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Viewpoint

The "bioeffect assessment index" (BAI) A concept for the quantification of effects of marine pollution by an integrated biomarker approach

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Abstract

The "bioeffect assessment index" (BAI) is based on the integration of several pathological endpoints measured in the liver of European flounder (*Platichthys flesus* (L.)) during a long term study of biological effects of pollution in the German Bight. The BAI represents a modification of the "health assessment index" since it includes solely validated biomarkers reflecting toxically induced alterations at different levels of biological organisation in order to quantify the effects of environmental pollution. The concept of the BAI is based on the observation of progressive deleterious effects from early responses to late effects. Specific "key events" were detected, representing progressive stages of functional deterioration. The biomarkers selected from a whole battery of cellular markers for the BAI calculation reflect deleterious effects of various classes of contaminants such as heavy metals, organochlorines, pesticides, PAHs, and therefore reflect general toxicity in an integrative manner. Selected biomarkers were: lysosomal perturbations (reduced membrane stability), storage disorders (lipid accumulation) as early markers for toxic effects of liver cells, and the size of macrophage aggregates and their acid phosphatase activity. The latter two markers are indicative for the modulation of non-specific immune response which represents longer time scale responses after chronic exposure.

Keywords: Environmental health; Index; German Bight; Flounder; Biomarker; BAI; HAI

1. Main objectives

Increasing disease prevalence (Dethlefsen et al., 2000), tumour frequencies (Koehler, 2004) and disturbances of reproductive success (von Westernhagen et al., 1981) including feminisation of males are only a few examples for the fact, that coastal areas are heavily impaired. The central question is: How to quantify biological effects and include these informations into an integrated risk assessment approach? Concepts which combine both, comprehensive information about the wide range of biological effects and easy applicability and accessibility of reliable test assays are badly needed (Bernstein and Weisberg, 2003; Schiff et al., 2002). These concepts should comprise information on the extent of toxically induced degradation of environmental quality such as acute pollution events and their recovery as well as acute and chronic exposure to complex mixtures of pollutants.

Monitoring "environmental health", the quality status of marine life conditions especially in coastal areas, requires reliable tools to demonstrate the effects of anthropogenic impact on biological systems. The term "environmental health" is used in this context because the evaluation of changes in ecosystems shares common features with the standard approaches of human medicine. The development of early warning indicators, risk

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approaches and tests of ecosystem fitness parallels closely developments in health sciences. While long-term effects at higher biological levels such as the population and ecosystem level have a much higher economic and ecological relevance, responses at the molecular and cellular level are rapidly detectable and highly specific for the environmental effects of contamination. In order to analyse the extent of disturbances of a biological system and to quantify the actual state, the integration of several biomarkers at different levels of biological organisation has been discussed by several authors (McCarthy and Munkittrick, 1996; Attrill and Depledge, 1997; Allen and Moore, 2004) as one of the most promising concepts. Results obtained from studies at the molecular and cellular level do not automatically allow predictions of stress responses at higher levels such as communities. Only, when the emergent properties of a system are fully described, a selection of variables at lower levels can be undertaken (Kerr, 1976). Thus, only a biomarker that is verified and calibrated in this way can be used as a valid bio-indicator, in case its response coincides with responses at the community level (McCarthy and Munkittrick, 1996). Therefore, integrated biological effects monitoring should comprise the implementation of different biomarkers which reflect pollution-induced effects at several levels of the biological organisation (see also Cairns, 1991).

The present concept is based on two large data sets on biomarker responses obtained from individual flounder (*Platichthys flesus*) of the German Bight and the Baltic Sea over a time period of 7 years. These data bases were available to implement a strategy for the integration of several biomarkers of biological effects in this fish species based on the health assessment index (HAI) devised by Adams et al. (1993), providing a quantitative index that allows statistical comparisons of "fish health" from different data sets. For this purpose a suite of biomarkers applied in the present study was analysed in each individual animal in order to define the physiological status of pollution-induced stress at various organisational levels.

