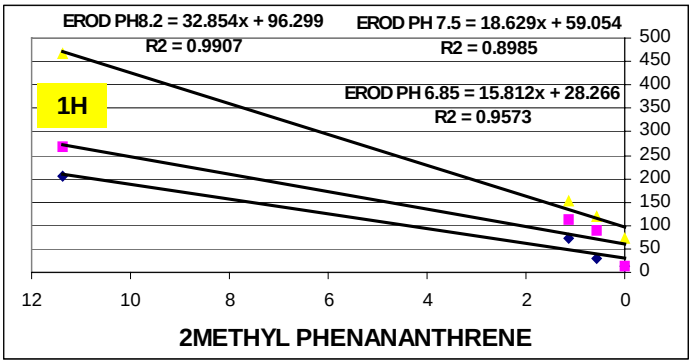
EROD activity with changed temperature, time and 2methylphenananthrene concentration

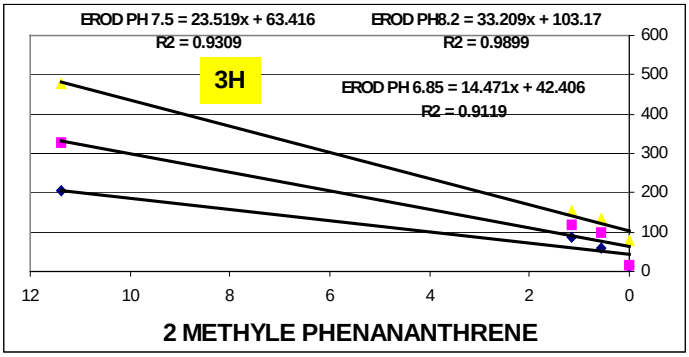


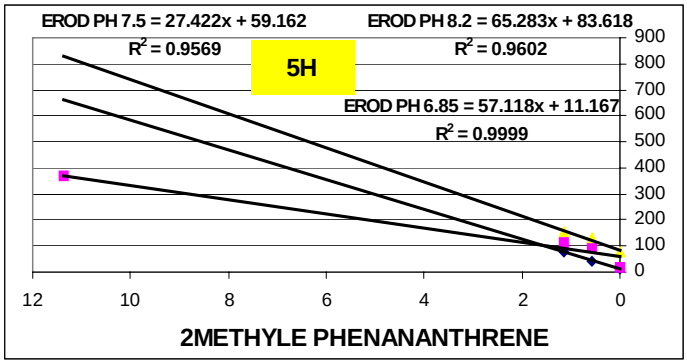




EROD activity with changed pH ,time, and 2methylphenananthrene concentration







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The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms*

by

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ABSTRACT

The paper outlines a 2-tier approach for wide-scale biomonitoring programmes. To obtain a high level of standardization, we suggest the use of caged organisms (mussels or fish). An "early warning", highly sensitive, low-cost biomarker is employed in tier 1 (i.e. lysosomal membrane stability and survival rate, a marker for highly polluted sites). Tier 2 is used only for animals sampled at sites in which LMS changes are evident and there is no mortality, with a complete battery of biomarkers assessing the levels of pollutant-induced stress syndrome. Possible approaches for integrating biomarker data in a synthetic index are discussed, along with our proposal to use a recently developed Expert System. The latter system allows a correct selection of biomarkers at different levels of biological organisation (molecular/cellular/tissue/organism) taking into account trends in pollutant-induced biomarker changes (increasing, decreasing, bell-shape). A selection of biomarkers of stress, genotoxicity and exposure usually employed in biomonitoring programmes is presented, together with a brief overview of new biomolecular approaches.

Thanks to the rapid advancement of the field of aquatic toxicology in recent years, we now have a greater understanding of the effects of pollutants on marine organisms at a molecular, cellular, tissue / organ, and organism level. Within the framework of the 2001-2004 EU Biological Effects of Environmental Pollution Program (BEEP), additional emphasis has been put on the importance of assessing physiological responses suitable for evaluating the reproductive performance of the animals of interest, linking observed changes in biomarker activity to putative contaminant effects at a population level.

Molluscs (mainly mussels, *Mytilus sp.*) and fish (*Mullus sp., Platichthys flesus* L.*, Zoarces viviparus, Perca sp.*) have both been employed as sentinel organisms in routine biomonitoring programs, both at a national and an international level (Med Pol, UNEP Mediterranean Biomonitoring Program; OSPAR Convention, RAMOGE., etc.). As a result, molluscs are now taken as the bioindicators of choice on the basis of their wide geographic distribution, their straightforward availability in the field and through aquaculture, and their suitability for caging experiments along coastlines. At the same time, the use of fish in monitoring programs is believed to be of importance despite logistic problems (the cost of sampling, caging, transportation, etc.) because of the key position of these organisms in the trophic chain and their high commercial

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value. Therefore, protecting fish populations from the adverse effects of sea pollution is not only an issue of conservation ecology but it is of great economic value.

In this paper, we focus on low-cost biomonitoring strategies that employ sentinel organisms and biomarkers while taking into account the recent advances in the field of aquatic toxicology. It should be noted here that an approach that makes use of biomarkers does not replace chemical and ecotoxicological monitoring strategies, but it integrates them providing an unique contribution in determining the toxicity of pollutants (even when they are present at low, sub-lethal concentrations).

Thus, this review attempts to describe in some detail the use of molluscs as sentinel organisms while providing some basic information for developing monitoring strategies that analyse pollutant effects on fish the latter being mainly studied as wild organisms for investigating the physiological status of natural populations.

The first two important issues to be considered are the means through which sentinel organisms are exposed to the pollutants and the choice of the biomarkers.

Molluscs, and mussels (*Mytilus sp.*) in particular, can be easily caged in adequate steel containers usually positioned 3-4 meters under the sea surface. They have been proved to be suitable sentinel organisms for biomonitoring coastal sea waters (Cajaraville et al., 2000; Viarengo et al., *in press*), lagoons and estuaries (Nasci et al., 2002). Caging the organisms as described for 3-4 weeks allows physiological parameters not to be affected by differential changes in the reproductive cycle although slight variations in temperature, food availability and salinity may take place at each site.

Under these conditions, observed changes in organism physiology are believed to be related to the effects of toxic chemicals present in water that are accumulated in the tissues of these filter-feeding molluscs.

Using caged organisms in biomonitoring studies, as well as in related research, makes it easier to standardize the results and to compare control organisms to animals collected from potentially polluted sites, making it possible to correctly measure the differential accumulation of chemicals in the tissues of control individuals and individuals caged in polluted areas. In fact, a relationship between biological effects and pollutant concentrations cannot be inferred utilising wild animals since harmful compounds may have a very different biological half-life in mussels: from days (copper and pesticides) to months (cadmium) and years (Viarengo et al., 1985; Poremski and Wiandt, 2000). Moreover, pollutants may be partially stored in the cells in non-toxic forms (Viarengo and Nott, 1993). For this reason, they are found in different amounts in individuals of different ages (Koehler, 1989).

In addition to this, animals from wild populations may be at different stages of gonadal maturity and therefore may show different biological responses and pollutant accumulation patterns in the various tissues; typical examples are polycyclic aromatic hydrocarbons (PAHs) and coplanar polychlorinated biphenyls (PCBs) contents in the egg lipidic envelop. It should be noted that the use of wild mussels can always integrate mussel watching with caged organisms. This is the case, in particular, when studying pollutant long-term effects that may be emphasized in mussels from natural populations. However, as mentioned above, the requirements concerning mussel sampling conditions should be always carefully satisfied (same specie sampled along the coast, similar age and similar gonad developmental stage, etc.).

It is important to point out that different tools for biomarker data integration and interpretation have been developed in recent years, aimed at drawing up indices to rank the different stages of pollutant-induced stress syndrome (Schmolke et al. 1999; Broeg et al., 2005). In particular, an "expert system" for mussel stress syndrome classification was developed within the BEEP framework (Viarengo et al., 2000; Dagnino et al., 2007). This approach is an important contribution in the attempt to sort out what biomarkers are useful to assess the organisms' health. Indeed, this

system assesses the development of pollutant-induced stress syndrome, "ranking" the different stages. To do so, some requirements need to be fulfilled. The suite of biomarkers must include:

1. biomarkers that are sensitive to stress at a molecular and a cellular level. These, being rapidly activated, should give early warning signals of toxic chemical effects on the animals.
2. biomarkers assessing pollutant damage at the tissue level.
3. biomarkers assessing stress at the organism level, giving indication of the potential survival capacity of the animals as well as their reproductive performance. These are essential to relate the effects of pollutants on individuals to the possible changes at the population level.

