INDICATORS FOR THE ASSESSMENT OF THRESHOLDS AND POINTS OF NON-RETURN

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THRESHOLDS PROJECT

(THRESHOLDS OF ENVIRONMENTAL SUSTAINABILITY)

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European Commission FP6, Contract no. 003933-2

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1. INTRODUCTION

Ecological indicators are used increasingly to assess the conditions and/or status of ecosystems. Historically, the first approach was to develop indices based on a particular species or components, e.g. macrophytes, zooplankton, etc. In general, such indices are not broad enough to reflect the complexity of the ecosystem, as they do not include information at the structural, functional and system levels. To cope with these aspects new indices have been develop (for recent reviews see Rapport, 1995; Jørgensen *et al*., 2005) that try to synthesyze information at all ecosystem levels. In this project we are interested in evaluating indicators in terms of their potential to detect thresholds and point of non-return in coastal ecosystem. Analyses have so far been mainly carried out using:

- *Specific species or ratio between species*. For example, vegetation cover and submerged versus floating plant biomassess (Scheffer and Carpenter, 2003);
- *Concentrations of chemical compounds*. Total phosphorous (TP) is one of the most used indicators for threshold detection, see Scheffer and Carpenter (2003) and Qian *et al*. (2003). Oxygen has also been proposed by Jørgensen (1997) to account for pollution in rivers and by Turner and Rabalais (1994) in the Gulf of Mexico. Dose-response curves used in toxicology are based on contaminant concentrations (e.g. Klepper and Bedaux, 1997; Brock *et al.*, 2004);
- *Biodiversity indices*. Carpenter (1996) suggested the existence of a threshold between ecosystem function and biodiversity, but he did not provide experimental evidence.

This report aims at providing a comprehensive overview of indicators with applicability to the assessment of thresholds of ecosystem integrity in coastal ecosystems. A preliminary screening has been performed and indicators suited for identification of thresholds have been described. The selected indicators have been divided into seven levels according to the classification presented in Jørgensen *et al*. (2005). The emphasis has been placed on these indicators that we believe will be able to detect thresholds and points of non-return due to eutrophication as well as contaminants effects, i.e. toxicity and bioaccumulation in the food chain, which are two of the main focuses of the project. No attempt has been made for covering the economic and social aspects.

2. CRITERIA FOR THE SELECTION OF ECOLOGICAL INDICATORS

Ecological status has been defined in Karr (1991) as referring to the system wholeness, including the presence of appropriate species, populations and communities and the occurrence of ecological processes at appropriate rates and scales, as well as environmental conditions that support these taxa and processes.

Ecological indicators need to capture the complexity of the ecosystem but yet remain simple enough to be easily and routinely used. Ideally, monitoring of ecosystem integrity should include indicators of the 3 components of the ecological system: the function, the composition and the structure. In practice, this is not always the case and indicators are developed having in mind specific problems or specific ecosystems. In any case, ecological indicators should meet the following criteria (Dale and Beyeler, 2001):

- Be easy to measure
- Be sensitive to stresses on the system
- Respond to stresses in a predictable manner
- Be anticipatory
- Predict changes that can be averted by management actions
- Be integrative
- Have a known response to disturbances, anthropogenic stresses and changes over time
- Have a low variability in response

The challenge, when assessing ecological status, is to derive a manageable set of indicators that together meet these criteria. In our case, we are interested on indicators or set of indicators that allow threshold detection in coastal ecosystems. However, at this point is not possible to define specific criteria other than the already general ones above mentioned. Only through the analysis of the different indicators using data sets from several case studies would be possible to develop further criteria concerning suitable indicators for thresholds detection.

3. CLASSIFICATION OF THRESHOLDS INDICATORS

The classification proposed by Jørgensen *et al*. (2005) has been adapted to the purpose of structuring the present report. The classification may be summarized according to seven levels. Level 1 is based on indicators that are applied to specific coastal species, e.g. presence or absence of some characteristic species; level 2 corresponds to the ratio between classes of organisms; level 3 uses concentrations of chemical compounds, e.g. total phosphorous (Scheffer *et al*., 2001) or biomarkers; level 4 applies concentration of entire trophic levels, e.g. Chlorophyll-a; level 5 is based on rates of processes in coastal ecosystem, e.g. primary production; level 6 covers composite indicators, e.g. respiration/production; level 7 is based on holistic indicators, e.g. buffers capacity; and, finally, level 8 considers thermodynamic variables able to enclose all ecosystem's characteristics, e.g. exergy.

