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ARE BIOMARKERS USEFUL IN ENVIRONMENTAL MANAGEMENT?

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The use of biomarkers (of exposure and/or effect to xenobiotic contamination) as surrogate measures of biological impact of contaminants within the environment has been studied in several European coastal areas. However, the incorporation of biomarkers into regulatory legislation for environmental risk assessment has rarely been used. Moreover, the approval of the Water Framework Directive by the European Union offers the potential for the incorporation of biomarkers as an integrated approach in environmental management. With this aim several biomarkers of exposure and/or effect were measured in several tissues of the mussel *Mytilus galloprovincialis* collected from several hot spots along the South Coast of Portugal. The biomarkers used were: superoxide dismutase (SOD), catalase (CAT), glutathion peroxidases (total and selenium dependent) cytochrome P450 (CYP450), glutathione-S-transferases (GST), acetylcholinesterase (AChE), metallothioneins (MT), δ-aminolevulinic acid dehydratase (ALA-D), lipid peroxidation (LPO) along with the condition index to assess the mussels health status. The data of this battery of biomarkers was used in an integrated manner to classify the health status of these invertebrates and consequently of this coastal ecosystem. A biomarker index was calculated using several approaches. The use of this index enabled to highlight that despite metals and organic contaminants present in their tissues, mussels from six of the eight sites, were in good health while those from the two sites were seriously affected by environmental contamination. Therefore, the use of this index provides a useful tool of assessment, since it integrates a suite of biomarkers of exposure and/or effect, facilitating the definition of risk sorting and ultimately offering an easier way for "decision makers" to assess the quality of the aquatic environment.

Introduction

Although the use of marine organisms was considered the most appropriate matrix for the evaluation of health risk, since they integrate the bioavailable concentration of contaminants in time and space, the increasing number and types of complex mixtures of potential pollutants entering the marine environment calls for novel strategies to access the health effects of these contaminants. Therefore, the use of biomarkers measured in body fluids, cells or tissues to detect the exposure to contaminants or other stressors (exposure biomarkers) or the mechanisms of action of the host response (effect biomarkers) is considered a promising approach since they respond to either (or both) exposure to and/or doses of xenobiotic substances and can give information if an organism was submitted to a particular environmental stress (biomarkers of susceptibility) (Depledge, 1994; Chambers et al., 2002, Handy et al., 2003). Biomarkers are affected by the presence of mixtures of different chemicals in contaminated areas giving rise to additive, synergistic and/or antagonistic effects. Moreover, the assessment of the biological effects should be based on a battery of different biomarkers, since no single biomarker can unequivocally measure environmental degradation (Handy et al., 2003; Galloway *et al.*, 2004; Broeg *et al.,* 2005; Hagger et al., 2006). Therefore the use of a suite of biomarkers at different levels of biological organization allows a better evaluation of the environmental hazard (Handy et al., 2003; Allen & Moore, 2004).

The use of biomarkers (of exposure and/or effect) as surrogate measures of biological impact of contaminants within the environment has been studied in several European coastal areas (Cajaraville et al., 2000; De Lafontaine etal., 2002; Aarab et al., 2004; Schiedek et al., 2005; Hoarau et al., 2006; Bebianno et al., 2007). Suites of biomarkers (a multibiomarker approach) combined with chemical analysis are used that detect short-term as well as long-term ecologically relevant end points to provide a weight-of-evidence approach for establishing relationships between environmental stressors and ecological effects and therefore are included in environmental management to assess the heath of the organisms in complex ecosystems (Galloway, et al., 2002; Hagger et al., 2006*).* Moreover, several biomarkers were included in environmental pollution assessment by several international organizations and governmental agencies (OSPAR, 2000; Galloway *et al.,* 2004; Schiedek *et al.,* 2005). However, the incorporation of biomarkers into regulatory legislation for environmental risk assessment is rare.

While these end points taken individually indicate the presence of potentially deleterious effects at the molecular and cellular levels, it is often difficult to combine these effects into the assessment of the status of an organism. One possible approach is to develop an index for environmental managers to evaluate the relative hazard at various aquatic sites. Several biomarkers-based indexes were developed for minimizing random errors and variations in order that biomarker can be used in Environmental Risk Assessment (ERA) (Narbonne et al., 1999; Chèvre et al., 2003; Bodin et al., 2004; Broeg et al., 2005; Hagger et al., 2006). Chèvre et al. (2003) developed an index that could integrate the data derived from a battery of biomarkers (MT, DNA strand breakage, LPO, vitellin-like proteins, phagocytosis and non-specific esterase activity in haemocytes) as end points for application to both spatial and temporal studies using the clam *Mya arenaria.* Narbonne et al. (1999) also developed a similar approach using a battery of biomarkers to evaluate marine environmental quality. Broeg et al. (2005) developed the "bioeffect assessment index" (BAI) based on several pathological endpoints measured in the liver of the European flounder (*Platichthys flesus*) using only validated biomarkers. Moreover, Dagnino et al. (2007) developed an objective decision-support or expert system capable of integrating a suite of biomarkers measured in marine mussels (*Mytilus spp.*) able to translate complex biological responses into a relatively simple easy to use five-level heath-status index.

