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Development of an expert system for the integration of biomarker responses in mussels into an animal health index

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Abstract

Biomarkers on sentinel organisms are utilised worldwide in biomonitoring programs. However, the lack of effective interpretational capacity has hampered their uptake for use for assessment of risk in environmental management. The aim of the present study was to develop and test an objective decision-support or expert system capable of integrating biomarker results into a five-level health-status index. The expert system is based on a set of rules derived from available data on responses to natural and contaminant-induced stress of marine mussels. Integration of parameters includes: level of biological organization; biological significance; mutual inter-relationship; and qualitative trends in a stress gradient. The system was tested on a set of biomarker data obtained from the field and subsequently validated with data from previous studies. The results demonstrate that the expert system can effectively quantify the biological effects of different levels of pollution. The system represents a simple tool for risk assessment of the harmful impact of contaminants by providing a clear indication of the degree of stress syndrome induced by pollutants in mussels.

Keywords: *Biomonitoring, biomarkers, risk assessment, stress syndrome, mussel, decision-support system, expert system, environmental management*

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Introduction

The relative dearth of effective and simple tools for aiding interpretation of pollutant effect biomarker data, in the context of risk assessment, has been a major limiting factor in the adoption of biomarker tests for use in environmental management. Parameters measuring biological effects are being used worldwide to monitor the quality of the marine environment (Cajaraville et al. 2000, Viarengo et al. 2000a, Chase et al. 2001, Nasci et al. 2002, Monirith et al. 2003, Fung et al. 2004, Kalpaxis

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et al. 2004, Banni et al. 2005, Devier et al. 2005). Assessment of the synoptic environmental condition is carried out using the traditional chemical analytical approach routinely coupled to a battery of biological analyses that measure the possible toxic or stressful effects on selected species (Den Besten 1998, Widdows et al. 2002, Lam & Gray 2003).

Over the past two decades a considerable effort has been made by the scientific community to develop and test several biochemical, cellular and physiological biomarkers in order to quantify the sub-lethal effects of contaminants and allow for the evaluation of the impact of environmental pollution in its developing phase. Consequently, they facilitate an efficient and early intervention, representing a fundamental contribution to ecological risk assessment and environmental management (WHO 1993, Decaprio 1997, Wedderburn et al. 2000, Van der Oost et al. 2003). Plenty of biomarker data have been obtained in both laboratory and field studies utilising marine bivalves as sentinel animals that are widespread along many coastal areas at various latitudes (Livingstone et al. 2000, Wedderburn et al. 2000, Nasci et al. 2002, Bebianno et al. 2004).

Research has also been focused on the standardisation of analytical protocols and intercalibration among laboratories in several international programmes (such as the BEEP project, Biological Effects of Environmental Pollutants on marine coastal ecosystems, UNEP-MAP, United Nations Environmental Programme – Mediterranean Action Plan, MedPol I, II and III Biomonitoring Programme, RAMOGE agreement intercalibration exercise).

However, the use of biomarkers has not yet been included in legislative technical procedures and in the activities of environmental agencies (Leonard 2002, Handy et al. 2003). Actually, a successful application of the biomarker approach by the environmental management is still hampered by the lack of a simple and reliable integration system able to overcome the obvious difficulties in relating changes in biomarker data to environmental quality and classifying the sites on a scale according to pollutant-induced changes in the health status of the organisms (Belaoussoff & Kevan 1998, Livingstone et al. 2000, Viarengo et al. 2000b, Allen & Moore 2004, Moore et al. 2004a). Although several attempts have been made in this direction (Astley et al. 1999, Narbonne et al. 1999, 2005, Beliaeff & Burgeot 2002, Li et al. 2002, Chèvre et al. 2003, Beliaeff & Bocquené 2004, Banni et al. 2005), the risk of underestimating the complexity of biological responses, thus leading to an inadequate description of environmental conditions, must be taken into account. In order to obtain a correct interpretation of complex results and a quantification of the biological response able to reflect the stress syndrome induced by pollutant exposure, potential algorithms must be capable of taking into account the biological meaning of various biomarker responses in selected sentinel organisms and the possible consequences that interactive effects between different biomarker responses may have on the interpretation of the data (Moore & Viarengo 1987, Hyne & Maher 2003).

In this study we are proposing a decision-support or expert system that utilises a suite of biomarker tests measured in marine mussels (*Mytilus* spp.) to translate complex biological responses into a relatively simple, easy to understand and objective evaluation of the changes in the organism physiology induced by pollutants. The expert system is based on a classification scale that considers the various characteristics of the biological responses to environmental stressors. It was developed and calibrated utilising biomarker data from a field study on mussels caged at a polluted

site along the Ligurian coast (Italy). It was subsequently applied on two sets of data, one obtained from mesocosm experiments performed within the framework of the BEEP project activities (see the BEEP website), the other from a field study carried out along a pollution gradient in the Langesundfjord, Norway (MEPS 1988).