3.1. Specific species

Level 1 covers the presence or absence of specific species. Indicators in this level are pelagic and benthic indicator species which appearance/absence, dominance/weakness, and or tolerance are related to the environmental deterioration and perturbation. Several studies have been conducted, suggesting different indicator species in different regions (e.g. the marine angiosperm *Posidonia oceanica* is an endemic species for Mediterranean ecoregion), although some higher taxonomic groups (i.e. genus and family) may still retain indicator value and show consistent trends across more than one region (e.g. the genus Zostera is the most widely distributed marine angiosperm in the Northern Hemisphere).

3.1.1. Pelagic species

Anthropogenic nutrient increase and change to the N:P:Si ratios is reflected in the composition of the phytoplankton community, although, specific phytoplankton indicator species of eutrophication have not yet been found in coastal areas (Smayda, 2004). However, it is recognized that high phytoplankton biomasses (often associated with anthropogenic nutrient enrichment) large phytoplankton cells dominate and these are almost invariably dominated by a single phytoplankton species (Irigoien *et al.*, 2004). The same authors concluded that on a global scale the dominant species at high phytoplankton biomass are generally a diatom, a dinoflagellate, a coccolithophorid (*Emiliana huxleyii*) or *Phaeocystis* sp..

In contrast to freshwaters, the indicative value of the Cyanobacterial communities is not evident in coastal areas and estuaries. Although, in some coastal areas and estuaries of low salinity such as those in the Eastern Gulf of Finland, Baltic Sea, the dominance of freshwater cyanobacteria species (e.g. *Planktothrix agardhii*, *Microcystis spp*), or the increasing frequency and intensity of late-summer cyanobacterial blooms in low-saline estuaries (e.g. *Nodularia spumigena*) (Kahru *et al.*, 1994, Finni *et al.*, 2001) indicate increased eutrophication status (Kauppila *et al.* 1995).

Phaeocystis blooms

The marine prymnesiophyte *Phaeocystis pouchetii* forms recurrent blooms North Sea coasts. It lives in either flagellated form, or forms gelatinous colonies when under nitrogen limitation (Riegman, 1995). During mass occurrences in spring and early summer, *Phaeocystis* produce foam that accumulates to the shoreline. Long-term monitoring has shown that the *Phaeocystis* blooms are connected to nutrient enrichment of the coastal area (Lancelot *et al.*, 1987, Cadée and Hegeman, 2002).

Blooms of haptophytes

Blooms of haptophytes, especially *Prymnesium parvum* and *Chrysochromulina polylepis* have been reported around the world in brackish and marine waters, including European coasts (Edvardsen and Paasche 1998). These haptophyte flagellates form massive blooms and can cause fish kills or even extensive ecosystem disasters along the coastal areas. As an example, such a disastrous bloom of *Chrysochromulina polylepis* occurred on the Norwegian coast and Kattegat in 1988 (Dahl *et al.*, 1989), and evidence suggests that this particular bloom was stimulated by anthropogenic nutrients (Aksnes *et al.*, 1995).

Cyanobacterial blooms

In the Baltic Sea, filamentous cyanobacteria regularly form late summer blooms, dominated by *Aphanizomenon flos-aquae*, and *Nodularia spumigena*, which are both able to fix atmospheric nitrogen. *N. spumigena* blooms are frequently toxic (Sivonen *et al.*, 1989). These blooms initiate in areas where different water masses meet and where inorganic phosphate is introduced to the trophogenic layer from below the thermocline (Kononen *et al.*, 1996), thus, giving the N-fixing cyanobacteria a competitive advantage in N-limited late-summer situation.

Dinoflagellate and diatom blooms

Most harmful algal blooms are caused by dinoflagellates. They may produce toxins causing paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), or compounds that cause mass mortalities of fish. The most regularly occurring taxons in European coastal waters, which are also listed as indicator species by the OSPAR Commission (OSPAR, 2003) are *Gymnodinum mikimotoi, Alexandrium* spp., *Dinophysis* spp. and *Prorocentrum* spp. (table 1). In addition, species of the diatom genus *Pseudo-nitzschia* can cause shelfish poisoning in European coastal areas (Moestrup, 2004).