The approval of the Water Framework Directive (WFD) by the Commision of the European Union offers (EU; 2000/60/EC) the potential for the incorporation of biomarkers as an integrated approach in environmental management. With this aim a battery of biomarkers of exposure and/or effect were measured in several tissues of the mussel *Mytilus galloprovincialis* collected from several sites along the South Coast of Portugal whose environmental characteristics are described elsewhere (Bebianno et al., 2007) to assess environmental changes and the impact on the health of these species. The biomarkers selected reflect deleterious effects of various classes of contaminants such as metals, organochlorine compounds, pesticides and polycyclic aromatic hydrocarbons (PAHs) and some of which are recommended by the Oslo and Paris Commission (OSPAR) and the International Council for the Exploration of the Sea (Hagger et al., 2006). The data of this battery of biomarkers was used in an integrated manner to establish a biomarker index a useful tool for “decision makers” to assess the quality of the aquatic environment.

Materials and Methods

Mussels *Mytilus galloprovincialis* were collected from eight sites on the South Portuguese Coast (Fig. 1) previously identified as hot spots of contamination. Mussels were kept alive at 4ºC and bring to the laboratory were the shells were measured and tissues weighted and when appropriate dissected into gills and digestive gland. Tissues were immediately frozen in liquid nitrogen (-80ºC) until further needed.

The biomarkers analyzed in mussel tissues were: superoxide dismutase (SOD) (MacCord & Fridovich, 1969), catalase (CAT) (Greenwald, 1985), glutathion peroxidases (total and selenium dependent) (Laurence and Burk, 1976), lipid peroxidation (LPO) (Erdelmeyer et

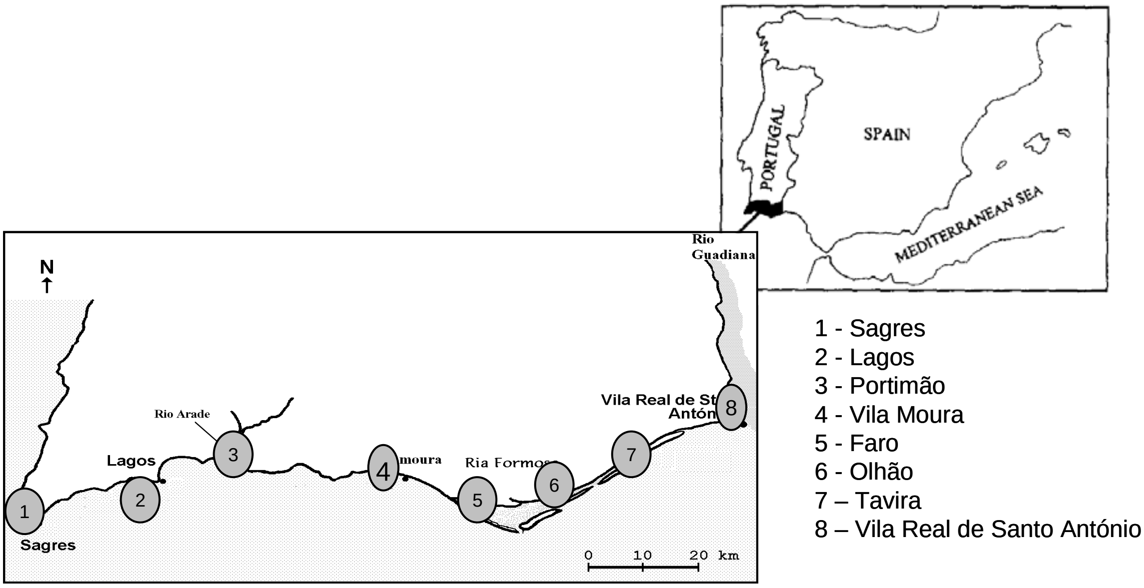

Figure 1. Sampling sites along the South Portuguese Coast

al., 1998), cytochrome P450 (CYP450) (Livingstone, 1988), glutathione-S-transferases (GST) (Habig et al., 1974), acetylcholinesterase (AChE) (Ellman et al., 1961), metallothioneins (MT) (Bebianno & Langston, 1989), δ-aminolevulinic acid dehydratase (ALA-D) (Berlin & Schaller, 1974), along with the condition index to assess the mussels health status.