Materials and methods

Animals and experimental protocols

Mussels (*Mytilus galloprovincialis* Lam.) obtained from the rearing plant of Palmaria (La Spezia, Italy) were caged at two different sites in potentially unpolluted (Portofino, Italy) and polluted (Genoa Harbour Oil Terminal, Italy) areas along the Ligurian coast, for different periods of time (3, 7, 14, 24 and 30 days). Each cage contained at least 800 mussels. Tissues from 20 mussels (for cytochemical analysis) and groups of 80 individuals (for the analysis of each of the other biomarkers) were collected at each sampling time point. Data obtained from mussels before caging (zero time) were utilised as controls.

For details on mesocosm and field experiments see the Aquatic Toxicology Special Issue (2006) and MEPS (1988).

Biomarker analyses

The following biomarkers were measured in this study:

- Lysosomal membrane stability (LMS) (UNEP 1999, Moore et al. 2004a);
- Neutral lipid (NL) and lipofuscin (LF) lysosomal content (Moore 1988);
- DNA damage (DND) and micronuclei frequency (MF) (UNEP 1999, Bolognesi et al. 2004);
- Catalase activity (CAT) (Viarengo et al. 1991);
- Metallothionein content (MT) (Viarengo et al. 1999);
- Acetylcholinesterase (AChE) and glutathione transferase (GST) activities (Regoli & Principato 1995);
- Lysosome/cytoplasm volume ratio (L/C) (Lowe 1988);
- Stress on stress response (SOS) (Viarengo et al. 1995).

Chemicals

All chemicals utilised were of analytical grade and were purchased from Sigma Aldrich Ltd (Milan, Italy).

Description of the expert system

Both blue (*Mytilus* spp.) and green mussels (*Perna* spp.) are widely used as sentinel organisms in marine coastal biomonitoring programmes (Viarengo & Canesi 1991, Moles & Hale 2003). The expert system contains a database of rules inferred from the large amount of biomarker data available in the literature mainly on blue mussels, obtained from both laboratory and field studies.

The expert system uses biomarker responses at different levels of biological organisation (from molecular to whole organism) to screen for the stress syndrome in its different phases of evolution. These data were categorised by considering that

the changes in individual biomarker values over a stress gradient yield characteristic trends, such as increasing, decreasing or bell-shaped response profiles (Figure 1). The system also considers mutual interferences that may occur between various biological responses under stress conditions.

Data obtained from control and exposed organisms were compared and statistical differences analysed by the non-parametric Mann–Whitney U test ($p < 0.05$) (Slingsby & Cook 1986). Significant differences were ranked by using threshold limits established on the basis of the qualitative response profile characteristic of each biomarker and of data obtained from previous studies utilising mussels in laboratory exposure experiments and in biomonitoring programmes. For example, these thresholds consider large differences in biomarker data, such as a two- or three-fold increase with respect to controls for biomarkers characterised by an increasing response profile (i.e. biomarkers of accumulation and certain biomarkers of genotoxicity), or a decrease below 50% or 15% of control values for those biomarkers showing a decreasing response profile (i.e. lysosomal membrane stability). Bell-shaped parameters (i.e. metallothionein content) were analysed following the same procedure utilised for the increasing ones; however, when in the same sample heavy alterations in the other biomarkers are observed, the system takes into account that data from bell-shaped parameters can be in the decreasing phase of their response profile.

This simple procedure allows the classification of each biomarker result into one of four classes or alteration levels (AL), from NA (no alteration) to $+++/---$ (large alteration) (Table I). The system also selects a 'guide parameter' (GP) (i.e. the most sensitive biomarker) and changes in this parameter are primarily considered in the subsequent integration of data.

A final algorithm based on a set of rules in the 'if ... then' form, ranks the samples into five levels for health status: A = healthy, not stressed; B = low stress; C = medium stress; D = high stress; E = pathological. These rules are based on the accumulated

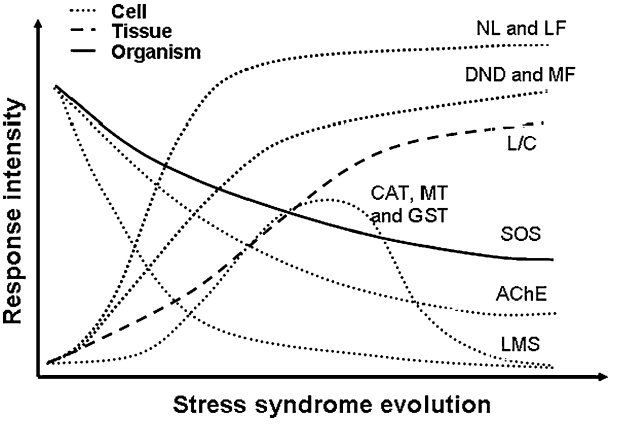

Figure 1. Different response profiles of biomarkers in a pollution gradient, related to the development of the stress syndrome. Decreasing: i.e. lysosomal membrane stability (LMS), acetylcholinesterase activity (AChE), stress on stress response (SOS). Increasing: i.e. neutral lipid lysosomal content (NL), lipofuscin lysosomal content (LF), DNA damage (DND), micronuclei frequency (MF), lysosome/cytoplasm volume ratio (L/C). Bell-shaped: i.e. catalase activity (CAT); metallothionein content (MT); GSH transferase activity (GST).