Using cellular biomarkers that can integrate input from different environmental stressors during the 3 - 4 week period of exposure would provide a clear insight in the development of the stress syndrome.

Among others, biomarkers currently employed that fulfil these requirements are the following: lysosomal membrane stability, lipofuscin and neutral lipid accumulation in lysosomes, and micronuclei frequency. These biological parameters have often been utilized in biomonitoring programs in the past years. In the light of recent advances, however, they have acquired a new relevance. It is important to point out that the above mentioned "expert system" takes into account the fact that the changes in individual biomarker values over a stress gradient show characteristic trends (i.e. increasing, decreasing, or bell-shaped response profiles). On the basis of this information and of the physiological significance of the different biomarkers employed, the system described takes into consideration the possible mutual interferences that may occur among various biological factors in a stress condition.

Another very important point to be addressed here regards the setting up of a “biological mussel watch”. It would be very expensive to make use of a full biomarker battery (at least 6-8 indices) to verify the potential effects of pollutants in tens or hundreds of sites along a sea coast.

Thus, to make the biomonitoring tool suitable for application purposes, we propose a two-tier approach aimed at developing a cost-effective strategy which Environmental Agencies can afford.

Tier 1. Screening: For the initial screening, we propose the use of a highly sensitive, low-cost biomarker such as lysosomal membrane stability. Mussel mortality rates will indicate areas characterized by high levels of pollution.

Tier 2. In sites where an alteration in lysosomal stability has been established, the level of the stress syndrome is then to be assessed by utilizing the full battery of biomarkers. The data collected should be then run through an appropriate approach of data integration (including the “expert system”) to obtain an objective assessment of the pollutant induced stress syndrome.

In the dataset of stressed animals, sampled in the polluted areas, biological effects are predicted to be related to the amount of toxic chemical compounds accumulated in their tissues during the caging period. The selection of biomarkers represents therefore a crucial issue in obtaining an integrated index for ranking the stages of stress syndrome advancement.

The present review describes in some detail a number of biomarkers of stress and exposure that can be measured in mussels and fish (both caged or wild), to render easier the selection of the tests to be utilized in a two tiers biomonitoring program.

Examples of suitable biomarkers to be utilized on bivalves or fish as sentinel organisms are listed in Table I and Table II, respectively.

Biomarkers of stress

Lysosome membrane stability

In the field of aquatic toxicology lysosomes have attracted considerable attention in recent years because they i) were shown to be the target for a wide range of contaminants, ii) are easy to visualize in blood cells and in reacted tissue cryosections, and iii) are present in all nucleated cells and therefore are not species-specific. Lysosomes are subcellular organelles surrounded by a semipermeable membrane that contains numerous hydrolytic enzymes involved in a range of cellular processes including digestion, defense, and reproduction (Moore, 1976; Pipe, 1993; Ferreira and Dolder, 2003). Enhanced catabolic activity and lysosomal damage can provoke, as an ultimate effect, the leakage of acidic hydrolases in the cytosol, possibly leading to a more severe damage and to cell death (Koehler et al., 2002). Recent findings suggest that toxic chemicals may affect these organelles not only by acting directly on the membrane, but also by activating (or downregulating) calcium- and tyrosine kinase-dependent cell signaling pathways (Burlando et al., 2002; Canesi et al., 2004; Marchi et al., 2004).

Initial studies about the toxic effects of contaminants on lysosomes were carried out to determine lysosomal membrane stability by using histochemical procedures applied on frozen tissue sections of fish liver or mussel hepatopancreas (Moore, 1976; Koehler, 1991). This methodology is currently chosen by many researchers; for example it was utilized in the BEEP EU project in the Baltic Sea as well as in the MARS project (Broeg et al., 1999a, 1999b, 2002; Koehler et al., 2002; for a review dealing with both fish and mollusc procedures see Moore et al., 2004).

In the cytochemical assay, serial sections of the tissue containing the lysosomes are exposed to a series of artificial labilisation periods in acid buffer. At the point when the lysosomal membrane is destabilized the substrate enters the lysosome and is then visualized by aid of a coupling reaction with an azo dye (such as fast violet B). In this method, animals are considered to be healthy if the lysosomal stability is >20 minutes; stressed but compensating if <20 but >10 minutes and severely stressed and exhibiting pathology if <10 minutes.

The advantage of the cytochemical approach using frozen sections is related to its potential to analyse a number of other parameters in parallel, such as lipofuscin and unsaturated neutral lipids contents, enzyme altered preneoplastic foci, and any protein of interest for which specific antibodies and/or cytochemical stain are available.

In fish, two peaks of lysosomal membrane destabilisation have been identified. The first peak corresponds to aged secondary cell lysosomes loaded with undegradable material (including chemicals as well as injured cell organelles). This first peak at <10 minutes clearly indicates the onset and progression of liver histopathologies (Koehler et al., 2002). The second peak is related to younger and more stable lysosomes and is reached at < 30 minutes. Among other things, it is also indicative for an assessment of liver damage. It correlates significantly with accumulated chemicals (POPs, heavy metals), thus reflecting the actual contamination (Diamant et al., 1999). Values for the second peak between ≤ 30 – 20 minutes indicate compensating stress and > 40 minutes reflect a healthy, unaffected state.

However, Lowe and Pipe (1994) developed an *in vitro* method that was originally set up for fish hepatocytes and subsequently further developed for blood cells of a range of invertebrate species living in water or soil (Weeks and Svendsen, 1996). This method, usually referred to as neutral red retention time (NRR or NRRT), uses the fact that damaged lysosomes showing membrane destabilization are an indication of a critical health status of the cells.

According to the *in vitro* methodology, the dye is sequestered into the lysosomal compartment when living cells are preloaded with neutral red (NR); if the lysosome membranes are damaged NR leaks out into the cytosol where it can be visualized under the microscope. The time taken for the dye to leak out into the cytosol is related to the degree of membrane damage. In case lysosome membranes are severely damaged the dye will leak out within 15 minutes of incubation,

whereas healthy lysosomes retain it for up to 180 minutes (Lowe and Pipe, 1994). The NRR assay has to be considered "a stress on stress test", in that neutral red is itself toxic for the cells and therefore further damages lysosomal membranes that are potentially already damaged. The additional damage caused by the dye results in a total membrane failure in the case of severely impacted cells, and also in membrane failure and leakage in healthy cells but in this case only after 150-180 minutes.

NRR as well as the cytochemical approach to measure lysosomal membrane stability was one of the core biomarkers selected for the biomonitoring approach within the BEEP program (EU VI Framework Program), that examined the usefulness and robustness of a range of biomarkers in studies carried out in different sites along the North Atlantic, Baltic and Mediterranean coasts, as well as in series of experimental exposure studies undertaken at the mesocosm facility at Rogaland Research in Stavanger (Norway) (see Aquatic Toxicology Vol. 78, Supplement 1, 2006). Lysosomal membrane stability assays were carried out accordingly to what reported by Moore et al. (2004), and the analysis of results demonstrated a good negative correlation between lysosome membrane damage and contaminant burdens at the different sites. Interestingly, samples taken from sites in the Baltic region indicated the need for appropriate saline solutions when working with mussels pre-adapted to a low salinity regime, i.e. 12-15 ppt. Therefore, the studies at the Baltic sites were carried out by replacing the usual physiological saline with 0.2 -filtered local sea-water (4 weeks being an adequate period of time for caged mussels to adapt to the different salinity environmental conditions).

As a good indicator of contaminant-induced lysosomal membrane damage, lysosomal membrane stability assays were employed in a number of other field studies with successful results using mussels (Lowe et al., 1995; Fernley et al., 2000; Castro et al., 2004; Einsporn et al., 2005; Schiedek et al., 2006) and also other invertebrate species including oysters (Hauton et al., 2001; Ringwood et al., 2002), scallops (Hauton et al., 2001), limpets (Brown et al. 2004), and crabs (Wedderburn et al., 1998) and fish (Koehler et al., 1992, 2002). The high sensitivity of this assay was confirmed during experimental studies within the BEEP program (EU VI Framework Program), by the fact that in some exposure experiments the levels of contaminants used were at concentrations lower than 1% of their published LC50 values. Nevertheless, they provoked a damaging effect on the lysosomal membranes of blood cells as well as in fish liver (Lowe and Koehler, unpublished result) that was clearly identified by NRR assay.

Reduced lysosomal membrane stability has to be considered as an indicator of a general physiological stress, i.e. non-specific. As such, it was used in a variety of other studies where a measure of stress was required; however, in a recent study by Harding et al. (2004) the effect of transportation of cultivated mussels was assessed using lysosomal damage as a biomarker of effect; moreover, the effect of temperature and salinity (Hauton et al., 1998, 2001) were also investigated in combination with other stress variables using this assay.