Indexes of environmental contamination were calculated for each site using the DiSAV Expert System (Dagnino et al., 2007). The expert system was developed and calibrated using biomarker data from active biomonitoring and subsequently applied on data sets obtained from either mesocosm experiments or field studies for calibration. It contains a data base of rules inferred from biomarker data available in the literature mainly on mussels (*Mytilus spp.*) from laboratory and field studies. It uses biomarkers at different levels of biological organization (from molecular to whole organisms) and considers mutual interferences that may occur between various biological responses. Details of the expert system and algorithm description are in Dagnino et al. (2007). The data considered the changes in individual biomarkers over a stress gradient yield characteristic trend such as:

increasing (LPO), decreasing (ALA-D, AChE) or bell-shaped (MT, CAT, SOD, GPx (total and Se-dependent), CYP450 and GST). The results were integrated into a five-level health status index such as A (high), B (good), C (moderate), D (poor) and E(bad) following the classification established by the WFD.

Statistical analysis

Data were compared and statistical differences analysed by a non-parametric Mann-Whitney U test ($p<0.05$). Results were ranked into five levels as described above.

Results and Discussion

An index was developed from the spatial data of ten biomarkers of exposure (MT, ALA-D, AChE, CYP450, GST), effect (AChE, LPO) and susceptibility (antioxidant enzymes) of mussel tissues from eight sited along the South Portuguese coast with different environmental characteristics. Biomarkers of non-specific toxic effects (biomarkers of susceptibility) were used because they respond to a variety of contaminants and integrate synergistic and cumulative interactions of various environmental stressors (Broeg et al., 2005). The results from the different biomarkers were integrated to evaluate the effects of pollution on the health of the mussels *M. galloprovincialis* from the South Coast of Portugal and constitute the first multibiomarker approach in this area. Data was run through the DiSAV Expert System and the results are in figure 2. Although not all of the biomarkers revealed differences among sites (Cravo et al., submitted), the combination of effects at the molecular, cellular and whole organism level allowed the system to recognize the pollution changes along the South Portuguese Coast and to discriminate between the sites with compelling evidence of environmental stress. Therefore, mussels from two (3

(Portimão) and 6 (Olhão)) of the eight sites studied showed that the impact of human activities was highest and the index revealed that mussels from these sites are in moderate conditions (health status 3 – Fig. 2). The mussels from all the other six sites are in high conditions (health status 1- Fig. 2) and therefore healthy. Site 3 is influenced by the Arade River, a system of ecological and economic importance that is under increasing anthropogenic pressure due to the existence of a large fishery harbour and a marina. The estuary receives treated wastewater of a population of around 45 000 inhabitants and in recent years, there has been an increase in fish farm practices that release nutrients and chemical compounds into the area (Fernandes et al., 2007). Other sources of contaminants are: the harbour substructure, involving vessel traffic and parking; fishery industrial activity (naval restoration, ice manufacture and canned fish); swine cultures, industrial effluents, agriculture and road runoff.

Site 6 is located in the Ria Formosa lagoon, a coastal lagoon of high economic and ecological relevance that supports several economically important activities, particularly shellfish and aquaculture. Water quality in the lagoon has deteriorated in recent years mainly due to unsustainable economic development. Site 6 is in an area directly affected by discharges from a population of about 42 500 inhabitants and from the most important fishing harbour in the South Coast of Portugal representing a discharge of around 14 000 ton of fish and shellfish per year.