Table 1. The elevated "nuisance bloom" or toxic assessment levels and their type of effects for some phytoplankton indicator species (this list is not exhaustive).

Indicator species	Level/threshold	Effect
Nuisance species		
Phaeocystis spp. (colony form)	> 10^6 cells/l (and 30 days duration)	Nuisance, Foam, Oxygen Deficiency
Noctiluca scintillans	> 10^4 cells/l (area coverage > 5 km^2)	Nuisance, Oxygen Deficiency
Toxic (toxin producing) species		
Chrysochromulina polylepis	> 10^6 cells/l	Toxic; Fish and Benthos Kills
Gymnodinium mikimotoi	> 10^5 cells/l	Toxic; Fish kills, PSP mussel infection
Alexandrium spp.	> 10^2 cells/l	Toxic; PSP mussel infection
Dinophysis spp.	> 10^2 cells/l	Toxic; DSP mussel infection
Prorocentrum spp.	> 10^4 cells/l	Toxic; DSP mussel infection

Shifts in the phytoplankton composition

It has been observed in long-term studies, that changes within the functional phytoplankton groups occur in the course of eutrophication and concomitant increase in total phytoplankton biomass. (Olenina, 1998) has reported shifts in both spring bloom diatom community as well as late summer cyanobacterial community towards "eutrophic" species in the Kuršiu Marios lagoon, the Baltic Sea. In her time series, *Stephanodiscus hantzschii* became dominant in the spring bloom, and *Planktonema lauterbornii* in the summer cyanobacterial assemblage.

Tikkanen and Willen (1992) have identified the following phytoplankton indicator species for eutrophication in the Baltic Sea:

- *Actinastrum hantzschii* Lagerheim
- *Pediastrum angulosum* (Ehrenberg) Meneghini
- *Pediastrum duplex* Meyen
- *Pediastrum tetras* (Ehrenberg) Ralfs
- *Coelastrum microporum* Nageli
- *Microcystis aeruginosa* Kutzing

OSPAR has also identified phytoplankton indicator species, setting as well abundance thresholds in the North-Eastern Atlantic. Region/area-specific phytoplankton eutrophication indicator species, such as nuisance species (*Phaeocystis*, *Noctiluca)* and potentially toxic (dinoflagellates) species (e.g. *Chrysochromulina polylepis*, *Gymnodinium* *mikimotoi*, *Alexandrium* spp., *Dinophysis* spp., *Prorocentrum* spp.) should remain below respective nuisance and/or toxic levels (table 1, OSPAR Commission, 2003).

Other authors (Hasle and Syvertsen, 1997; Libby *et al*., 2003) have set the same threshold for *Alexandrium* spp. (100 cell/l). Another non-toxic species whose blooms have caused anoxic events (at abundances approaching those) is *Ceratium tripos*. Potentially toxic species of the diatom genus is *Pseudo-nitzschia*. *Pseudo-nitzschia pseudodelicatissima* has been associated with domoic-acid toxicity in the sea (Hasle and Syvertsen, 1997). It is unclear whether abundances of *P. pseudodelicatissima* within the threshold levels should cause alarm, when these thresholds were originally established for what is identified with light microscopy as *Pseudo-nitzschia "pungens*". This designation can include both non-toxic *P. pungens* as well as the identical-appearing (at least with light microscopy) domoic-acid producing species *P. multiseries*. Resolving the species identifications of these two species requires scanning electron microscopy. Libby *et al*., 2003 have proposed indicator species and thresholds for Massachusetts waters (see table 2).

Table 2. Contingency plan threshold values for water column monitoring (Libby *et al*., 2003).

Species	Time Period	Caution Level
Phaeocystis pouchetii	Winter/spring	2,020,000 cells l^{-1}
	Summer	334 cells l^{-1}
	Autumn	2,370 cells l^{-1}
Pseudo-nitzschia pungens	Winter/spring	21,000 cells l^{-1}
	Summer	38,000 cells l^{-1}
	Autumn	24,600 cells l^{-1}
Alexandrium tamarense	Any nearfield sample	100 cells l^{-1}