2. Strategy and results

2.1. Questions

- Is it possible to define the physiological status of pollution-induced alterations by a suite of validated biomarkers at different levels of organisation on an individual basis?
- Is a progression of these alterations detectable by the biomarker response?
- How can different biomarker responses be integrated and combined and these datasets are compared?
- Is it possible to quantify responses to "environmental deterioration" using an integrative biomarker index?

2.2. Biomarker selection

Biomarkers can be divided into markers of *exposure* and *toxic effects*. Biomarkers of *exposure* represent responses such as induction or inhibition of specific enzymes involved in biotransformation and detoxification as a consequence of chemical exposure. In most of the cases, these responses are early biomarkers for specific toxicants at a low level of biological organisation, the molecular or cellular.

The advantages of *biomarkers of exposure* are their early response and their specificity of reaction. The latter may also be regarded as disadvantageous since the complex contamination situations are not reflected. Thus *biomarkers of exposure* are useful for the monitoring of hot spots of pollution or clearly defined point source inputs as well as for the characterisation of chronic unknown chemical input.

Biomarkers of toxic effects reflect pathological endpoints and are determined at each level of the biological organisation. In contrast to the *biomarkers of exposure* these effects mostly cannot be attributed to the impact of single contaminants and therefore serve as integrative markers of complex toxicities.

The advantages are the high ecological relevance of biomarkers at high levels of organisation (individual, population and community level) and the general picture of the status of environmental deterioration that can be obtained by applying this kind of biomarkers. The disadvantage is that in most of the cases the quality of contamination remains speculative.

Therefore, only a combination of both kinds of biomarkers provides sufficient information for the assessment of responses reflecting the quality *as well as* the quantity of environmental deterioration.

The following biomarker data are integrated in the present study (Fig. 1).

2.2.1. Molecular level

As a biomarker of exposure at the molecular level the activity of the cytochrome P 450-dependent monooxygenase EROD (Phase I of the biotransformation system) was analysed. The measurement of the activity of mixed-function oxygenases as a measure of increased activity of the biotransformation system is suited well to detect exposure to classes of organic pollutants such as co-planar PCBs, polyaromatic hydrocarbons, planar dibenzodioxines (CCD) and dibenzofurans (CDF) (Stegeman and Hahn, 1994; Goksoyr et al., 1996; Bucheli and Fent, 1995; Livingstone et al., 1993; Förlin, 1980).

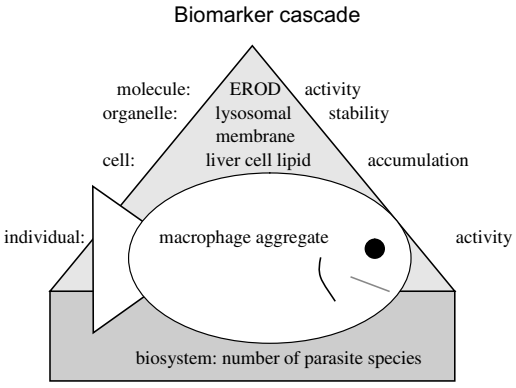

Fig. 1. Applied biomarkers at different levels of biological organisation.

2.2.2. Subcellular level

At the subcellular level the integrity of the lysosomal membranes of hepatocytes was tested as a marker for non-specific toxic responses. The lysosomal system is involved in the intracellular recycling of macromolecules and other exogenous material, including xenobiotics. Metals and organic pollutants are known to induce alterations in the lysosomal structure (Moore, 1985; Viarengo et al., 1985; Moore et al., 1984; Cajaraville et al., 1995; Regoli, 1992; Koehler et al., 2002). The destabilisation of the lysosomal membrane is one observed response due to the influence of, for example, (polycyclic) aromatic hydrocarbons (redox cycling/ ROS formation), heavy metals and organochlorines and their interaction. The stability of lysosomal membranes is an integrative marker which reflects the breakdown of the adaptive capacity of the fish liver to toxic injury. Release of lysosomal hydrolases and accumulated chemicals finally induces irreversible pathological alterations including cell death. Its sensitivity to anthropogenic impact is able to show pollution induced alterations in affected areas (Koehler, 1991; Koehler et al., 1992, 2002; Broeg et al., 1999, 2002).