In conclusion, the lysosomal membrane stability, assessed either by NRR or by the histochemical technique, provides a robust Tier 1 screening biomarker for Environmental Impact Assessments (EIAs).

Oxidative stress biomarkers and lipofuscin lysosomal content

Toxic effects of pollutants often depend on their capacity to increase the cellular levels of reactive oxygen species (ROS). This can happen either by the straighforward activation of processes that lead to their synthesis or indirectly acting on enzymes (including superoxide dismutase, catalase, etc.) and scavengers (both hydrophilic such as GSH, ascorbate and MT, as well as lipophilic such as vitamin E and carotenoids), and thus decreasing cell defences. When ROS levels production exceeds antioxidant defences, cells experience oxidative stress which causes, among others, membrane lipid peroxidation (Viarengo, 1989).

The peroxidation end-products are accumulated in lysosomes as insoluble granules containing autofluorescent pigments and are usually referred to as lipofuscins. The bulk of lipofuscin granules is constituted by oxidatively modified proteins and lipid degradation products, along with carbohydrates and metals (George and Viarengo, 1985; Viarengo and Nott, 1993; Terman and Brunk, 2004). Although lipofuscin composition may be variable, all lipofuscin pigments are not degradable, probably because of the presence of peptides that are cross-linked by aldehydes into plastic-like, insoluble structures (Kikugawa et al., 1989).

The accumulation of these pigments in the lysosome vacuolar system of fish hepatocytes or in digestive gland cells of molluscs represents an indication of the oxidative stress level in the cells and it is related to the level of membrane lipid peroxidation (Viarengo and Nott, 1993).

During the exposure of mussels to pollutants, this biomarker typically shows a continuously increasing trend , which reaches a maximum level that is determined by the rate of secretion of lipofuscin-rich residual bodies into the external fluids. This rate is characteristically different for each tissue in different animal species (George and Viarengo, 1985; Viarengo and Nott, 1993). Overall, the use of lipofuscins as a cellular biomarker of oxidative stress seems to be more appropriate than the use of the malondialdehyde (MDA) or thiobarbituric acid (TBA) reactive compounds. In fact, these compounds are intermediate products of lipid peroxidation and, as reactive toxic metabolites, they are usually rapidly degraded (Esterbauer, 1985). On the other hand, lipofuscins represent an end point in the lipid peroxidation process and their accumulation is easily detectable in cells of stressed organisms. Recent studies by Moore (personal communication) indicated that lipofuscin levels are strongly correlated to lysosomal damage.

The method proposed for the determination of lipofuscin levels in molluscs and fish is simple and inexpensive although it requires a cryostat for obtaining 10 m sections of frozen tissue. Sections are stained by Schmorl´s reaction (Moore, 1988) and observed under a microscope. Lipofuscin content is then usually quantified by image analysis.

The mechanism underlying most assays employing biomarkers of oxidative stress that are in use is related to the change in the activity of the ROS defences system. Catalase, superoxide dismutase (SOD) and GSH transferase activities (GST) are often modified in response to cellular oxidative stress (Viarengo et al., 1988; Orbea et al., 2002b; Regoli et al., 2002; Geret et al., 2003; Orbea and Cajaraville, 2006).

In particular the assessment of catalase and GST activities, easily carried out by a low-cost enzymatic test, has often been used in laboratory studies and biomonitoring programmes for both fish (Romeo et al., 2000) and mussels (Romeo et al., 2003a, 2003b). These enzymatic tests have been proved to be suitable for monitoring the effects of pollutants on sentinel organisms. The enzyme response to toxic chemicals shows however a bell-shaped trend, with an initial increase due to the activation of enzyme synthesis followed by a decrease in enzymatic activity (due to the enhanced catabolic rate and/or a direct inhibitory action of toxic chemicals on the enzyme molecules). For this reason, enzyme assays should be used in association with other biomarkers such as the lysosomal membrane stability and lipofuscin accumulation assays, which follow the development of the pollutant-induced stress syndrome and therefore may help to correctly interpret the “physiological meaning” of changes observed in antioxidative enzymatic activities.

Similarly, the concentration of scavengers such as GSH, can be utilized per se as a biomarker of oxidative stress. In this case, a decrease in GSH concentration is usually associated to enhanced peroxidation processes in the cell membrane (Ziegler, 1985; Viarengo et al., 1990). It is important to mention that cells tend to maintain a constant level of reduced glutathione. For this reason, this biomarker is considered not sufficiently sensitive and thus it is not utilized routinely in biomonitoring programs. On the other hand, it is important to point out that protein oxidation, often under investigation in proteomic studies, has been recently proposed as a biomarker of oxidative stress (Sheehan, 2006). Because of the importance of this biochemical change in relation to cell physiology alterations, this stress biomarker should be considered for future large-scale biomonitoring applications.

As mentioned above, changes in the activity of the antioxidants present in cells provide information on organism responses to prooxidant pollutants. On the other hand, this is not sufficient to assess the overall efficiency of the antioxidant system. A measurement of the total oxyradical scavenging capacity (TOSC) may be utilized to quantify cellular resistance to different oxyradicals (Regoli and Winston, 1999; Regoli, 2000). TOSC provides therefore a useful indication of the contribution levels of oxidative stress to the pollutant induced alteration of the organisms' physiological status. This biomarker was recently employed both in laboratory and field studies (Gorbi and Regoli, 2003).

Neutral lipid accumulation

The effects of pollutants are often associated with an unbalanced fatty acid metabolism and with the accumulation of neutral lipids in the lysosomal vacuolar system (Lüllman-Rauch, 1979). The lysosomal storage of neutral lipids in mussel digestive glands and fish hepatocytes has been found to be a useful indicator of a change in the physiology of the cells (Koehler et al., 1992, 2002; Koehler, 2004).

As reported by Moore (Moore, 1988), a build-up of these substances in mussel digestive gland cells may be described as a form of lipidosis induced by toxic chemicals. In fact, cytochemical data clearly indicate an initial stockpiling of neutral lipids in the cytoplasm of cells of pollutant-exposed organisms. Lipids (probably in form of droplets) are then internalised into the lysosomes by autophagic uptake. It is important to note that such increase in the lysosomal storage of neutral lipids may be related to an increase in the cytosolic lipids content or to a decrease in fatty acid processing.

As with lipofuscins, an assay assessing the build-up of neutral lipids is a simple and low-cost biomarker, which also requires a cryostat to prepare 10 m sections of frozen tissue. Fatty acids are then specifically stained, observed under a microscope (Moore, 1988; Dondero et al., 2006a), and finally, they are quantified by image analysis.

Biomarkers of genotoxicity

DNA damage

Marine pollutants produce multiple consequences at the organism, population and ecosystem levels, affecting organ function, reproductive status, species survival, population size and ultimately biodiversity. Among these, carcinogenic compounds are of particular interest, and tumours have indeed been described in marine fish and shellfish (Mix, 1986; Malins et al., 1988; Bolognesi, 1990; Gopal and Pathak, 1993).
Recently, a concern about persistent organic pollutants (POPs) has been proved to be substantiated (Siu et al., 2004). These substances are usually present in the sea at very low concentrations. However, because of their persistance, they are accumulated in tissues of marine organisms at high concentrations, which are several orders of magnitude higher than that of their environment.

POPs bring about not only general toxic effects, but they are also known to be genotoxic, i.e. they can alter the integrity of the DNA structure, either directly or through their metabolites (Shugart, 1995). Genotoxic compounds such as POPs may cause mutagenesis (Siu et al., 2004). It should be noted that POPs chronic effects can be studied more appropriately when employing wild mussels in the tissue of which these persistent pollutants can accumulate and act for years. In this case, pollutants will affect the physiology of the organisms in the long term.

DNA alterations induced by chemical and physical agents include single and double strand breakages, modified bases, DNA-DNA crosslinks, and DNA-protein crosslinks.

Strand breakages may also be induced indirectly by an interaction with oxygen radicals, or by the action of excision repair enzymes, and finally as a consequence of the apoptosis or necrosis processes (Eastman and Barry, 1992; Speit and Hartmann, 1995).

Direct evidence of the genotoxic effects induced by different classes of pollutants is DNA adduct formation, detectable by using the 32P-postlabeling technique (Malins et al., 1990; Stein et al., 1992; Venier and Canova, 1996; Harvey et al., 1999; Ericson et al., 2002). However, this powerful technique is rather complex and is not usually employed in large biomonitoring programs.