Among the zooplankton species, rotifers such as *Asplanchna brightwelli, Brachionus angularis, Brachionus falcatus, Filinia terminalis*, *Euchlanis dilatata, Trichocerca spp., Acanthocyclops vernalis* and *Polyarthra remata* are good indicators of eutrophic conditions. The rotifer genus *Brachionus* has proved to be a better indicator organism for these environmental gradients than the entire zooplankton assemblage. Hence, this taxon can be considered a target taxon for more intensive monitoring and conservation planning. The used method to assess the indicator properties of species assemblages and to select target taxa can be widely applied in any aquatic ecosystems to any group of organisms, spatial and temporal scales, and environmental gradients (Attayde and Bozelli, 1998). The high concentration of the cladocera species *Penilia avirostris* is an indicator of alterations in the food web components. *P. avirostris* has the capacity to quickly build dense populations and significantly influence the food web structure and the fate of the primary production. It feeds mostly on nanoplankton (2-20 μm) (Turner *et al.*, 1988) and thus plays a different role in the pelagic food web than the other marine cladocera species. Hence, *P. avirostris* is an important link between bacterioplankton and higher consumers because of its predation on bacterivorous flagellates. The uncertainties arise not only from the non-specific response to eutrophication, but also to climate changes. More, *P. avirostris* is an invasive species in the North Sea (Ærtebjerg *et al*., 2002). For its high frequency of distribution and density, the copepod *Acartia clausi* is also used as indicator for elevated eutrophication (Petran and Rusu, 1990). Eutrophication is believed to cause an increase in the relative importance of gelatinous zooplankton vs. crustacean zooplankton (main element of fish food spectrum). Thus density of jellyfish species could be used as a reliable indicator of eutrophication. Further, jellyfish blooms could be seen as a sign of ailing seas. Energy and organic matter that could otherwise be channeled into harvestable organisms is turned into non-utilizable jelly and gelatinous branch of the food web (Robinson and Connor, 1999). Because of the strong year-to-year fluctuations of the gelatinous plankton, individual years may be identified as “poor”, “normal” or “rich” (Buecher, 2001). For each of the European Seas typical specific species, but also common gelatinous, regularly bloom (equal to “rich” year, based on the long-term variability assessment) and thus could be used as a indicator.

3.1.2. Benthic species

Benthic macroinvertebrates are long-term indicators of environmental quality; they integrate water, sediment, and habitat qualities (USEPA, 1990). The presence/absence of several sensitive benthic species is used in all European Seas as an indicator for hypoxia/anoxia.

The other Ecological Quality Objective (EcoQO) identified for the benthic community, the presence of imposex in the dog whelk, *Nucella lapillus*, is an indicator of the effects and persistence of organotin compounds on benthic organisms, and is supported by sample data from coastal waters in several European countries (Gibbs *et al*., 1987; Stroben *et al*., 1995).

Examples of indices based on diversity values of benthic species for marine coastal and transitional waters are:

- AZTI marine biotic index (AMBI, Borja *et al*., 2000a);
- BENTIX index (Simboura and Zenetos, 2002).

The AMBI, or, in short, the BI, was designed to establish the ecological quality of European coasts, investigating the response of soft-bottom communities to changes in water quality. It allows classifying a particular site, representing the health of the benthic community (sensu Grall and Glemarec, 1997). The theoretical basis of the BI is that of the ecological strategies of the *r*, *k* and *T* (Pianka, 1970) and the progressive steps in environments stressed, for example, by organic enrichment (Bellan, 1967; Pearson and Rosenberg, 1978). Most of the concepts developed within the AMBI are based upon previous proposals: the species should be i) classified into five ecological groups (EG) (Glemarec and Hily, 1981; Grall and Glemarec, 1997); and ii) index values range from 0 to 7 (Hily, 1984; Majeed, 1987); in brief, the ecological theory of the BI is based upon sensitivity/tolerance to pollution (disturbance, such as drill cutting discharges, submarine outfalls, harbour and dyke construction, heavy metal inputs, eutrophication processes, diffuse pollutant inputs, recovery in polluted systems under the impact of sewerage schemes, dredging processes, mud disposal, sand extraction and oil spills). Five EG can be established:

EG I: Species very sensitive;

EG II: Species indifferent;

EG III: Species tolerant;

EG IV: Second order opportunistic species;

EG V: First order opportunistic species.