Table I. Determination of the alteration level (AL).

Decreasing parameters		Increasing and bell-shaped parameters		
Threshold	AL	Threshold	AL	Biological relevance
AF >0.80	NA	AF <1.20	NA	Small differences (±20%) with respect to controls; although statistically significant, they are not considered of biological relevance
AF <0.80	–	AF >1.20	+	Larger than 20%, statistically significant differences with respect to controls. The magnitude of changes indicates a first physiological response of the organisms
AF <0.50	– –	AF >2.00	++	Large differences with respect to controls; the change, however large, falls within the range of alterations induced by strong natural stressors
AF <0.15	– – –	AF >3.00	+++	Differences that largely overcome those induced by natural stressors indicate pathologically altered health conditions

AF, alteration factor; NA, no alteration.

knowledge of biomarker responses in mussels and take into account the criteria discussed below.

Some biomarkers are extremely sensitive to pollutant-induced stress and therefore are able to describe directly the stress syndrome during all its phases of evolution (from early responses to the pathological conditions): this in particular applies to certain biomarkers characterised by an increasing or decreasing response profile such as LMS. The expert system selects as a GP the biomarker that shows the highest sensitivity to pollutant-induced stress and interprets the other data in the light of the AL reached by the GP. Small changes in the GP (AL = + or –) in the absence of significant changes in the other parameters in the same sample reflect the fact that the selected GP actually represents the earliest and most sensitive index of stress; accordingly, the level assigned by the system is A; if the GP alteration is accompanied by changes in other biomarkers, then samples are classified as a level of stress ranging from B to E, according to a set of rules generated by the following criteria.

The number of altered biomarkers increases with the evolution of the stress syndrome, according to both the sensitivity of each parameter in the battery and the progressive activation of the various biological stress response mechanisms. For each 20% increase in the percentage of the total number of parameters showing significant changes with respect to controls, the classification shifts to the next higher level of stress (e.g. for >20% from A to B; for >40% from B to C).

For some biomarkers, characterised by an increasing or decreasing response profile such as LF, NL, MF or LMS, the amplitude of certain changes observed with respect to controls corresponds to well known biological changes that reflect profound alterations in cell and tissue physiology (Moore et al. 2006). Again, LMS is an example of this: moderate decreases in LMS (up to 50% of control values) indicate activation of the lysosomal system and membrane fusion events, not necessarily

related to toxicity, and limited changes in lysosomal protein catabolism; decreases in LMS between 50 and 15% correspond to increased autophagic processes; values below 15% of controls indicate increased protein catabolism that overcomes the rate of protein synthesis. Moreover, below this threshold lysosomal membrane damage and leakage of hydrolytic enzymes occurs, leading to gross morphological and physiological alterations at the cellular and tissue levels (Moore & Viarengo 1987). Changes in LMS are directly related to physiological scope for growth (SFG), an indicator of the mussel health status at the organism level (Allen & Moore 2004); the importance of LMS as a powerful prognostic biomarker for whole animal health has been thoroughly investigated (Moore et al. 2006).

Different biomarker responses are characterised by mutual interferences. This aspect is again linked to LMS: since large decreases in LMS reflect increased protein degradation (Moore & Viarengo 1987), the level of the protein classes involved in the stress response (i.e. heat-shock proteins, MT, components of the MFO-mixed oxygenase function system, etc.) may be similar to, or even lower than, that of controls. The absence of changes in the level of these proteins (or in the enzyme activity) may reveal an altered state in protein metabolism (the catabolic rate overcomes the anabolic rate) that affects the organism's capacity to build up a stress response rather than being due to a direct effect of pollutants on net protein synthesis. In these conditions, although these biomarkers show values similar to those of controls, they can be considered as affected by pollutants (because of either an increased catabolic rate, or a direct inhibitory effect due to high pollutant concentration). Therefore, when LMS is below 15% of controls (AL = − − −), bell-shaped parameters are considered to fall within the decreasing part of the response curve (Figure 1). Samples that show strong alterations at all levels of biological organisation (cell, tissue and organism) but not significant changes in the bell-shaped parameters are thus classified as E.

The level of biological organisation reached by the stress syndrome determines the relevance of the exposure in terms of potential ecological risk. In fact, alterations of organism parameters can have possible consequences on the population and/or community. The expert system classifies as D and E only those samples that show alterations in biomarkers at the tissue or organism level. Moreover, when biomarkers at the organism level show large alterations, the E level of stress is attributed irrespectively of other criteria, since these alterations are always associated with dramatic changes in the survival capacity of the organism.

The classification system was initially developed utilising a battery of nine biomarkers of stress at different levels of biological organisation: seven at the cellular level (LMS, LF, NL, DND, MF, CAT, GST), one at the tissue level (L/C) and one at the organism level (SOS). Alterations at the tissue level are reflected by histological biomarkers, such as L/C and nuclear–cytoplasmic volume ratio. Biomarkers measured at the organism level, such as SOS and SFG, and also mortality in caged mussels, reflect physiological alterations that impair the individual's capacity to cope with other natural environmental stressors (such as major changes in temperature, oxygen and salinity) (Allen & Moore 2004). The battery is completed by two biomarkers of exposure (MT, AChE), reflecting the response to particular classes of pollutants.