Among the methods usually adopted in biomonitoring programs to detect DNA damage, three tests seem to stand out. The first to be discussed here is alkaline elution, based on the evidence that the rate at which DNA single strand fragments pass through a membrane filter under alkaline conditions is related to the length of the DNA strand itself (Kohn et al., 1976). This method has been successfully employed to evaluate genotoxic effects of pollutants in fish and molluscs exposed to chemical compounds (Batel et al., 1994; Bolognesi et al., 1996, 1999, 2004, 2006).

Another technique is the COMET assay, now widely used to assess the genotoxic effects of pollutants. In this technique, individual cells are directly embedded in agarose, where the nuclear DNA is then electrophorized through the gel, in which the cleaved DNA fragments migrate away from the residual chromatin nucleosomal core structure. The DNA stained with a fluorescent dye shows a “comet” in which the distance of DNA migration from the core (i.e. the comet tail length) reflects the level of double strand DNA breakage (Siu et al., 2004). This technique has been successfully employed with DNA extracted from fish and mollusc cells (Lee and Steinert, 2003; Frenzilli et al., 2004; Regoli et al., 2004; Siu et al., 2004).

A third method employed to assess DNA strand breakages monitors the rate of DNA unfolding under alkaline conditions by measuring the rate at which a fluorescent dye is incorporated in the double stranded DNA. Recently, a "micromethod" estimating DNA damage in fish and invertebrate cells and tissues has been developed (Batel et al., 1999). In this technique, a fluorescent dye, Picogreen binds to the intact double stranded DNA with an high affinity and the fluorescence response is monitored, while little background activity is picked up since the unbound dye has virtually no fluorescence allows estimation of minimal levels of DNA fragmentation caused by genotoxic compounds. The characteristics of this not very used methodology should allow an accurate assessment of the levels of DNA fragmentation caused by genotoxic compounds, even at low levels. Experimental conditions, such as the time set for allowing the DNA to unfold and the pH of the denaturation media have to be established on the basis of the complexity of the DNA molecule, which differs considerably among different taxa (Jaksic and Batel, 2003). The analysis of data on DNA damage may show a bell shape trend depending on the level of pollutant contamination and on the time of exposure.

Chromosomal damage: Micronucleus Test

The micronucleus test is a well known cytogenetic technique commonly used for the assessment of genotoxic effects caused by environmental stressors. Micronuclei appear when cells fail to incorporate complete or fragment chromosomes in the daughter nuclei during cell division. These are instead incorporated in small additional nuclei where they remain throughout the life of the cell . The presence of micronuclei is an indicator of chromatin breakage which may be caused by clastogens or spindle dysfunctions, ultimately caused by toxic compounds (Heddle et al., 1983; Carrano and Natarajan, 1988).

The test consists in the scoring of cells containing one or more cytoplasmic micronuclei in addition to the main nucleus; this procedure is technically easier and more rapid than the analysis of chromosomal aberrations during metaphase This is one of the reasons why this biomarker is widely utilized in biomonitoring programs. The micronuclei assay just requires a microscope: no fancy equipment. The assay is however time-consuming, involving the scoring of a high number of cells in each sample.

Micronuclei frequency can be considered a good indicator of genetic damage accumulated during the cell life span. Taking into account the life span of each cell type and their mitotic rate in a particular tissue, micronuclei frequency may provide an indication of what is the extent of the genotoxic effects of pollutants.

The micronucleus test has been widely applied in wild and caged marine invertebrates (Majone et al., 1990; Scarpato et al., 1990; Bolognesi et al., 1999, 2004; Siu et al., 2004). Caged mussels exposed to sea water polluted by aromatic hydrocarbons (such as the Petroleum Harbour in Genoa) displayed a continuous increase of micronuclei frequency in gill cells reaching a plateau after a month of caging (Bolognesi et al., 1996).

Micronuclei in fish can be visualized in different cell types such as gill, kidney, hepatic cells and fins (Al-Sabti and Metcalfe, 1995; Arkhipchuk and Garanko, 2005; Barsiene et al., 2006). At any rate, to assess cytogenetic damage in fish, the use of peripheral erythrocytes is more widespread because it avoids the complex procedures of cell preparation and the killing of animals (Al-Sabti, 1986a, 1986b; Grisolia and Starling, 2001; Llorente et al., 2002; Bolognesi et al., 2006). In mussels, micronuclei are usually assessed both in hemolymph and gill cells. However, gill cells seem to be the best target tissue for micronuclei determination in caged as well as in free-roaming mussels. This biomarker assay shows a typically continuously increasing trend in animals exposed to increasing pollutant concentrations and/or times of exposure.

Biomarkers of exposure

Tissue levels of metallothioneins

The battery of biomarkers used for environmental biomonitoring programs usually includes biomarkers of exposure, i.e. parameters whose changes can be related to the organism exposure to a specific class of pollutants.

Metallothioneins (MTs) are low-molecular-weight cytosolic proteins rich in SH groups, with a high affinity for IB and IIB metal ions, known to be involved in heavy metal homeostasis and over-expressed in organisms experiencing high metal concentrations in their environment (Viarengo et al., 1989, 1999b). They show a peculiar aminoacid composition characterized by high levels of cystein (up to 30% of total aminoacid content) and the absence of aromatic aminoacids and histidine. These metalloproteins are normally expressed in animal tissues, and are highly upregulated as a consequence of heavy metal exposure both in fish as well as in mussels (Bremner, 1987; Webb, 1987; Viarengo et al., 1999b). Their expression in tissues is therefore regarded as an indicator of metal contamination and widely used as a tool for biomonitoring programs (Viarengo et al., 1999b).

Several methods quantifying MT concentration have been developed. Different methodological approaches have thus been used: for example, spectrophotometric evaluation (Viarengo et al., 1997a), metal substitution assays (Piotrowski et al., 1973; Eaton and Toal, 1982; Scheuhammer and Cherian, 1986; Lobel and Payne, 1987; Martinez et al., 1993), electrochemical analyses (Olafson and Sim, 1979; Thompson and Cosson, 1984), and radioimmunological techniques (Nolan and Shaikh, 1986; Roesijadi et al., 1988; Hogstrand and Haux, 1990). In addition to these, chromatographic separations of soluble cytosolic MT-containing fractions have been utilized together with an assessment of metal concentrations, HPLC-AAS (Suzuky, 1980; Lehman and Klaassen, 1986) and HPLC-ICP (Sunaga et al., 1987; Mason et al., 1990; Mazzucotelli et al., 1991).

It has however been pointed out that MTs play a number of functions in relation to heavy metal cation homeostasis (Bremner, 1987). Moreover, they display ROS scavenger activity as part of the antioxidant defence system of the cells (Sato and Bremner, 1993; Viarengo and Nott, 1993; Viarengo et al., 1999a), and act as regulators of the activity of Zn finger proteins in modulating gene expression (Zeng et al., 1991; Roesjadi et al., 1998). At the same time, it is important to note that a number of studies also indicated that MTs are induced not only by heavy metals but also by

organic aromatic compounds able to cause oxidative stress in fish cells (Pedrajas et al., 1995; Kling et al., 1996). Therefore, when considering fish, the tissue level of MTs may be used as a biomarker of general stress responses to environmental pollutants, notwithstanding their expression being highly upregulated by heavy metals. Studies on the MT gene structure further support this: the MT promoter of the A gene coding MTs in trout indeed contains AP1 sequences typical of oxidative stress regulation, and also contains other sequences typical of heavy metal and hormone regulation (Bonham et al.,1987; Olsson et al.,1995).

Studies focussing on the structure of the promoters of MT genes in mussels indicated the presence of nucleotide sequences with a high homology to the AP1, GRE and MRE sequences previously identified in fish (Dondero et al., unpublished results). Further studies using real-time Q-PCR utilizing specific probes for the MT10 and MT20 genes suggested that MT10 is a constitutively activated gene (activated mainly by Zn and Cu), while MT20 is expressed at low levels in control conditions (and is typically induced by Cd, Hg, to a lower extent by Cu, and minimally by Zn) (Dondero et al., 2005). The MT20 gene is also activated by the synthesis of oxyradicals (Dondero et al., 2005). On the basis of this information, MTs could mistakenly considered biomarkers of general stress responses for studies in mussels too. In these organisms, however, organic aromatic chemicals are weak MFO inducers (Ade et al., 1984; Livingstone et al., 1989) and thus they only give rise to a low-level cytosolic ROS production, which is not sufficient to activate MT20 expression. This hypothesis is supported by the fact that no increase in MT levels was observed in digestive glands of mussels caged for 30 days in the Petroleum Harbour of Genoa, which is highly polluted by PAHs. Mussels exposed to millimolar concentrations of paraquat in laboratory experiments did not show a significant increase of MT (Viarengo, unpublished data).