BI is calculated as follows:

$$BI = [(0 \times \%EGI) + (1.5 \times \%EGII) + (3 \times \%EGIII) + (4.5 \times \%EGIV) + (6 \times \%EGV)] / 100.$$

The formulation of the index allow for continuous values, with several thresholds in the scale, based upon the proportions amongst the five EG (figure 1). Some of the differences in this BI, in relation to those adopted previously, are based upon the use of a formula to obtain a continuous value of an index, called the Biotic Coefficient (BC). This is referenced to a BI, representing the quality of the bottom conditions in a discreet range from 0 (unpolluted) to 7 (extremely polluted). Although this index was based on the paradigm of Pearson and Rosenberg (1978), which emphasises the influence of organic matter enrichment on benthic communities, it was shown to be useful for the assessment of other anthropogenic impacts, such as physical alterations in the habitat, heavy metal inputs, etc. (Borja *et al*., 2000a). On the other side, it would be possible to extend the use of the BC to all European coastal areas, under the condition that new species be assigned to the

EC already designated. A program for calculation of both BI and BC are available free of charge at www.azti.es/ingles, along with a continuously updated list of species and their corresponding EG, currently encompassing over 2700 taxa.

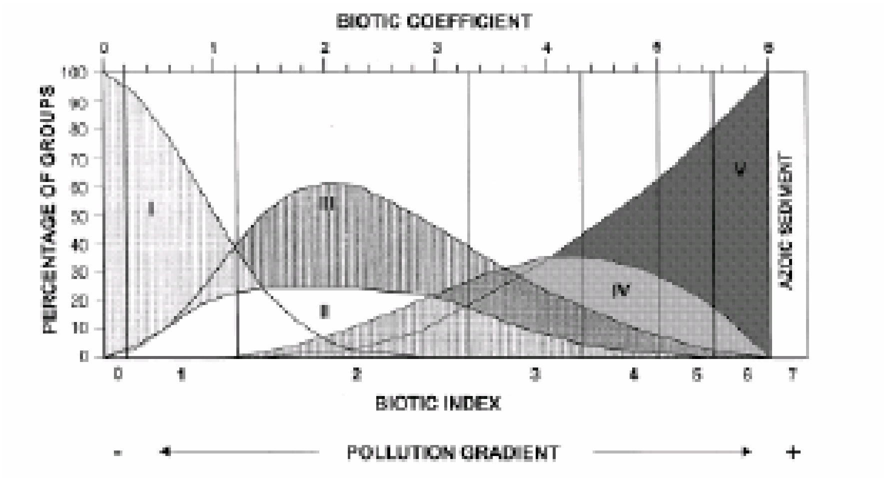

Figure 1. Classification of soft bottom macrofauna species into five ecological groups (I: very sensitive species; II: indifferent species; III: tolerant species; IV: second-order opportunistic species; V: first-order opportunistic species), according to their sensitivity to increasing pollution gradients (Borja *et al*., 2000a). The relative proportion of abundance of each group in a sample produces a discreet Biotic Index with 8 levels (0-7) and an equivalent continuous Biotic Coefficient (0-6).

The index examined the response of soft-bottom benthic communities to natural and man-induced disturbances in coastal and estuarine environments. It has been successfully applied to different geographical areas and under different impact sources, with increasing user numbers in European marine waters (Baltic, North Sea, Atlantic, Norwegian Sea and Mediterranean, all in Europe, but also in Hong Kong, Uruguay and Brazil). The AMBI has been used also for the determination of the ecological quality status (EcoQ) within the context of the European Water Framework Directive (WFD). In this contribution, 38 different applications including six new case studies (hypoxia processes, sand extraction, oil platform impacts, engineering works, dredging and fish aquaculture) are presented (see table 3).

The results show the response of the benthic communities to different disturbance sources in a simple way. Those communities act as ecological indicators of the 'health' of the system, indicating clearly the gradient associated with the disturbance.

Although the AMBI is particularly useful in detecting time and spatial impact gradient, its robustness could be reduced when only a very low number of taxa (1 to 3) and/or individuals are found in a sample. The same could occur when studying low-salinity locations (e.g. the very inner part of the estuaries), naturally-stressed locations (e.g.

naturally organic matter enriched bottoms), or some particular impacts (e.g. sand extraction, some locations under dredged sediment dumping, or physical impact). For problems associated with the use of AMBI, see Borja *et al.* (2004), and the protocol for the use of AMBI contained in the free-ware software for its calculation (www.azti.es). In the above mentioned particular cases Borja *et al.* (2004) recommend the use of AMBI, together with other metrics, in order to obtain a more comprehensive view of the benthic community, being also recommended a more detailed analysis and discussion of the results.