The expert system has been developed using Microsoft® Visual Studio.NET®.

Data analysis

Data analysis is essentially performed in four steps:

Choice of the suite of biomarkers. The system contains a default list of biomarkers including those most widely utilised and applied in international biomonitoring programmes; in particular, the list is based on the biomarkers developed and validated in the framework of the BEEP project. Among these, the user can select the parameters to create the battery of choice (Figure 2). It is also possible to add a new biomarker not yet included in the default list; in this case, the system requires information on its characteristics (i.e. level of biological organisation, biomarker type – 'of stress' or 'of exposure', response profile in a stress gradient) (Figure 3).

It is possible to feed the data as mean values or as single measurements. In the former case, the user has to perform the statistical analysis separately and tick the box indicating which data has a significant difference from the control; in the latter case, the software processes the data utilising the non-parametric Mann–Whitney test ($p < 0.05$).

Calculation of the alteration factor. For each biomarker, the system calculates the alteration factor (AF), i.e. the ratio between mean values of unknown samples (m_u) and that of control samples (m_c) (1).

$$AF = m_u / m_c \tag{1}$$

Determination of the alteration level. If the difference between unknown sample and control is statistically significant ($p < 0.05$), AFs are compared to specific threshold values on the basis of the qualitative stress-response profile characteristic of each biomarker. This identifies four different alteration levels (ALs) that are reported as

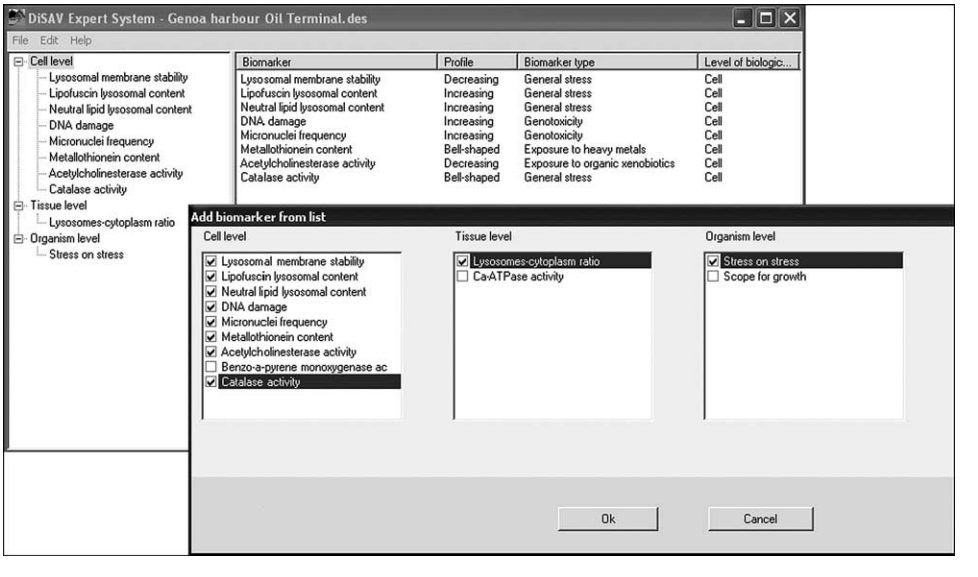

Figure 2. Selection of biomarkers from the default list in the expert system software.

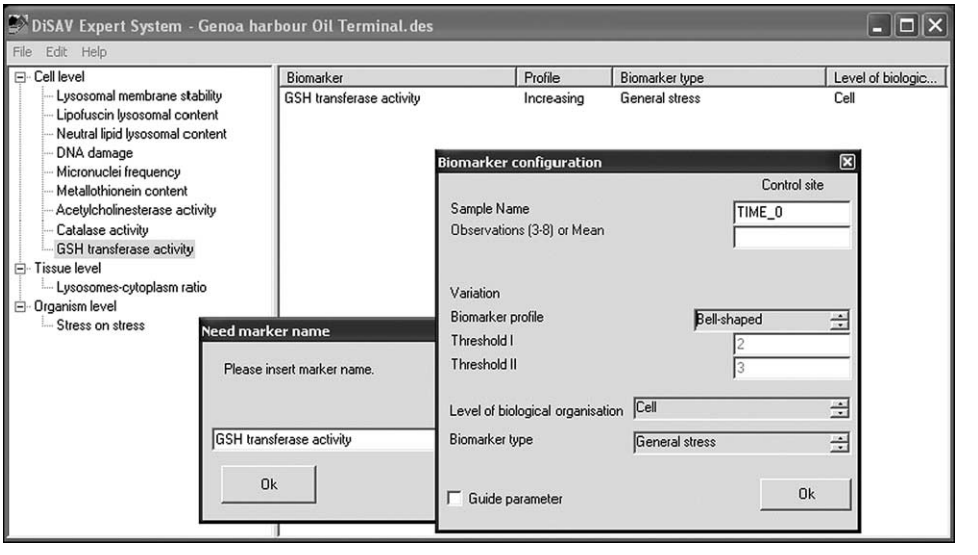

Figure 3. Addition of a new biomarker to the default list and its characterisation in terms of 'biomarker type' (biomarker of stress, biomarker of exposure), 'stress response profile' (increasing, decreasing, bell-shaped) and 'level of biological organisation' (cell, tissue, organism).

symbols of alteration (Table I). Changes in biomarker values $\pm 20\%$ of controls, although statistically significant, are not considered as biologically significant, in analogy with toxicological tests. As previously mentioned, bell-shaped parameters are analysed in the increasing part of their response profile curve.