As a final point, recent studies (Dondero et al., 2006b; Banni et al., *in press*) clearly showed that the use of quantitative RT-PCR provides more specific information about mussel responses to heavy metal contamination than quantifying metallothioneins. Thus, assessing mRNA levels of the mussel MT10 and MT20 genes may be important for integrating the information on MT content as a biomarker of exposure in mussels.

Inhibition of cholinesterase activity

Cholinesterases (ChEs) represent a well-known class of serine hydrolases (Walker and Thompson, 1991). They are considered ubiquitous enzymes whose physiological function is to remove acetylcholine from synaptic clefts. Increasing use of organophosphate (OP) and carbamate pesticides (two classes of compounds that are well-known inhibitors of ChE activity even at very low concentrations) has posed the problem of the possible effects of these neurotoxic compounds on wildlife (Weiss, 1964; Bocquené et al., 1990; Sturm et al., 1999).

Pesticides enter waterways along with agricultural and urban waste, thus reaching estuaries and marine coastal waters. These toxic compounds are known to be hydrolyzed quite rapidly in the environment, their half-life being in the range of hours or days (Barron and Woodburn, 1995). In spite of this, because of the increase of pesticide concentrations found in the wild, there is a need for an evaluation of their potential toxic effects on organisms living in marine coastal environment. In the attempt to do this, ChE inhibition seems the appropriate choice for a biomarker.

Two ChE isoforms have been identified in vertebrates: acetylcholinesterase (AChE, which preferentially hydrolyses acetyl esters such as acetylcholine), and butyrycholinesterase (BChE, which preferential acts on butyrylcholine) (Chang and Opperman, 1991; Massouliè et al 1993). AChE is thought to be mainly involved in neurotransmitter hydrolysis. On the other hand, no specific physiological substrate has been identified so far for BChE, which can however hydrolyze acethylcholine. This enzyme is thought to be involved in detoxification of natural compounds (Massouliè et al., 1993).

ChEs have often been found as polymorphic enzymes in invertebrates (Talesa et al., 2001). Two forms of ChEs have been identified in *Ostrea edulis* and *Mytilus spp*., namely AChE and BChE (Bocquené et al., 1997), while AChE and propionylcholinesterase (PChE) have been found in *Corbicula fluminea* (Mora et al., 1999). Biochemical characterizaton of *O. edulis* AChE, BChE and

PChe has recently been reported (Valbonesi et al., 2003). ChEs with different substrate preferences and sensitivities to pesticides are present in molluscs. Thus it has been suggested that in these organisms enzymatic activity can be assessed by calculating the difference between total AChE activity and activity following incubation in the presence of 1 mM paraoxon, a well known AChE inhibitor (Bocquené et al., 1997).

An assessment of AChE activity can be achieved with the simple and low-cost use of a specrophotometer. The analysis is usually performed on the gills or the whole body as reported for mussels (Mora et al., 1999; Galloway et al., 2002; Rickwood and Galloway, 2004), *O. edulis* (Valbonesi et al., 2003) and *Crassostrea gigas* (Bocquené et al., 1997). At any rate, certain bivalve species are thought to have a minimal AChE activity. This is the case for *Tapes philippinarum,* where AChE activity has proved to be undetectable when utilizing the usual methodologies (Valbonesi et al., 2003).

Several reports have been published addressing the use of this biomarker of exposure in different vertebrates from fish to mammals (Sturm et al., 1999; Sanchez-Hernandez and Moreno-Sanchez, 2002; Rodriguez-Fuentes and Gold-Bouchot, 2004).

While vertebrate AChE activity is extremely sensitive to neurotoxic pesticides, in the *Mytilus sp*. (and generally in molluscs) this biomarker can be employed for exposures to high concentrations of pesticides. Recent reports (Galloway et al., 2002; Rickwood and Galloway, 2004) pointed out that AChE activity in marine mussels is significantly reduced by pesticides only at high concentrations. It has been suggested, therefore, that a typical biomarker of stress (for example lysosome membrane stability) should be used as the first step for assessing the cumulative effects of pesticides and of the other environmental pollutants. Unless further evidence is put forward, AChE has to be considered, for the time being, a low-sensitivity biomarker of stress in bivalve molluscs, whose variation in activity is to be related to neurotoxic effects at the organism level (e.g. changes in valve closure cycle, gill beating**,** muscle movements, and so on), and as a high-sensitivity biomarker of exposure in vertebrates.

Mixed function oxygenases (MFO) and EROD activity

In most animal groups examined to date the presence of an enzymatic system whose activity is related to the oxidative metabolism of endogenous lipophilic compounds such as steroids has been demonstrated (Goksøyr and Förlin, 1992; Stegeman et al., 1992; Goeptar et al., 1995). This enzymatic mixed function oxygenase (MFO) system is associated with the membranes of the smooth endoplasmic reticulum (Stegeman et al., 1992; Bucheli and Fent, 1995), where it catalyzes the oxidation of lipophilic substrates by utilizing O2 and NADPH. The MFO activity is usually found at a low level, since it is related to the metabolism of endogenous lipophilic compounds; however, in organisms exposed to polycyclic aromatic hydrocarbons (PAHs) or coplanar polychlorinated biphenyls (PCBs) certain iso-enzymes may be enhanced 10-100 fold (Payne, 1976, 1984; Stegeman and Hahn, 1994; Bucheli and Fent, 1995; Goeptar et al., 1995).

Cytochrome P450 is the terminal component of the MFO system. It exists in many isoforms having different functions in the metabolism of endogenous and xenobiotic compounds (Goksøyr and Förlin, 1992; Stegeman et al., 1992). Among these, the CYP1A1 isoform has been studied in a number of fish species using immunoblotting, while its expression has been confirmed by the corresponding mRNA and DNA coding sequences (Stegeman and Hahn, 1994). Early studies measured CYP1A1 induction through the increase of benzo[a]pyrene (BaP) hydroxylase activity (Payne, 1976). The most widely used parameter now is the quantification by 7-ethoxyresorufin-*o*-deethylase (EROD) activity, easier to carry out and not requiring the use of the carcinogenic BaP substrate (Stegeman et al., 1988; Stegeman and Lech, 1991; Gorbi et al., 2005).

CYP1A activity is greatly upregulated by the presence of organic xenobiotics, so an assessment of BaP hydroxylase and/or EROD activities in fish liver has usually been reported as a biomarker capable of detecting the biological effects of many aromatic xenobiotic compounds present in water and accumulated in the organism tissues (Payne, 1976; Sijm and Opperhuizen, 1989;

Stegeman and Lech, 1991; Stegeman et al., 1992; Stegeman and Hahn, 1994; Bucheli and Fent, 1995).

Although an assessment of EROD activity is the simplest way to investigate the biological response of fish to organic aromatic xenobiotic pollutants, it has also been demonstrated that the protein amount (quantified by using specific antibodies) (Stegeman et al., 1988) or the level of mRNA coding for CYP1A1 are extremely powerful tools for biomonitoring activity (Goksøyr and Förlin, 1992; Goksøyr, 1995).

Biological factors (including animal age and sex) and environmental parameters such as sea water temperature are believed to affect EROD activity (Goksøyr, 1995). Moreover, heavy metals (including Cd, Cu and Hg) (Viarengo et al., 1997b), oxidative stress (Barouki and Morel, 2001), and excess of substrate/contaminants (Goksøyr, 1995), significantly inhibit MFO activity (Omura and Sato, 1964; Viarengo et al., 1997b). Finally, different pollutants that activate the lysosomal vacuolar system have been reported to indirectly affect EROD activity (Koehler and Pluta, 1995). This biomarker, like others related to protein expression, shows a characteristic bell-shaped response to increasing concentrations and times of exposure. This situation limits the possibility of assessing biological responses to a certain range of contamination levels (Koehler and Pluta, 1995).

At any rate, EROD activity remains one of the most powerful biomarkers for detecting the effects of aromatic xenobiotic compounds in fish. All the same, this biomarker of exposure should always be used together with a battery of biomarkers of stress. They would thus more likely help uncover all possible changes in liver physiology, even those masking specific effects induced by aromatic pollutants.

Despite intensive research carried out during the last two decades, no CYP1A gene has been identified in the nuclear DNA of mussel cells. On the other hand, no single CYP iso-protein has been isolated from mussel tissues. Various attempts to purify endoplasmic reticulum cytochromes from mussel digestive glands and produce antibodies against them did not give the expected results, e.g. the expression of proteins cross-reacting with antibodies raised against CYP2 and CYP4-peptides has not been affected by exposure to PCBs (Jonsson et al., 2004) or crude oil or PAHs (Jonsson et al., 2006). Furthermore, EROD activity is not detectable in mussel preparations and BaP hydroxylase activity is often near the detectable limits (Livingstone, 1994; Michel et al., 1995a, 1995b). This parameter has been found to be weakly affected by organic pollutants such as PCBs and PAHs only in a few studies (Suteau et al., 1988; Michel et al., 1995a, 1995b).