2.2.3. Cellular level

At the cellular level the toxically induced accumulation of neutral lipids in hepatocytes of those fish species which store glycogen in the liver under normal conditions was applied as an indicator of toxically induced disturbance of liver fat metabolism (Couch, 1975). The pollution-induced generation of free radicals provokes membrane damage of cell organelles. Membrane damage of the endoplasmic reticulum of liver cells impairs synthesis and secretion of VLDL followed by the accumulation of neutral lipids (NL) (Karlson et al., 1982). Intralysosomal storage of NL as a storage site of lipophilic contaminants is closely correlated with reduced latency of lysosomal enzymes due to membrane damage (Koehler et al., 2002; Broeg et al., 1999).

2.2.4. Individual level

As a marker of pollution-induced modulation of the cellular immune response, the macrophage aggregate activity test was applied (Broeg et al., 1999; Broeg, 2003). Macrophages are phagocytotic cells of the immune system and play a key role in the maintenance of fish health, because they represent the first line of defence against pathogenic agents. They reside in tissues and body cavities as a quiescent population until appropriate stimulation, summarised under the general term ''activation''. The generation of reactive oxygen species and the activity of the enzyme acid phosphatase are two parameters, which reliably reflect the activity status of macrophages. Several studies indicated that chemical pollutants provoke a variety of immunomodulatory effects in cellular immune functions (Dunier and Siwicki, 1993). The phagocytotic activity of macrophages in fish is suppressed by various chemicals, such as chlorinated and polyaromatic hydrocarbons or tributyltin (Weeks et al., 1989; Roszell and Anderson, 1996). Therefore, chronic exposure to sublethal concentrations of these xenobiotics may predispose fish to higher disease susceptibility.

2.2.5. Community level

In order to obtain information about disturbances at the community level, the fish as individual was taken as a habitat for its parasite fauna (Schmidt et al., 2003; Broeg et al., 1999). For this purpose, different indices were measured to observe changes in species diversity and prevalences of potential parasitic indicator species (Khan and Thulin, 1991; D'Amelio and Gerasi, 1997; Kennedy, 1997; Marcogliese and Cone, 1997). The number of different parasite species and the *Trichodina* infection intensity showed the most consistent results with respect to the pollution of a specific study area and the contaminant burden of flounder. For example, the number of different parasite species was lower and *Trichodina* infection intensity higher in flounder from the polluted Elbe estuary compared to reference locations (Broeg et al., 1999; Schmidt et al., 2003).

2.3. Indicator species/ biomarker methodology

Target species in the German Bight was the flounder (*Platichthys flesus*). For detailed information of the biomarker methodology see Moore et al. (2004), Koehler (1991), Koehler et al. (2002), Broeg et al. (1999) and Broeg (2003). All parameters, except the whole body parasite analysis were performed in the liver of fish, the main organ for biotransformation, detoxification, food metabolism, and synthesis of the yolk precursor proteins during reproduction.

Lysosomal membrane stability, neutral lipid accumulation and MA acid phosphatase activity were assessed histochemically utilizing a single quick-frozen piece of

the central part of the liver for all three tests. With the histochemical approach a combination of several parameters could be realised which is cost-effective, practicable and easy to apply (Broeg et al., 1999; Broeg, 2003; Koehler et al., 1992, 2002; Van Noorden et al., 1997).

2.4. Areas under investigation

Data were obtained from flounder of three locations in the German Bight (1995–2000), representing a contamination gradient with highest contamination in the mouth of the River Elbe (1) followed by the Outer Eider estuary (2) and the Tiefe Rinne near Helgoland (3) (Wahl et al., 1995; Landwüst et al., 1996) (Fig. 2). Additional analyses of standard organochlorines and heavy metal residues in liver and muscle tissue of individual flounder confirmed this gradient (Broeg et al., 1999, 2002).