Table 3. Different impact sources and geographical areas for which AMBI has been applied in recent years. Key: p.c. = personal communication.

Impact Sources	Locations (Countries)	Seas	Author
Various sources along UK	(United Kingdom)	Atlantic Ocean	A. Miles, A. Prior (p.c., 2003)
Outfall and harbour	Brittany (France)		Borja *et al.*, 2003a
Engineering works (dyke)	Basque Country (Spain)		Borja *et al.*, 2000, 2003a
Sewerage works	Basque Country (Spain)		Borja *et al.*, 2000, 2003a; Gorostiaga *et al.*, 2004)
Harbour construction	Basque Country (Spain)		Muxika *et al.*, 2005
Submarine outfall	Basque Country (Spain)		Borja *et al.*, 2000; 2003b
Harbour and river inputs	Basque Country (Spain)		Muxika *et al.*, 2003
Various sources	Tejo estuary (Portugal)		M.J. Gaudencio (p.c., 2003)
Eutrophy	Mondego estuary (Portugal)		Salas *et al.*, 2004
River inputs	Guadalquivir (Spain)		AZTI (unpublished data)
Heavy metals	Huelva (Spain)		Borja *et al.*, 2003a
Estuarine inputs	Cádiz (Spain)		A. Rodríguez-Martín (p.c., 2003)
Various sources	(Morocco)		H. Bazairi (p.c., 2003)
Various sources	(Brazil, Uruguay)		Muniz *et al.*, in press
Various sources	Latvia	Baltic Sea	V. Jermakovs (p.c., 2004)
Anoxia-hypoxia	Sweden		Muxika *et al.*, 2005
Dredging mud disposal	Sweden		S. Smith (p.c., 2003)
Various sources along Sweden	Sweden		M. Blomqvist (p.c., 2003)
Various sources in a lagoon	Smir (Morocco)	Mediterranean Sea	A. Chaouti (p.c., 2003)
Dredging in harbour	Ceuta (Spain)		Muxika *et al.*, in press
Diffuse pollution (mines, agriculture,...)	Almería and Murcia (Spain)		Borja *et al.*, 2003a
Aquaculture cages	Murcia, Valencia (Spain)		AZTI (unpublished data)
Mining debris	Mar Menor (Spain)		L. Marín (p.c., 2004)
Submarine outfall	Catalonia (Spain)		M.J. Cardell (p.c., 2003)
Marina	Catalonia (Spain)		S. Pinedo (p.c., 2003)
Wastewater discharge in a lagoon	France		G. Reimonenq (p.c., 2003)
Inputs to a coastal lagoon	Adriatic Sea (Italy)		Caselli *et al.*, 2003
Various sources	Adriatic Sea (Italy)		Forni and Occhipinti Ambrogi, 2003
Industrial and urban pollution	Port of Trieste (Italy)		Solis-Weiss, *et al.* (in press)
Submarine outfall	Gulf of Trieste (Italy)		Solis-Weiss (p.c., 2004)
Various sources	Adriatic Sea (Italy)		R. Simonini (p.c., 2004)
Submarine outfall	Saronikos Gulf (Greece)		Borja *et al.*, 2003a
Aquaculture cages	3 locations (Greece)		Muxika *et al.*, in press
River inputs	Thames (United Kingdom)	North Sea	M. Davison (p.c., 2002)
Oil-based drilling muds (oil platforms)	11 locations (UK)		Muxika *et al.*, 2005
Impacts on sandy shores	(Netherlands)		S. Mulder (p.c., 2003)
Dredged sediment dumping	(United Kingdom)		H. Rees (p.c., 2004)
Ester-based drilling muds (oil platforms)	North Sea (Netherlands)		Borja *et al.*, 2003a
Re-opening of brackish lake to sea	Veerse Meer (Netherlands)		V. Escaravage (p.c., 2004)
Sand extraction	Belgium		Bonne *et al.*, 2003; Muxika *et al.*, 2005
Dredged sediment dumping	Hong-Kong (China)		Nicholson and Hui, 2003

Occhipinti *et al*. (2005) tested different approaches for Quality Assessment using the Benthic Community in the Northern Adriatic Sea, in Italy. The two biotic indices applied on these data were: the AMBI index by Borja *et al.* (2000a) and the BENTIX index by Simboura and Zenetos (2002). The contrasting quality levels which emerge from the two indices appear mainly due to the different assignment of the species to the ecological groups, but also to the different number and weight of the ecological groups in the formula calculation. In all our stations, AMBI correlates better than BENTIX both with the community structure and the chemical-physical parameters and seems more appropriate in describing the variations observed in our environment.