In summary, the use of MFO parameters as biomarkers of exposure to organic chemicals is not recommended for studies focussing on mussels. Recent investigations have suggested that the assessment of peroxisome proliferation and multixenobiotic resistance (MXR) may be more appropriate as biomarkers of exposure to organic xenobiotic compounds in molluscs. In experiments using fish as sentinel organisms the use of a combination of biomarkers is an appropriate method for monitoring both the organism response to organic xenobiotics and pollutant genotoxic effects.

The levels of aromatic fluorescent metabolites eventually accumulated in the bile of organisms can also be measured (Hellou and Payne, 1987; Lin et al., 1996; Stagg, 1998; Endre et al., 2000). This gives a clear indication of whether or not organic xenobiotics have been metabolized by the MFO system. In addition to this, as mentioned above, the amount of DNA adducts (i.e. the amount of organic chemicals that are biotransformed and become able to bind the DNA molecule affecting its integrity) can be quantified at the nuclear level.

Peroxisome proliferation

Peroxisomes are membrane-bound organelles involved in a range of cellular functions including lipid metabolism and ROS homeostasis (Mannaerts and Van Veldhoven, 1993; Singh, 1996). In vertebrate and mollusc cells these organelles contain antioxidant enzymes including catalase, SOD and glutathione peroxidase (GPX) (Dhaunsi et al., 1992; Singh, 1997; Orbea et al., 2000).

Different toxic organic compounds stimulate peroxisome proliferation in vertebrates (Beier and Fahimi, 1991; Gibson and Lake, 1993; Reddy and Mannaerts, 1994; Ortiz-Zarragoitia and Cajaraville, 2005). Similarly, chemical-induced peroxisome proliferation has been found to occur in various invertebrate species (Fahimi and Cajaraville, 1995; Cajaraville et al., 1997; Krishnakumar et al., 1997; Cancio et al., 1998; Orbea et al., 2002a, 2002b; Ortiz-Zarragoitia and Cajaraville, 2006).

Recent transplant studies have demonstrated that peroxisome proliferation is a rapid (i.e. two days) and reversible response to pollution by PAHs and PCBs in mussels (Cajaraville et al., 2003; Orbea and Cajaraville, 2006).

When organisms are exposed to organic xenobiotics, an increase in volume and number of peroxisomes is observed. These changes are often associated to an increase of enzyme activities involved in fatty acid oxidation, such as acyl-Co A oxidase (AOX). All evidence seems to suggest that peroxisome proliferation is an important biomarker that can assess the effects of exposure of both fish and mussels to organic xenobiotics (such as PAHs and oil derivatives, PCBs, phthalate ester plasticizers, polybrominated flame retardants, certain pesticides, bleached kraft pulp and paper mill effluents, alkylphenols and estrogens) (Cajaraville et al., 2003; Cajaraville and Ortiz-Zarragoitia, 2006). The fact that metals such as Cd (Orbea et al., 2002a) or Cu (Cajaraville and Ortiz-Zarragoitia, 2006) do not elicit peroxisome proliferation in mussels is believed to imply that this response is specific for organic xenobiotics.

Assessments of peroxisome proliferation have long been carried out by measuring peroxisomal volume density after cytochemical staining of catalase activity in cryostat sections (Cajaraville et al., 1997; Orbea et al., 2002a, 2002b). Analysis of recent data collected within the BEEP framework seem to suggest that the assessment of AOX activity usually carried out in whole homogenates of mussel digestive glands (Cajaraville et al., 2003) represents a simple approach for quantifying peroxisome proliferation. In spite of this, since changes in peroxisomal volume and peroxisomal enzyme activities do not always follow a parallel pattern, the best way to go is to perform the two complementary methods simultaneously. Very recently, new tools to assess peroxisome proliferation in mussels based on proteomic (Mi et al., 2005) and genomic (Bilbao et al., 2006) approaches have been developed.

MXR transport activity

P-glycoproteins (P-gps) are ATP-dependent drug efflux pumps that transport a wide variety of compounds through the cell membranes and mediate the processes of “multidrug resistance” or “multixenobiotic resistance" (MDR/MXR), originally discovered in human cancer cells (for a review see Sauna et al., 2001).

P-gps are members of the ABC transporter gene family along with the bile salt export pump (BSEP) and the multidrug resistance-related protein (MRP), both of which contribute to MDR.

Transporters of drugs or xenobiotics have been found in aquatic invertebrates (mussel, oyster, shore crab, sea urchins) and fish (flatfish, blenny, mummichog) (Bard, 2000; Smital et al., 2000; Koehler et al., 2004). A large number of anthropogenic pollutants such as PAHs, PCBs, DDT and their metabolites can either induce or inhibit the expression of these transporters in fish, invertebrates and mammals (Minier et al., 1993; Luedeking and Koehler, 2002, 2004). In the case of inhibition, they are referred to as chemosensitisers (Cornwall et al., 1995; Wadkins and Houghton, 1995; Waldmann et al., 1995; Smital et al., 2003).

During the Norwegian field campaign of the BEEP program, site-specific differences in mussel MXR-related gene expression were detected. In non-tidal habitats, contamination with polyaromatic hydrocarbons reduced P-gp-related protection in digestive glands, while contamination with copper enhanced MRP-related mechanisms (Luedeking and Koehler, 2004; Einsporn et al., 2005). Data on MXR transport activity analysed by the Calcein-AM assay were

consistent with those on expression levels in mussels at the Norwegian fjord sites, and confirmed inhibition at the PAH-polluted sites and stimulation at the copper-contaminated site (Luedeking and Koehler, 2004).

Furthermore, it was found that MXR-transport activity is highly temperature-regulated in moderate and cold climate zones, while in Mediterranean areas it operates at a much wider temperature range (Einsporn and Koehler, in preparation). Experimental short-term exposure to PAHs significantly induced MXR transport activity, while chronic exposure to a PAH-polluted site lead to the inhibition of xenobiotic export. MXR transport and gene expression were recommended as biomarkers of effect by ICES but more intercalibration exercises are needed and a generally agreed joint protocol has to be established for transport activity assays. More recent and detailed studies indicate that expression of MXR-related genes and their functional status, either inhibition or induction, can serve as appropriate biomarkers to determine hazardous effects of chemicals in contaminated marine habitats, when natural environmental factors are correctly taken into account (Luedeking and Koehler, 2004).

Tissue Damage

Cellular biomarkers act as early warning signals of stress suffered by organisms exposed to contamination. The measure of parameters whose values show a trend characterized by a continuous increase/decrease with time of exposure to pollutants (or to different concentrations of chemicals) may provide information on the level of the developing stress syndrome from starting biological effects to the impact on cell physiology. However, only when changes at the cellular level are as severe as to alter the tissue/organism function(s) it is possible to infer a real development of the stress level in the organism and possibly understand which organ(s) loses its functional capacity.

A number of studies, (including those carried out by Lowe (1988) and Cajaraville et al. (1990, 1992) identified and quantified morphological changes indicative of detrimental changes in mussel digestive tissues exposed to contaminants. Several years before Langton (1975) reported that the mussel digestive process has four morphologically distinct phases which he termed resting, digestive, excretory and reconstituting. A cross section of a mussel digestive gland can indeed show examples of all 4 phases and their intermediates at any time, with proportions of different phases varying depending on food availability and digestion.

Lowe (1988) observed that exposure to contaminants provoked a reduction or loss of digestive synchrony, whereby almost all tubules resembled what morphologically looked like the reconstituting phase, i.e. a very thin tubule epithelium. Associated with alteration at digestive gland level an increase in the number of epithelial basophil (secretory) cells was observed (some researchers argue that these are digestive cell precursors), and the appearance of lipid vacuoles in both digestive and basophil cells. Similarly, exposure of mussels to the water accommodated fraction of different oils provoked changes in proportions of digestive tubule phases, thinning of digestive epithelium and increased volume density of basophilic cells (Cajaraville et al., 1990, 1992).

This range of observations about the morphological changes produced by contaminant exposure was then expanded within the BEEP program to include new possible indicators such as loss in cell-to-cell adhesion, changes in the basophil to digestive cells ratio, and disruption of the basement membranes. When results were tested using principal component analysis they indicated that a reduction of the digestive epithelium thickness was the greatest contributor to differences between exposed/reference groups. This simple measure of effect is consistent with previous studies in mussels (Lowe, 1988) and oysters (Winstead, 1995) and provides a robust biomarker of stress at tissue level.