2.5. Integration of data

Based upon the biomarker responses, two clusters of individual flounder from the German Bight could be separated by the application of the *k*-means analysis, a special kind of cluster analysis (Schmolke et al., 1999). Grouping of the samples was achieved by minimising the within-group variance and maximising the between-group variance. Prior to this the data matrix was *z*-transformed (standardised on a parameter mean-value 0 and standard deviation 1). One of these clusters delivered the information "unimpaired", including animals with low EROD activity and low neutral lipid accumulation as well as high lysosomal membrane stability and high acid phosphatase activity in macrophage aggregates. The other cluster providing the information "impaired" consisted of animals with high induction of EROD activity and high neutral lipid accumulation as well as reduced lysosomal membrane stability and low MA acid phosphatase activity. For this set of biomarkers the differences between the two clusters were statistically significant (ANOVA). These initial results delivered the argument to analyse a progression of pollution-induced alterations in more detail.

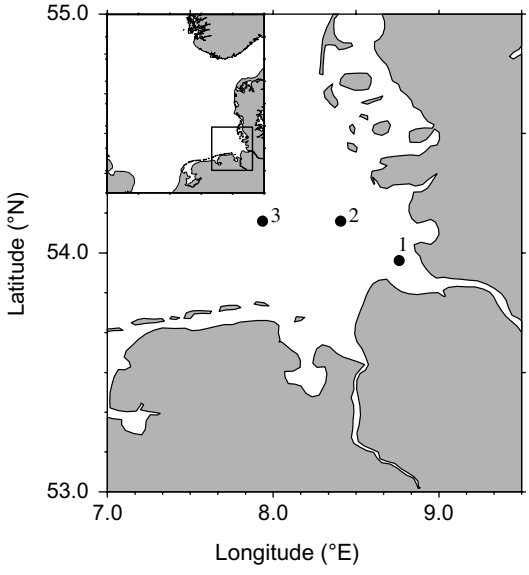

Fig. 2. Map of the sampled locations. (1) Elbe estuary (polluted), (2) Outer Eider estuary, (3) Helgoland.

2.6. Scale of biomarker response

Lysosomal membrane stability represents the most integrative parameter which reflects the onset and progression of pollution induced changes of liver histopathology in flounder (Koehler, 1991; Koehler et al., 2002; Koehler, 2004). Thus, lysosomal membrane stability served as a benchmark against which the other parameters were assessed. Four categories of lysosomal membrane stability from 'high' to 'low' membrane stability (stages 1–4) were established:

Stage 1	Destabilisation time 20 min and longer (reference)	Healthy
Stage 2	Destabilisation time >10 to <20 min	Minor diseased
Stage 3	Destabilisation time 5–10 min	Reversible, progressively
Stage 4	Destabilisation time <5 min	Irreversible, degenerative

A long time period needed to destabilize the membrane represents high membrane integrity (stage 1). Stage 4 is indicative for the highest effects of pollution-induced stress. The expressions of all other parameters were superimposed on (viewed against) these classes (Fig. 3). Decreasing lysosomal membrane stability was accompanied by *increasing* EROD activity, neutral lipid accumulation and "*Trichodina* infection intensity" as well as *decreasing* acid phosphatase activity in macrophage aggregates and "number of different parasite species". EROD activity and neutral lipid accumulation responded already between stage 1 and 2 and may therefore be used as early markers of toxic exposure respective toxic effects. The parasitological parameters "*Trichodina* infection intensity" and "number of different parasite species" (inhabiting the individual host), located at a high level of biological organisation, are responding late, between stage 3 and 4. These markers of high ecological relevance were correlated with the activity of acid phosphatase in liver macrophage aggregates (MA-AP), the suppression of the non-specific immune response. Thus, MA-AP can also be regarded as a marker of toxic effects after chronic exposure with high ecological relevance.