The BENTIX Index is a newly developed tool, based on macrozoobenthos of soft substrata, aiding the assessment of ecological quality status conforming to the requirements of the WFD. The zoobenthic species are classified into three EGs and assigned a score from 1 to 3 according to their response to organic pollution (i.e., the tolerance of various levels of dissolved oxygen):

EGI: includes species sensitive to disturbance in general;

EGII: includes species tolerant to disturbance or stress, whose populations may respond to enrichment or other source of pollution by an increase of densities (slightly unbalanced situations);

EGIII: includes first order opportunistic species (strongly unbalanced situations), pioneers, colonizers, and species tolerant to hypoxia.

BI is regionally specific. Potential benthic macroinvertebrates metrics are number of taxa (reducing under stress), % contribution of dominant taxon (elevated under stress). Following calculations, validation and testing with data from Hellenic ecosystems, an algorithm was developed giving different weight to the presence/abundance of each group:

$$BENTIX = [(6 \times \%\ EGI) + 2 \times (\%\ EGII + \%\ EGIII)]/100$$

A classification system (table 4) appears as a function of BENTIX including five levels of ecological quality status (EQS) in accordance with the needs of the WFD.

Table 4. Classification of EcoQ according to range of the Biotic Index (Simboura and Zenetos, 2002).

Pollution Classification	Bentix	EQS WFD	Bentix in physically stressed muds
Normal/Pristine	4,5 < Bentix < 6	High	4 < Bentix < 6
Slightly polluted, transitional	3,5 < Bentix < 4,5	Good	3,0 < Bentix < 4,00
Moderately polluted	2,5 < Bentix < 3,5	Moderate	2,5 < Bentix < 3,00
Heavily polluted	2 < Bentix < 2,5	Poor	
Azoic	Azoic	Bad	

Use of the BENTIX can produce a series of continuous values from 2 to 6, being 0 when the sediment is azoic (all groups zero). Numeric values between 2 and zero are nonexistent in the scale because if EG1 is zero the BENTIX index is 2. A classification system of soft bottom macrozoobenthic communities is proposed based on the BENTIX index and including five levels of ecological quality. The boundaries between classes were set keeping equal distances among classes limited only by the two extremes of the scale (2-6) and were tested using data from various sites with known environmental pressures. The BENTIX index applies to all kind of marine soft bottom benthic data.

The BENTIX index has been applied in:

- In **Saronikos** Gulf, receiving the sewage effluents of the Metropolitan city of Athens a Primary Treatment Plant started working in 1994. The benthic communities' ecological quality status is followed the years 1999 to 2004. As shown by Simboura and Zenetos (2002) the EQS is improving with the distance from the sewage outfall. A reference "high" quality status zone is limited in the more coastal areas.
- In **E-SE Attiki** characterized by touristic development of the coastal zone, disturbance is attributed primarily to organic pollution (wastes of coastal villages, ports etc). The offshore areas of E. Attiki (Petalioi Gulf) are important fishing grounds for bottom trawlers. The EQS of the E, SE coasts of Attiki appears to be good to high.
- In **Izmir** Bay, mean values of the BENTIX is increasing from the inner towards the outer bay and so is EQS (figure 2). The poor quality of the inner Bay, which is subject to a combination of pollution sources, is reflected in all parameters (figure 2).