In the assessment of lysosomal membrane stability in mussel digestive gland cells using a cryostat (as described above), image analysis can be employed to determine the ratio between the lysosome/cytoplasm volumes (Lowe et al., 1981). This parameter can be used to find out whether

or not stress syndrome development affects the physiology of the whole organ, since an increased lysosome/cytoplasm ratio indicates that the digestive gland cells are becoming catabolic (and therefore losing their proper functionality). A higher lysosome activity due to contaminations provokes autophagy, which with time makes the cytoplasm of digestive gland cells shrink, while the epithelium tends to reduce its thickness.

Recently, a biochemical test has been developed allowing an assessment of biological damage at the tissue level in mollusc (Yawetz et al., *in press*). This test is based on an appraisal of GST activity in the haemolymph of the mollusc *Patella caerulea*. The level of the GST activity is normally very low in haemolymph soluble fractions. When analyzing the haemolymph of metals stressed organisms GST activity displays a great increase as the enzymes are released from the cytosol of damaged cells of different tissues. The use of this biomarker is simple, effective and low-cost. It is the only biochemical approach currently available to assess the physiological status of tissue(s) and organs of the organisms exposed to pollutants. It should be important in the next future to verify the sensitivity of this biomarker also in mussels exposed to both inorganic and organic chemicals.

An assessment of fish health status monitoring liver lesions in free-roaming populations has recently been recommended as a consequence of several studies performed in European and North American sea waters (for a review see Feist et al., 2004). In addition to this, several laboratory and mesocosm studies demonstrated causal links between exposure to xenobiotics and development of toxipathic liver lesions (Vethaak et al., 1996). The diagnostic criteria applied for pathological analysis at the macroscopic and microscopic level have been recently agreed upon during the international intercalibration workshops on liver histopathology of flatfish within the EU BEQUALM project (1999). At the same time, a ranking catalogue of lesion types related to pathological relevance was developed as a guide for histological diagnosis (Koehler, 2004).

Fish liver has a role in food conversion, biotransformation of xenobiotics and vitellogenesis for reproduction purposes. Thus, impairment of liver functions has a number of negative consequences for growth, health and reproductive success of individuals, and may therefore adversely affect whole populations as well. Worldwide, increasing frequency of toxipathic lesions and liver cancers in fish have been reported in areas exposed to anthropogenic discharges from industrial and agricultural sources (Koehler, 1990; Koehler et al., 1992; Stein et al., 1992; Stentiford et al., 2003; Koeler, 2004).

Recent studies analysing male and female samples of flounder during the past 5 years at 3 stations from the highly polluted Elbe estuary along the German Wadden Sea coast found significantly higher rates of malignant cancers in female individuals (Koehler and Van Noorden, 2003). These findings have been related to the higher susceptibility of females due to a lower availability of reducing power needed for biotransformation reactions (Winzer et al., 2001, 2002), oxyradical scavenging, and the seasonal increase of 17-ß estradiol which is a highly potent tumor promotor (Koehler and Van Noorden, 2003). In national and international research projects (Fish diseases in the German Wadden Sea and in the North Sea, MARS, EU- BEEP) liver lesions have been diagnosed in various fish species such as wrasse, flounder and perch (Broeg et al., submitted for publication) on the basis of internationally accepted diagnostic criteria. The ranking scheme developed by Koehler (2004) will serve as a basis to introduce liver histopathology into the "expert system" as a stress biomarker (Broeg et al., 2005).

Biomarkers at the organism level

The tipical ecotoxicological end point to establish the effects of pollutants on living organisms are parameters able to indicate whether an effect at population level is hypotizable. The use of caged mussels can easily become very informative when quantifying: a) the number of mussels surviving a 30 day-caging period in polluted waters, and b) survival time when exposing animals to air (Viarengo et al., 1995; Pampanin et al., 2005).

The physiological significance of the latter test assumes particular importance when taking into consideration the definition of “stress”. In fact, stress is usually defined as a measurable alteration of biochemical and/or physiological parameters induced by a change in the environment which results in a reduced capacity to adapt to further environmental challenges (Bayne, 1986).

Therefore, the time of mussel survival in air should be thought as a “stress on stress” response. This biomarker can be employed to assess the effects of environmental stressors at the organism level to clearly determine whether contaminants have affected the capacity of molluscs to survive to further environmental change (e.g. air exposure).

An assessment of "stress on stress" responses in mussels is extremely simple and inexpensive and, together with "scope for growth" biomarker (Widdows and Donkin, 1992; Widdows et al., 2002), it is, at present, the only truly informative biomarker in understanding the effects of pollutants at the organism level, while also providing an indication of what the potential effects at the population level might be. Several publications have recently emphasized the importance of using this simple biomarker to assess the effects of PAH, as in the case of biomonitoring the aftermath of the Exxon Valdez (Thomas et al., 1999) and the Halifax Harbour disasters (Hellou and Law, 2003), and also to study the effects of pollutants present in untreated sewage (Moles and Hale, 2003). In fish, a stress on stress exposure test has yet to be developed and therefore only survival data may be obtained from caging experiments.

Gonad development and endocrine disruptors

Endocrine disruptors and gonad development are fundamental aspects of research related to the biological effects of pollutants. These have been recently reviewed in terms of both vertebrate and invertebrate responses (COMPRENDO, 2006; Goksoyr, 2006; Porte et al., 2006).

Parameters assessing changes in gonad development are often used for both molluscs and fish studies. Contaminant effects on gonad development are well documented. In fact, biomarkers assessing gonad development (such as simple gonad index or changes in structure of gonad tissue) may give an indication of the possible effects of pollution on the reproductive performance of animals (Lowe and Pipe, 1985; Kime, 1995; Minier et al., 2000; Van der Oost et al., 2003; Aarab et al., 2006).

Biomarkers such as vitellogenin (VTG) levels, zona radiata proteins (Haux et al., 1988; Spies et al., 1990) and steroid hormones balance (Karels et al., 1998) have been proved to be useful for an assessment of endocrine disruption caused by chemicals in fish (Armstrong, 1990; Martin-Skilton et al., 2006). Among these, vitellogenin is probably the most utilized ED biomarker in fish. Several methods have been developed for quantifying protein amounts by making use of specific antibodies (radioimmunoassay, ELISAs, western blot and immunohistochemistry) and analyzing VTG mRNA levels (Denslow et al., 1999; Arukwe and Goksøyr, 2003). As for the effects of ED chemicals on fish, a number of studies have pointed out that the synthetic steroid ethinylestradiol present in contraceptive pills, induces feminization of male fish dwelling in areas close to sewage treatment plants (Petersson et al., 2006). Moreover, investigations on fish communities living in water neighbouring pulp mills (Sandström and Neuman, 2003) showed a pattern of disturbed reproduction and growth. Effects such as slight induction of EROD, DNA damage, delayed sexual maturity, and masculinisation of fish living in waters closer to the discharge point suggest that fish were exposed to effluents containing toxic ED compounds. The masculinisation of fish by pulp and paper mill effluents is of special ecotoxicological concern. There are indications that the androgens present in the effluent, possibly derived from plant sterols, are responsible for these effects (Larson et al., 2002; Hansson et al., 2006).

Recently, studies using western blotting and ELISA have reported vitellogenin and zona radiata proteins in serum samples of adult males of two top predator marine species (swordfish *Xiphias gladius* and bluefin tuna *Thunnus thynnus thynnus*), both caught in the Mediterranean Sea. These data clearly indicate that the two species are affected by ED chemicals accumulating in their tissues (Fossi et al., 2002).

As for molluscs (and generally invertebrates), we would like to point out that, although there is evidence that EDs may affect gonad development and vitellogenesis, there is not much information about the toxic chemicals' biochemical patways of action. To this moment, there are no specific antibodies available for quantifying vitellogenin or zona radiata protein levels in mussel species (*Mytillus galloprovincialis, Mytilus edulis*).

This notwithstanding, the measurement of phosphoproteins using alkali-labile phosphate quantification (ALP) has recently been in into practice for both molluscs and fish (Kramer et al., 1998; Blaise et al., 1999; Pampanin et al., 2005). Studies focussing on fish have reported ALP values that were tightly correlated to the observed levels of VTG (in turn evaluated by immunoassay using specific antibodies) (Robinson et al., 2004; Versonnen et al., 2004; Ortiz-Zarragoitia, 2005). For this reason, an assessment of ALP values may be a simple and low-cost biomarker for biomonitoring programmes. In mussels, at any rate, further studies are needed for ALP to be accepted as a ED biomarker.