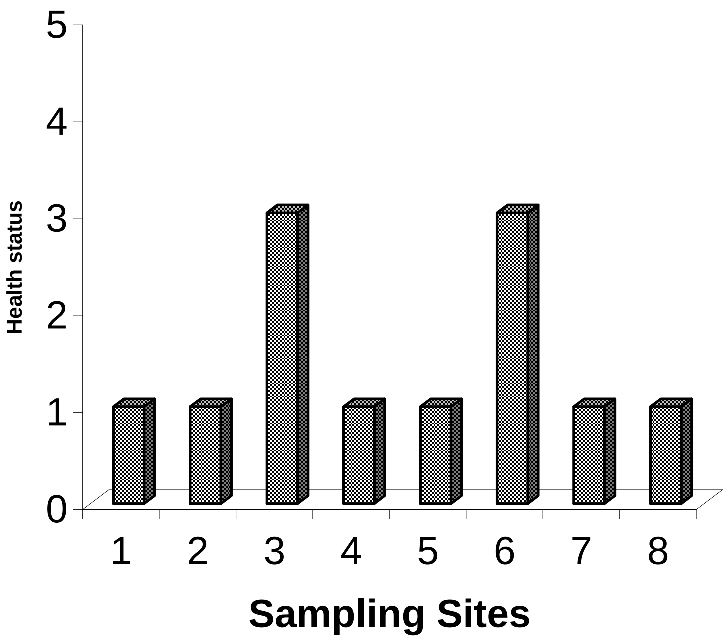

Figure 2. Health status of mussels *Mytilus galloprovincialis* from the South Coast of Portugal. The results were obtained by integrating biomarker data listed on Materials and Methods

Analysis using sterols as biomarkers of fecal contamination revealed that site 3 is highly contaminated by sewage (Mudge *et al*, 1998). Metal concentrations (Cd, Cu, Ni and Zn) in mussels whole soft tissues were highest (3.1±0.7 μg/g d.w., 6.8±0.8, 0.77±0.24 and 398±147 μg/g d.w. respectively) (Bebianno & Machado, 1997) than at site 6 and are directly related with sewage but also with other diffuse sources like agriculture run off, swine cultures and port activities (Bebianno and Machado, 1997; Hoarau et al., 2006; Bebianno et al., 2007; Fernandes et al., 2007). However, PAHs levels (451.5± 16.1 ng/g w.w.), halyphatic hydrocarbons (2.6 – 4-3 μg/g w.w.) in mussels whole soft tissues and PCBs levels (11.5 ng/g w.w. in the gills) were lower than at site 6. This site directly imopacted by the most important fishing harbour, is marked by high levels of PAHs (860.8 ± 196.1 ng/g w.w.) and PCBs (20.8 and 53.7 ng/g w.w. in the gills and digestive gland respectively) and lower metal levels (0.36 ± 0.04, 6.4± 1.2; 0.36 ± 0.12; 193± 46 μg/g d.w.

for Cd, Cu, Ni and Zn respectively (Bebianno & Machado, 1997; Morgado & Bebianno, 2005; Hoarau et al., 2006; Bebianno et al., 2007).

In what concerns the biomarker levels, CYP 450 in mussels digestive gland was high at both sites (3 and 6) but highest at 6 (61.9 ± 11.8 pmol/mg prot). Similarly the activities of NADPH-cit *c* reductase and NADH-cit b_5 reductase followed the same pattern as CYP450 and were also highest at site 6 (14.1 ± 1.5 nmol/min/mg prot and 84.9 ± 7.8 nmol/min/mg prot respectively). GST activity in the mussels digestive gland was lowest at site 3 (8.21 ± 1.49 nmol/min/mg prot) and highest at site 6 (23.8±5.6 nmol/min/mg prot) (Hoarau et al., 2006; Bebianno et al., 2007). Moreover, MT levels were higher at site 3 as well as CAT and TGPx and ALA-D lowest (inhibited most), indicating that biomarkers of metal exposure were more relevant at site 3 while biomarkers of PAHs and PCBs were more relevant at site 6. Conversely, the biomarker index was able to discriminate that both these groups of contaminants were the major driving force for the moderate conditions of the mussels at these two sites.

Similarly, the expert system was also applied to assess the health status of mussels *Mytilus galloprovincialis* caged at a polluted site in the Ligurian Sea based on a suite of eleven biomarkers some of which different from those used in the present study and the index was able to discriminate between the temporal evolution of the pollution effects in the organisms caged (Dagnino et al., 2007). Furthermore, the system was also applied to mussels exposed in the laboratory to crude oil and crude oil spiked with alkylated phenols and polycyclic aromatic hydrocarbons and bisphenol A using a battery of six biomarkers and once more the system was able to discriminate the effect of these contaminants in mussels in relation to controls (Dagnino et al., 2007). Moreover, the system was also applied to a biomonitoring study carried out in the Langsundfjorf using eight biomarkers and here again the system was able to recognise the existent pollution gradient (Dagnino et al., 2007)

These results illustrate how biomarkers are common end points that can be easily integrated in indexes easy to use by environmental managers and therefore the introduction of the multibiomarker approach into official monitoring programmes and its integration in ERA is therefore recommended.

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