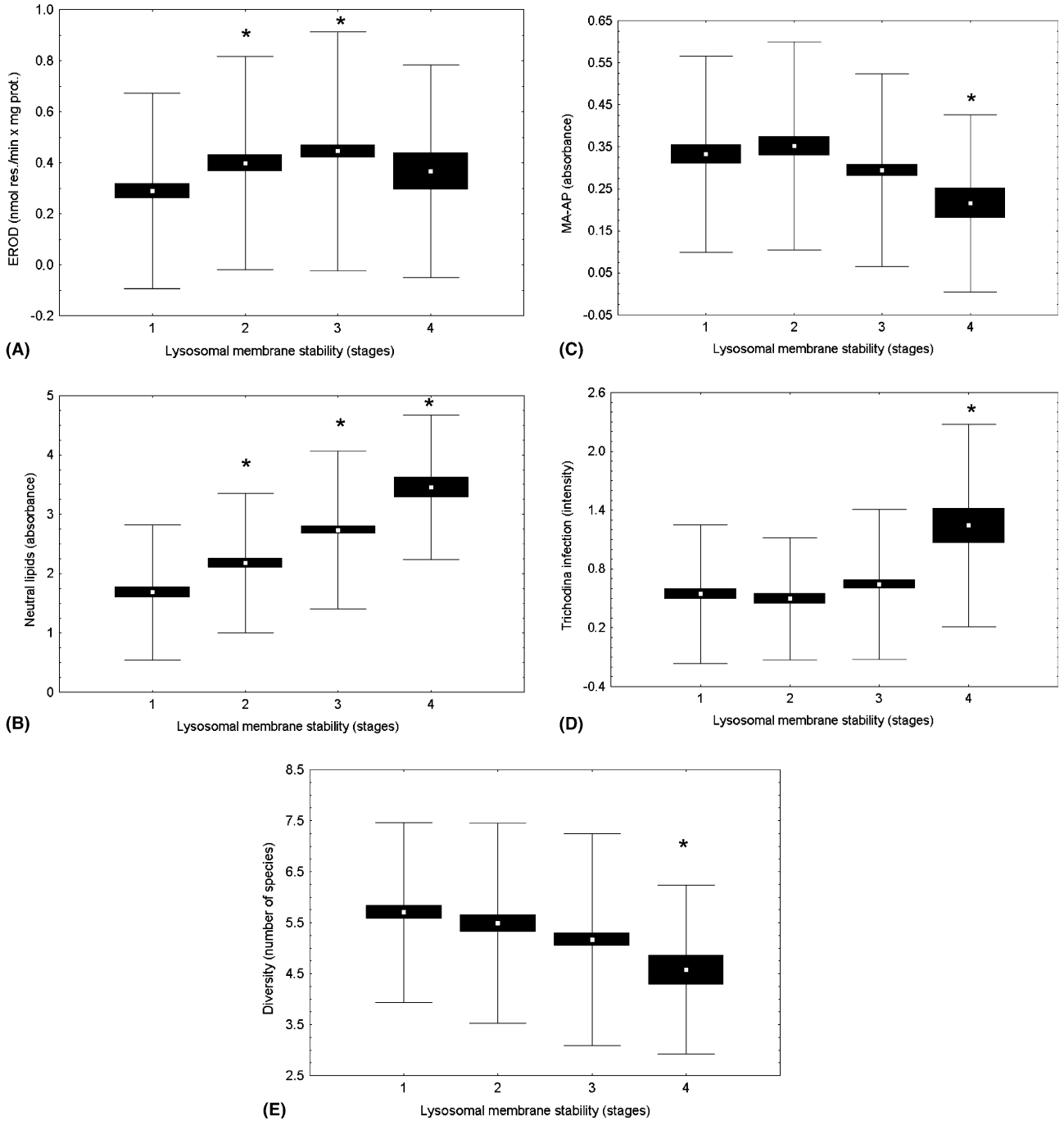

Fig. 3. (A–E) Biomarkers grouped by the four categories (stages) of lysosomal membrane stability from high (1/reference) to low (4) membrane stability. *: difference compared to reference (1) statistically significant. (Kruskal Wallis test, $p < 0.05$). Data obtained from 900 individual flounder caught in the German Bight from 1995 to 2000.

When integrating these results, a scale of biomarker responses (stages 1–4) was established (Fig. 4). The stages are characterised by the following "key-events":

Stage 1 defines baseline values for all parameters in relatively unimpaired fish with high lysosomal membrane stability (20 min destabilisation time and higher): EROD not induced, no lipid accumulation, high non-specific immune response, high parasite diversity and low *Trichodina* infection intensity.

Stage 2 is characterised by increasing EROD induction and the beginning of pathologically induced accumulation of neutral lipids in the liver related to minor histopathological changes.