Application of an algorithm based on a set of rules in the 'if . . . then' form and ranking of the samples into five levels of health status. The algorithm applied to rank the samples in the A–E scale has a three-step structure:

- Selection of the GP: on the basis of the body of data for a certain sample, the system will select the parameter most suitable to 'guide' the first step of data integration.
- Characterisation of the stress syndrome: the system calculates different coefficients related to both the number and type of parameters altered. The first coefficient, C, simply represents the percentage of altered parameters (n_a) with respect to the total number of parameters (n_t) (2).

$$C = (n_a/n_t) \times 100 \tag{2}$$

A second type of coefficient, C^{bo}, is calculated, representing the percentage of altered parameters at each level of biological organisation (bo) (C^c, C^t, C^o, respectively, at cell, tissue and organism level) (3).

$$C^{bo} = (n_a^{bo}/n_t^{bo}) \times 100 \tag{3}$$

C^{bo} = *ratio of altered parameters at each level of biological organization (bo)* (C^c, C^t, C^o)

n_d^{bo} = *number of altered (a) parameters at each level of biological organization (bo)* (n_a^c, n_a^t, n_a^o)

n_t^{bo} = *total (t) number of parameters at each level of biological organization (bo)* (n_t^c, n_t^t, n_t^o)

Three coefficients of weighted alteration (wa), are also calculated, C_{wa}^{rp}, that correspond to the percentage of altered biomarkers in each qualitative response profile (rp) class, C_{wa}^{i}, C_{wa}^{d}, C_{wa}^{bs}, respectively, for increasing (i), decreasing (d) and bell-shaped (bs); the AL of each biomarker is weighted by applying specific factors (0, 1, 1.5 and 2 for NA, +/−, ++/− − and +++/− − −, respectively) (4).

$$C_{wa}^{rp} = \left(\sum_i \alpha^i \times n_a^{rp-1} / n_t \right) \times 100 \tag{4}$$

C_{wa}^{rp} = *coefficient of weighted alteration (wa) in each response profile (rp) class* (C_{wa}^{i}, C_{wa}^{d}, C_{wa}^{bs})

α^i = *weighting factor of alteration specific of the ith class of alteration* ($\alpha^{NA} = 0$, $\alpha^{I} = 1$, $\alpha^{II} = 1.5$, $\alpha^{III} = 2$)

n_a^{rp-i} = *number of altered (a) parameters in each response profile (rp) class that have reached the ith class of alteration* (n_a^{i-NA}, n_a^{i-I}, n_a^{i-II}, n_a^{i-III}; n_a^{d-NA}, n_a^{d-I}, n_a^{d-II}, n_a^{d-III}; n_a^{bs-NA}, n_a^{bs-I}, n_a^{bs-II}, n_a^{bs-III})

The comparative analysis of these coefficients allows the system to rank the stress syndrome in a five-threshold scale from A to E in the final data integration step (Tables II and III).

- Final data integration (Table II): if the sample shows significant changes in more than one biomarker, than the system applies the set of rules summarised in Table II. If the GP shows a low or medium AL (+/++ or −/− −), samples are classified from A to C or from B to D, respectively, according to the percentage of changed parameters (C) and the level of biological organization reached by the stress syndrome (C^{bo}). If the GP shows maximal alteration (+++/− − −), then the system first compares the weighted alteration coefficients C_{wa}^{rp} to ad hoc percentage thresholds, in order to verify if: (1) the increasing and decreasing parameters as a whole show strong alterations; (2) the bell-shaped parameters show values in the decreasing part of the response profile; samples are then classified according to the level of biological organization concerned (from C to E). If these conditions are not satisfied, samples are classified simply on the basis of C and C^{bo} (from C to D). When no parameter is selected as a GP, the system can also rank the sample from A to E, utilising the same coefficients described above (Table III).

  As already mentioned, irrespectively of the percentage of altered biomarkers in the battery when large alterations of the parameters at the organism level (i.e. −50% for SOS) or significant differences in survival are measured, the system directly attributes the highest stress level (E) to the sample. Such alterations generally correspond to extremely stressful conditions. The expert system software can be freely downloaded at www.disav.unipm.it.

Results

The expert system was first applied to a battery of 11 biomarkers evaluated in caged mussels (*Mytilus galloprovincialis*) exposed for 3, 7, 14, 24 and 30 days at a polluted

Table II. Final data integration.