Recent advances in the biomarker field: from cell signalling to the biomolecular approach

Recent studies have suggested that changes in cellular signalling can be monitored for investigating pollutant effects on mussel gonads (Viarengo et al., 2002; Dailianis et al., 2003; Burlando et al., 2006). Believing that we are in the position to find new biomarkers among the several actors involved in signal transduction is premature, however. This is the case for a number of reasons, including the fact that cell signalling cascades are influenced by several endogenous factors. A deeper understanding of sentinel organism physiology is therefore needed to correctly interpret the responses observed. Moreover, we have previously stressed that the most important requirement for a biological parameter to be employed as a biomarker is to be easy and cheap: this is not the case for the assessment of most intracellular messengers.

Identifying one or more cell signalling steps as biomarkers is nevertheless an interesting idea and a challenge. It is in some ways surprising that the effects of environmental stressors on cell signalling have been neglected for a long time, since they are thought to impair physiological functions and, as a final step, they can lead to the death of the organism. At any rate, they are the subject of growing interest at the moment, especially because biomarkers assessing changes in signalling in gonad cells could link the effects on single individuals to those at population level. It is out of the scope of this paper to review studies concerned with contaminant effects on cell signalling, and we will only refer to a few of them.

When dealing with the issue of pollutant effects on signalling pathways one should mention the well-known fact that both inorganic and organic chemicals can stimulate ROS production and affect calcium homeostasis (Marchi et al., 2000; Viarengo et al., 2002). Different pollutants have been found able to induce a sustained increase in cytosolic free calcium concentration in protozoa (Viarengo et al., 1996; Dondero, 2006c), molluscs (Viarengo et al., 1995; Marchi et al., 2000), fish (Burlando et al., 2003) and mammalian cells (Orrenius et al., 1985). The deregulation of cytosolic [Ca^{2+}] homeostasis is an important component in the development of pollutant-induced stress syndrome (Schanne et al., 1979; Marchi et al., 2004). In this context, a simple cytochemical method assessing Ca-ATPase activity (an enzyme which is fundamental in the maintenance of cytosolic calcium homeostasis) has been developed (Pons et al., 2002) and successfully employed as a stress biomarker when assessing heavy metals (Burlando et al., 2004) and in field studies (Dondero et al., 2006a).

It has been recently shown that both heavy metals and organic aromatic xenobiotics (such as PCBs) can alter the tyrosine kinase phosphorylation cascade in different organisms, although the probable consequences in terms of cell/organ physiological effects are not entirely known (Canesi et al., 2003; Burlando et al., 2004, 2006).

The adenylylc cyclase/cAMP system is well-known for regulating several functions in molluscs (Valbonesi et al., 2004). In particular, cAMP guarantees the availability of glucose as a fuel for gonadal maturation (Fernandez et al., 1998; Diaz Enrich et al., 2003).

In light of this, the recent work by Dailianis et al. (2003) carried out within the BEEP framework is quite interesting. In this study, cAMP levels have been reported to be higher in the gills, digestive glands, and mantle of mussels collected from several marine areas subjected to pollution along the Greek coast with respect to those of mussels from a reference site. Tissues were found to be polluted by a mixture of chemical compounds. Based on this evidence, a possible use of cAMP as a biomarker has been suggested.

Further studies (Giannaccini et al., 2004) showed that adenylate cyclase activity in the mantle of mussels collected from polluted sites along the Italian Thyrrenian coast was similar to that of reference animals. The apparent discrepancy between these results could be due to other mechanisms influencing cAMP levels in individuals chronically exposed to environmental pollutants, including its hydrolysis by phosphodiesterases. More recently, another study (Fabbri and Capuzzo, 2006) showed that the adenylate cyclase/cAMP system is activated in mussels exposed to heavy metals for one week. These observations were made using mussel gill tissue. Further studies are needed to understand whether cAMP levels are modulated for recovering homeostasis or compensating pollutant effects, and whether or not they are significant for organism survival and reproduction.

Finally, it should be noted that in the last few years the array of molecular biology approaches has been enriched with the development of (i) more accurate and sensitive techniques, such as quantitative-PCR (Q-PCR) (often mentioned in this paper in relation to different biomarkers) and (ii) high-throughput applications, such as microarrays. The former is an extremely sensitive and accurate technique able to quantify expression levels of toxicological relevant genes, even low abundance transcripts, as in the case of the mussel MT20 metallothionein gene (Dondero et al., 2005). This technique seems to offer several advantages over conventional molecular approaches. For example, it has a high specificity (thus allowing researchers to discriminate between the expression of homologue genes) and a wider linear range of quantification (usually over three orders of magnitude) (Bustin, 2002; Dondero et al., 2005). As already mentioned, the use of Q-PCR for the expression analysis of two mussel (*M. galloprovincialis*) metallothionein homologue genes provided clear evidence that their transcription can be differentially activated by different heavy metals. These findings supplied the basis for a considerable advance in the use of metallothionein as a biomarker of exposure, an approached recently employed by our research group in field studies along the Tunisian costal areas (Banni et al., *in press*), where Q-PCR provided an accurate, sensitive and robust approach to assess biological responses to heavy metal contamination.

DNA microarrays have been introduced some ten years ago to enable the simultaneous quantification of a large pool of gene transcripts (Schena et al., 1995; DeRisi et al., 1997). In the last few years, the interest on microarrays for ecotoxicological purposes has been growing exponentially. These are not just extremely powerful tools for predicting the toxicological modes of action of anthropogenic substances on host physiology. In fact, they may also provide an identification of gene sets predictive of toxic outcomes together with diagnostics in relation to the exposure to generic stress and to specific classes of pollutants (Thomas et al., 2001). Several studies have been validating the use of microarrays applied to ecological relevant species, such as fish and molluscs. For example, a study employed a DNA expression array to detect toxic stress response in the European flounder (*Platichthys flesus*) sampled from the estuaries of the Tyne and Alde rivers in the UK, identifying a number of transcripts that were differentially expressed because of different pollution levels (Williams et al., 2003). Another study used a high-density cDNA microarray in studies focussing on the responses of rainbow trout at sublethal ranges of β-naphthoflavone, cadmium, carbon tetrachloride, and pyrene, identifying specific responses to the different contaminants (Koskinen et al., 2004).

A low-density oligonucleotide array encompassing 24 mussels genes involved in both basal and stress response functions and a high-density microarray derived from cDNA libraries have been generated for studies on mussels. They have both been used for assessing changes in gene expression profiles caused by pollutants in model situations (e.g. exposure to heavy metals and organic xenobiotics). These tools allowed researchers to correctly discriminate the degree of pollution at sites where mussels were transplanted, suggesting that their use in biomonitoring projects might be useful (Dondero et al., 2006a; Venier et al., 2006).

As a final point, we would like to point out that research approaches such as proteomics and metabolomics are now starting to be applied for studying the effects of environmental pollutants on both fish (Samuelsson et al., 2006) and molluscs (Rodriguez-Ortega et al., 2003; Manduzio et al., 2005).

These molecular approaches are of great interest, as they open up new possibilities for a deep understanding of molecular mechanisms in the development of pollutant-induced stress syndrome. Proteomics and metabolomics are at an initial stage of development. In the next years, researchers will need to understand what is their real value in terms of costs and benefits, and whether or not they are to be applied in biomonitoring programmes.

Table I Examples of biomarkers included in biomonitoring programmes utilising bivalves.

List 1 **Cytochemical methodologies**		**List 2** **Biochemical methodologies**
	Cellular level	
Lysosome membrane stability		Catalase activity
Lysosomal lipofuscin content		Malondialdehyde
Lysosomal neutral lipid		Total Oxidant Scavenging Capacity
CaATPase activity		Acetylcholinesterase activity
	Genotoxicity Biomarkers	
Micronuclei		
DNA damage		DNA damage
	Tissue level	
Lysosome / cytoplasm ratio		Glutathione-S-transferase in haemolymph (*)
Tissue depth		
	Organism Level	
	Stress on stress	
	Scope for growth	
	Mortality	

Metallothioneins and / or Peroxisome proliferation as Biomarker(s) of exposure to specific classes of chemicals

(*) To be proved to be a useful biomarker of tissues damage in mussels (see in the text ***"Tissue damage"*** **paragraph**

Table II Examples of biomarkers included in biomonitoring programmes utilising fish.

Lysosome membrane stability
Lysosomal lipofuscin content
Neutral lipid content
Lipid peroxidation
Glutathione-S-transferase
Glutathione peroxidase
Glutathione reductase
Protein oxidation
DNA damage / Micronuclei
DNA adducts
Histopathology
Gonads Morphology (e.g. intersex) / Atrophy
Larvae Sex ratio
Larvae development and malformation

CYP1A Ethoxyresorufin-O-deethylase (EROD), Vitellogenin, Steroid hormones, Acetylcholinesterase activity, Metallothioneins as Biomarkers of exposure to specific classes of chemicals

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ANNEX

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