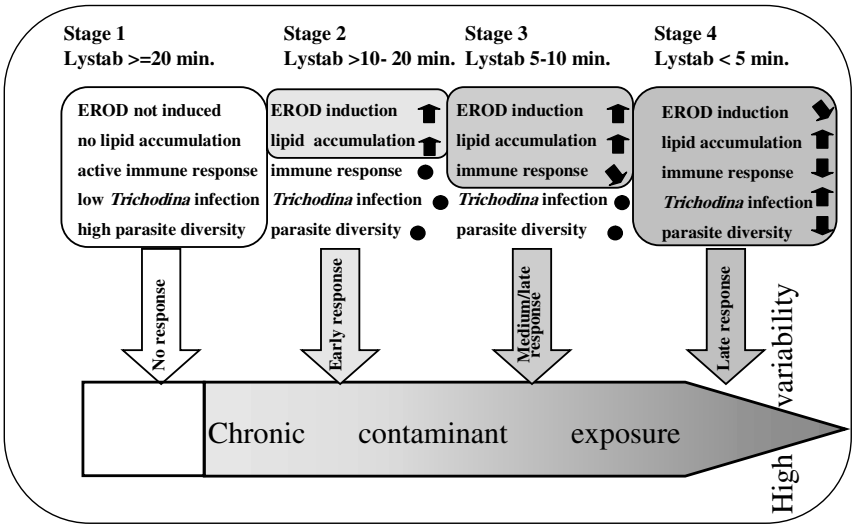

Fig. 4. Scale of biomarker responses evolved from the integration of biomarker data obtained from flounder of the German Bight.

Stage 3 is marked by a decline of the non-specific immune response (MA acid phosphatase activity).

Stage 4 is accompanied by a high variability of data in all parameters which is supposed to be caused by the break down of homeostasis in liver metabolism. In this stage significantly decreased MA acid phosphatase activity and parasite diversity represent an alteration with a high ecological relevance (individual risk/ecological risk). In many cases a decline of EROD activity could be observed.

2.7. The "bioeffect assessment index"

(BAI)—A modification of the "health assessment index" (HAI, Adams et al., 1993).

In order to be able to quantify toxically induced deviations from the normal condition of the fish physiology, a modification of the health assessment index (HAI, Adams et al., 1993) was employed. This index is an extension and refinement of a previously published field necropsy system. The HAI is a quantitative index that allows statistical comparisons of fish health among data sets. Index variables are assigned numerical values based on the degree or severity of damage of an organ or tissue caused by environmental stressors. The HAI has proven to be a simple and inexpensive tool for the rapid assessment of general fish health in field situations. The HAI was originally targeted to give general information about the health status of fish populations. In our concept, the aim is to concentrate on toxically induced alterations by the consideration of specific biomarkers of toxic effects exclusively. The information obtained from this "bioeffect assessment index" (BAI) is therefore more general on one hand side since the selected biomarkers respond to a broad range of different chemicals. On the other hand, it is conceived to diagnose a disturbance of the biosystem with respect to toxic injury by using fish as representative indicator. The BAI was designed as an index for the assessment of the multifactorial contamination situation of coastal areas. Thus, it includes only biomarkers of general toxicity.

In a following step, provided that a disturbance of the biosystem has been detected by using this first screening procedure, more specific tests of several biomarkers of specific exposure may help to identify the quality of contamination. Analogous concepts are used in medicine. A set of generalized tests is used on a patient to diagnose a disease. For further diagnosis, more specific measures are applied (Adams et al., 1993).

The use of the BAI allows:

- Statistical comparison of large data sets obtained from sampling of different geographical areas.
- Integration of different parameters by substituting each parameter value with a numerical value: 10 = stage 1, 20 = stage 2, 30 = stage 3, 40 = stage 4.
- the calculation of individual BAI-values and mean values for the single locations by summing up all individual fish BAI-values and dividing them by the total number of fish in the sample whereas higher BAI values indicating a poorer health condition.

The numerical values for all applied biomarkers are given in Table 1.