GP AL	Rules		Health status	Comments
NA			A	No significant changes in the guide parameter (GP); samples are classified as healthy (A)
+ or –	$C \leq 20\%$		A	Significant changes in less than 20% of biomarkers; samples are classified as healthy (A)
	$20\% < C \leq 40\%$		B	Significant changes in more than 20% of biomarkers; samples are classified as low stress (B)
	$C > 40\%$	$C^o = 0$ and $C^t = 0$	B	Significant changes in more than 40% of biomarkers. Samples showing alterations only at the cellular level are classified as B; those showing alterations at the tissue or organism level are classified as medium stress (C)
		$C^o \neq 0$ or $C^t \neq 0$	C	
+ + or – –	$C \leq 40\%$		B	Significant changes in less than 40% of biomarkers; samples are classified as B
	$40\% < C \leq 60\%$	$C^o = 0$ and $C^t = 0$	B	Significant changes in more than 40% of biomarkers. Samples showing alterations only at the cellular level are classified as B; those showing alterations at the tissue or organism level are classified as C
		$C^o \neq 0$ or $C^t \neq 0$	C	
	C > 60%	$C^o = 0$ and $C^t = 0$	C	Significant changes in more than 60% of biomarkers. Samples showing alterations only at the cellular level are classified as C; those showing alterations at the tissue or organism level are classified as high stress (D)
		$C^o \neq 0$ or $C^t \neq 0$	D	
++ + or – – –	$C_{wa}^{bs} < 0.2$ and $\frac{C_{wa}^{i} * n^{i} + C_{wa}^{d} * n^{d}}{(n^{i} + n^{d})} > 1$	$C^o = 0$ and $C^t = 0$	C	Strong alterations in the increasing and decreasing parameters and no significant changes in the bell-shaped biomarkers as a whole. Samples are classified between level C and pathological (E), according to the level of biological organization affected by the stress syndrome
		$C^o = 0$ and $Ct \neq 0$ $C^o \neq 0$	D E	

Table II (*Continued*)

GP AL	Rules		Health status	Comments
	$C \leq 60\%$		C	Large alterations in the most sensitive biomarker (GP); however, the occurrence of a highly stressful condition is not supported by significant changes in other biomarkers. Samples are classified as C
	$C > 60\%$	$C^o = 0$ and $C^t = 0$	C	More than 60% of biomarkers show significant differences with respect to controls, indicating a general physiological alteration. Samples are ranked between C and D, according to the level of biological organization affected by the stress syndrome
		$C^o \neq 0$ or $C^t \neq 0$	D	
Any AL	$C^o = 1$		E	Large alterations of biomarkers at the organism level, indicating that the stress syndrome has affected the organism as a whole. This situation is particularly at risk because of its possible consequences at population-community level

site (Genoa Harbour Oil Terminal, Italy). Biomarker data obtained at different times of exposure were compared with those at zero time (controls) and the results are reported in Figure 4. The expert system clearly recognises the temporal evolution of the stress syndrome in the organisms caged at the polluted site, from B (OT_03, 3 days) to E (OT_30, 30 days) with respect to the controls.

Figure 5 shows the results of a biomonitoring study carried out about 20 years ago (UNESCO–IOC/ICES Oslo GEEP Workshop held in 1986) (MEPS 1988). In this study, a different battery of eight biomarkers was utilised as input data (Table IV). As shown in Figure 5, the system recognises the pollution gradient along the Langesundfjord. The sites in the inner zone of the fjord (sites 3 and 4), where the impact of human activities was highest and the seawater exchange rate very low (MEPS 1988), were identified as the sites at which the sampled organisms showed the greatest degree of stress (E level).

Further validation was undertaken with the results of the application of the expert system utilising the set of data from laboratory experiments performed at the RF Rogaland Research Institute (Stavanger, Norway) within the activities of the BEEP framework. Mussels (*Mytilus edulis*) were exposed for 3 weeks to crude oil (Statfjord B, 0.5 ppm) and crude oil (0.5 ppm) spiked with a mixture of both alkylated phenols (total concentration of phenols: 0.1 ppm) and polycyclic aromatic hydrocarbons (total PAHs concentration: 0.1 ppm); in addition, another group of mussels was exposed for the same period of time to bisphenol A (59.4 $\mu g\ l^{-1}$); animals were sampled at 7 and 21 days of exposure. Data on exposed organisms were compared to data collected from control mussels (kept in filtered seawater, temperature 10–12°C, salinity 34‰). The expert system recognises the development of a stress syndrome in pollutant-exposed mussels, that reached the D stress level at the longest exposure time (21 days) (Figure 6). Although the battery of

Table III. Rules in the final data integration when no biomarker is selected as guide parameter (GP).

Rules		Health status
$\frac{C^i_{wa} * n^i + C^d_{wa} * n^d}{(n^i + n^d)} > 1$ and $C^{bs} < 0.2$	$C^o = 0$ and $C^t = 0$	C
	$C^o = 0$ and $C^t \neq 0$	D
	$C^o \neq 0$	E
$C \leq 0.2$		A
$0.2 < C \leq 0.4$		B
	$C^o = 0$ and $C^t = 0$	B
$0.4 < C \leq 0.6$		
	$C^o \neq 0$ or $C^t \neq 0$	C
	$C^o = 0$ and $C^t = 0$	C
$C > 0.6$		
	$C^o \neq 0$ or $C^t \neq 0$	D

biomarkers utilised here was quite different from that of the two previous examples (six biomarkers) (Table IV), the expert system was able to evaluate correctly the mussel stress syndrome, thus confirming its flexibility and reliability.

Discussion

The expert system was developed on the basis of the extended knowledge accumulated over the past two decades from biomonitoring studies in marine coastal ecosystems and on the biological effects of pollutants on blue mussels; this extensive experience has facilitated consideration of the problem of data integration and global stress evaluation starting from a solid scientific base and a relatively good knowledge of the mechanisms underlying the development of the stress syndrome in these organisms.

When the results of the various laboratory and field experiments are compared, the system was able to characterise correctly the different pollution pressures, describe a

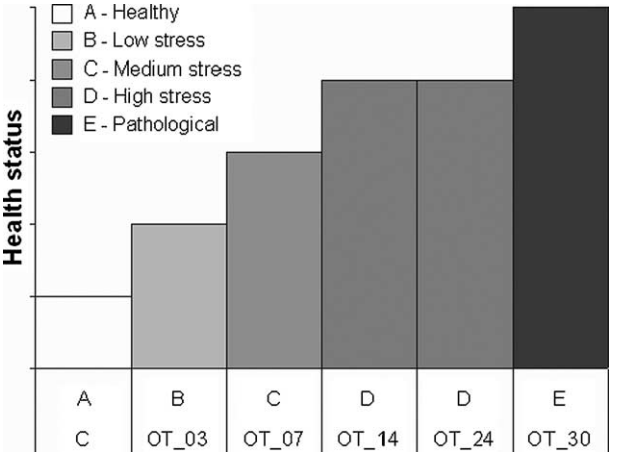

Figure 4. Health status of mussels caged at a Ligurian sea site for different periods of time. Mussels were caged for 3, 7, 14, 24 and 30 days at the Genoa Harbour Oil Terminal (OT); C, control (time 0). The results were obtained by integrating biomarker data listed in Table IV (I).

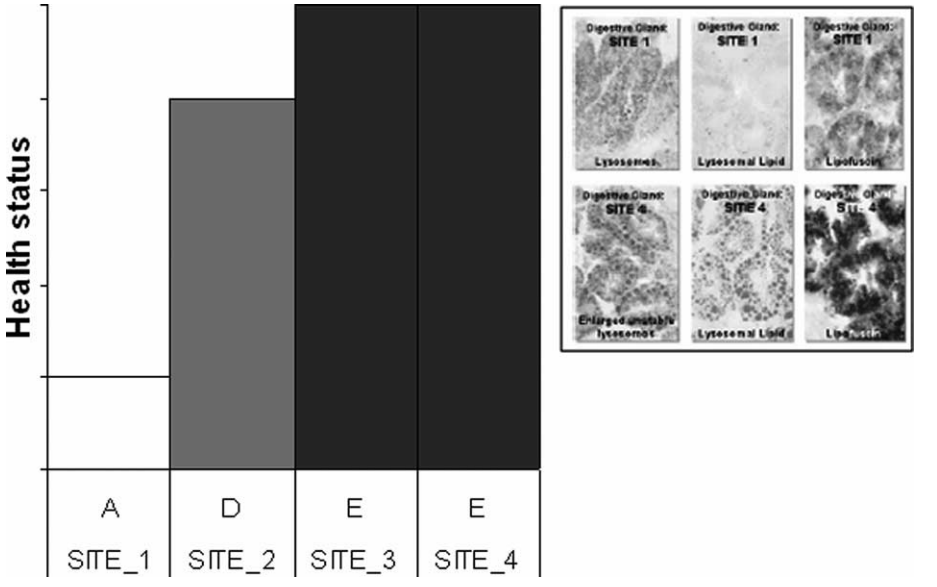

Figure 5. Health status of mussels sampled along the Langesundfjord in Norway. Data from sites 2, 3 and 4 are compared to those from the reference site (site 1, Langesund Bay, Oslo Fjord) located outside the fjord (MEPS 1988). The results were obtained by integrating biomarker data listed in Table IV (II). Lysosomal biomarker responses are illustrated for lysosomal enlargement, lysosomal lipid and lysosomal lipofuscin and are shown for the reference site (site 1) and the most contaminated site (site 4); data from Moore (1988).

clear stress gradient and discriminate between the sites characterised by various pollution levels.

Moreover, the results show that the system, developed utilising a battery of 11 biomarkers, is able to describe the evolution of the stress syndrome even utilising a smaller number of parameters (Table IV). However, when all the biomarkers at the organism level are eliminated from the data set, the maximum level of stress that the expert system is able to recognise is D.