In the examples presented, the best BAI value reached by an individual flounder was 11.7, the worst was 38.5. For the classification of limits of the BAI scale, the mean value 25 was defined on the basis of the scale of biomarker responses as critical value to distinguish

Table 1
Numerical values (BAI) assigned for the applied biomarkers

Lysosomal stability (destabilisation period in minutes)	⩾20	>10 to <20	5–10	<5
Numerical value BAI	*10*	*20*	*30*	*40*
Neutral lipid accumulation (mean absorbance)	1	2	3	>3
Numerical value BAI	*10*	*20*	*30*	*>40*
Acid phosphatase activity in MA (mean absorbance)	0.5–0.6	0.4–0.49 > 0.6	0.3–0.39	<0.3
Numerical value BAI	*10*	*20*	*30*	*40*

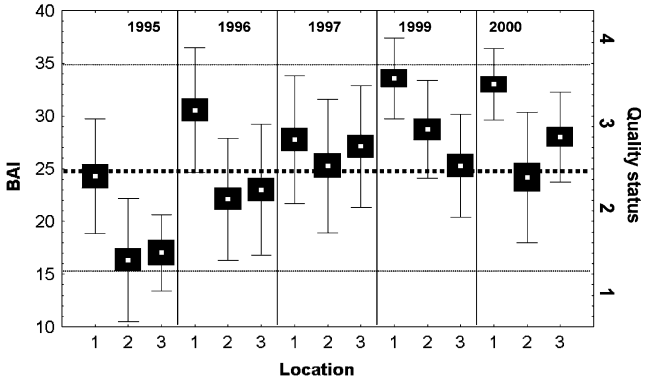

Fig. 5. BAI values of three different locations in the German Bight from 1995 to 2000. (1) Elbe estuary (polluted), (2) Outer Eider estuary, (3) Helgoland.

between locations with impaired and less impaired conditions. Mean values higher than 30 are only reached when all parameters are located in stage 3 or 4 which represents an advanced state of pollution-induced deterioration of health condition, reflecting a decline of environmental quality.

The results of the index calculation are presented in Fig. 5.

In 1995, all three locations in the German Bight displayed mean BAI values below 25 which indicate a less impaired health condition of flounder (see above). Only at the most polluted Elbe location, single flounder revealed mean BAI-values beyond 25. The two other locations harboured individuals that displayed very low (good) BAI-values. One year later, the situation changed dramatically at all locations caused by a pollution event that occurred in the River Elbe in early spring 1996 (for references see Broeg et al., 1999, 2002). As demonstrated by the BAI, environmental deterioration of the reference areas 2 and 3 progressed in 1997 and no sustained recovery could be observed until the year 2000 at all three locations.

3. Conclusions and perspective

The combined application of a set of several biomarkers in individual organisms provides reliable databases for the assessment of life quality in the environment of the German Bight. A progression of toxically induced alterations in central metabolic organs from the cellular level to the whole fish could be observed and integrated by the application of the BAI. Thus, a sequence of histochemical biomarker responses from early warning to late responses could be identified. The latter proved to be correlated with parasitological indices of high ecological relevance (Schmidt et al., 2003). The test parameters used for the implementation of the BAI are rapidly applied as well as inexpensive which are the basic requirements for the implementation of a biomarker approach in biological effect monitoring. The lysosomal membrane stability is a test that is recommended by the ICES Working Group on Biological Effects of Contaminant (WGBEC, ICES, 2002; Moore et al., 2004) for the application in routine monitoring programmes, while the other parameters included in the BAI are presently tested on a large scale range within the EU-project "BEEP" (MPB special issue, in preparation: Baršiene et al., Broeg et al., Pempkowiak et al., Schiedeck et al.). Here the major aim is the compilation of standard operating procedures (SOPs) for the sampling, preparation and analysis of the other tests and the comparability of biomarker responses from various coastal zones. As indicated above, for flounder sampling in the autumn gave the most consistent results since the food availability is high (no effects of starvation) and fish are immature. In the Baltic Sea the applicability of the BAI was also tested successfully for other fish species such as eelpout (*Zoarces viviparous*) (Broeg et al., in preparation).

We suggest the BAI as a quantitative biomarker index, which allows statistical comparisons of toxically induced-alterations among data sets obtained at different geographic areas with varying contamination situation and amount of input. Since the BAI comprises biomarkers of non-specific toxic effects it responds to a variety of different contaminants and integrates synergistic and cumulative interactions of various environmental stressors.

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