A crucial point emerging from the application of the expert system, is that the use of biomarkers characterised by different stress response profiles had a different impact on the general determination of the stress syndrome. Biomarkers characterised by increasing or decreasing trends, (such as LMS, LN, LF, MF, AChE) are more suitable for characterising the evolution of the stress syndrome from its early phase to the development of pathological conditions (Moore 1988, Moore et al. 2004a,b). On the other hand, the bell-shaped biomarker responses are able '*per se*' to describe the development of the stress syndrome until the cells can still compensate in order to maintain homeostasis; at higher levels of cellular stress, when protein synthesis is overwhelmed by catabolism and/or enzyme inhibition prevails, these parameters are no longer useful for correctly describing the development of the stress syndrome, since in these conditions they show values comparable to controls (Moore & Viarengo 1987) (Figure 1). On the other hand, using LMS data, the expert system is able to interpret correctly the real meaning of the bell-shaped parameter values (i.e. if the value is in the ascending or in the descending part of the curve). It is evident that use of this kind of biomarker in the suite can unduly affect the final response of the expert system in the absence of data related to changes in LMS or in other sensitive

Table IV. Biomarkers utilised as input data in the evaluation of the mussel stress syndrome in different studies (I, caged mussels along the Ligurian sea coast; II, mussels sampled from field sites in Langesund Bay; III, Stavanger mesocosm experiments).

Biomarker	Stress response profile	Biological response	Level of biological organisation	Study
Lysosomal membrane stability	Decreasing	General stress	Cell	I, II, III
Neutral lipid – lysosomal content	Increasing	General stress	Cell	I, II, III
Lipofuscin –lysosomal content	Increasing	General stress	Cell	I, II, III
DNA damage	Increasing	Genotoxicity	Cell	I
Micronuclei frequency	Increasing	Genotoxicity	Cell	I
Catalase activity	Bell-shaped	General stress	Cell	I
GSH transferase activity	Bell-shaped	General stress	Cell	I, II
Metallothionein	Bell-shaped	Heavy metal exposure	Cell	I, II, III
AChE activity	Decreasing	Exposure to carbamates-organophosphates/ general stress	Cell	I
NADPH-cyt c reductase	Bell-shaped	General stress	Cell	II
Lysosome/cytoplasm ratio	Increasing	General stress	Tissue	I, II, III
Stress on stress	Decreasing	General stress	Organism	I, III
Scope for growth	Decreasing	General stress	Organism	II

biomarkers used as indicators of the health status at the organism level (e.g. SFG, SOS, mortality).

Using data from field experiments, the expert system was able to identify a well-defined stress gradient along the study area, Langesundfjord (Figure 5), with the mussels sampled from the sites positioned in the inner part of the fjord being classified as the most stressed. Moreover, the results obtained from the mesocosm experiment confirm that the system is also able to classify the stress syndrome correctly utilising only six biomarkers (Table IV). From these results it is possible to draw some general rules to obtain reliable ranking of the mussel stress syndrome utilising a reduced

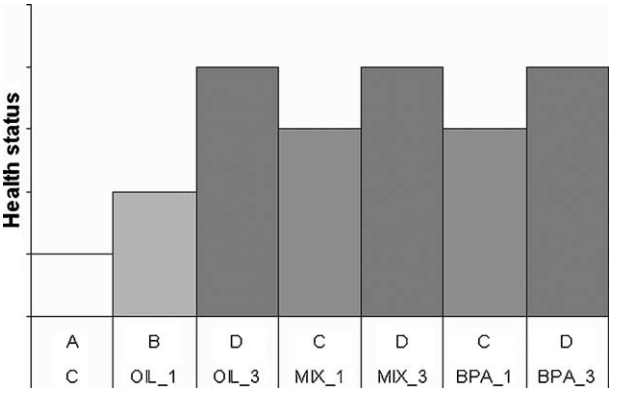

Figure 6. Health status of mussels from the Stavangen mesocosm experiments (BEEP project). Mussels were exposed to crude oil for 1 (OIL_1) and 3 (OIL_3) weeks, crude oil spiked with a mixture of phenols and PAHs for 1 (MIX_1) and 3 (MIX_3) weeks or with bisphenol A for 1 (BPA_1) and 3 (BPA_3) weeks. The results were obtained by integrating biomarker data listed in Table IV (III).

biomarker battery. At least three biomarkers of stress with an increasing or decreasing trend and at least two biomarkers at tissue/organism level should be utilised; furthermore, the battery must include LMS, as the most representative biomarker of the development of the stress syndrome. These findings are in agreement with the conclusions of the application of the 'Bioeffect Assessment Index' (BAI), recently developed for the integration of biomarkers responses in fish (Broeg et al. 2005). Obviously, new biomarkers can be added to the battery; however, only biomarkers well characterised in terms of sensitivity, stress response profile and biological level can be utilised.

Conclusions

The expert system can evaluate the biological effects of different levels of pollution by using suites of biomarkers. It represents a tool for an objective assessment of risk for the harmful impact of contaminants by providing a clear indication of the degree of stress syndrome induced by pollutants in marine mussels.

A key aim of environmental toxicology 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. 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 physicochemical characteristics of compounds (the QSAR-based approach), and from laboratory-based toxicity tests (Moore et al. 2004a). Although these procedures constitute a low-cost and pragmatic means of ranking the toxicity of potentially hazardous chemicals, they do not directly evaluate the sub-lethal 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 (Moore et al. 2004a).

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 (Moore et al. 2004a).

The system can be easily used by environmental managers and not just by expert scientists. This procedure for data integration and interpretation should permit the introduction, in the near future, of the biomarker approach into the body of official monitoring procedures and ecological risk assessment.

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