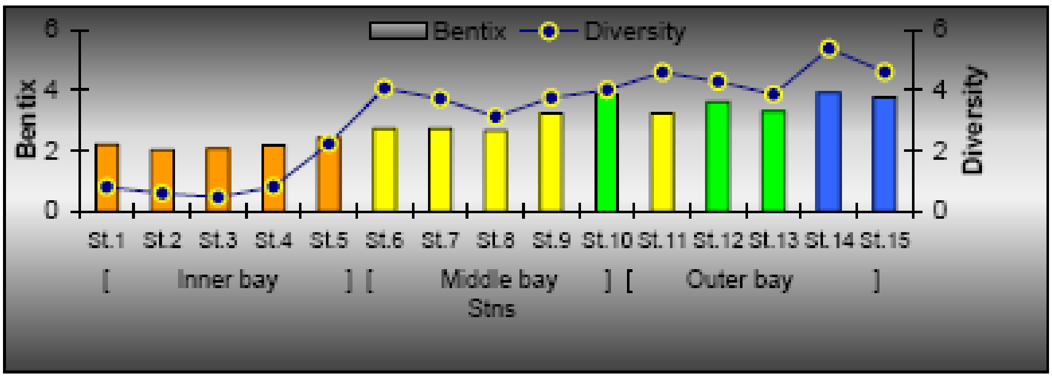

Figure 2. Mean values of BENTIX and H' along a pollution gradient in Izmir Bay. Colours correspond to EQS classes as defined in the WFD (Source: Dogan, 2004).

- **Edremit** Bay is one of the most important fishery regions of the Turkish Aegean Sea. However, the innermost region of the Bay is partly affected by increasing human settlements (tourist resorts) and this is reflected in species richness of the zoobenthos (Albayrak, pers. com.). The EQS according to BENTIX appears to be moderate to high.
- In **Augusta** Bay, qualitative and quantitative studies based on polychaetes and mollusks confirmed a degradation of the ecosystem between 1983 and 1985 (Di Geronimo, 1990). BENTIX revealed a degradation of the shallower coastal sites (closer to LBS) and an improvement of the deeper stations.
- In **Portman** Bay (Spain) the main stressor is dumping coarse metalliferous waste. The assessments derived by the BENTIX did not match at all. According to Marín-Guirao *et al.* (2005) the indicator species lists proposed by Simboura and Zenetos (2002) are based on organic pollution literature and therefore, its application in the case of purely toxic pollution was not successful.
- In **Iskenderun** Bay, where the main stressor is a pipe line and a power plant, BENTIX produced similar results with Shannon-Wiener index in 60 % of the cases.
- In **Banias**, a very impacted area along the Syrian coasts, results obtained by BENTIX was inconclusive. With the exception of the most polluted station (site of sewage treatment plant) the assessment was always contradictory: polluted status according to H', high status according to BENTIX. This may be explained by two reasons: a) sampling was semi quantitative (dredges) and b) most important the fauna was very poor and inadequately identified to species level.

Conclusively, BENTIX appears to work successfully (different ecological quality classes corresponding to different stress) mostly in the eastern Mediterranean provided that a certain taxonomic effort is exerted (specimens assigned mostly to species level). Results were independent of mesh size used, but were misleading when based on semi qualitative data from dredges. In any case, EQS assessments should be based on a combination of indices as the results may be misleading according to case (i.e. heavy metal pollution). Moreover, further development of this type of environmental tool requires the consensus of scientists in the assignation of species to a particular ecological group. The Geographical Intercalibration Groups (GIGs) for Mediterranean Member States has provided an update on the intercalibration exercise including the BENTIX index as tool index to test.

3.1.3. Pelagic and benthic invading species

Species invasions are irreversible events, shifting the ecosystem from one to another state. Towards that point, the presence or absence of invasive species and their number are indicators for a threshold of non-returning point. The concentration of invaders must be monitored, and the range of their variability and dispersal could be used for prognoses and management scenarios for future invasions (Gollasch and Leppakoski, 1999; Moncheva and Kamburska, 2002). The world shipping fleet is transporting approximately 10 bill.t. of ballast water around the globe/year - on average more than 3000 species of plants and animals transferred daily around the world. It was estimated that every 28 weeks there was a new record of nonindigenous species in Baltic Sea (Gollasch and Leppakoski, 1999) and every 44 weeks in the Black Sea (Kamburska and Moncheva, 2003). The invader species may behave as a "biological time-bomb" either by promoting the colonization of other aliens, or unforeseen direct and indirect ecological and socio-economic impacts.

Table 5. Summary for level 1 indicators.

Indicator/ Index				
Easiness to measure				
Sensitiveness to pressure				
Predictable response				
Anticipatory				
Predict changes due to management actions				
Integrative				
Known response to disturbances , pressures and changes over time				
Low variability				

3.2. Ratio between classes of organisms

Level 2 indicators use ratios between classes of organisms. A characteristic example of such a biological indicator of water quality is the Nyggard index (Nygaard, 1949), it is a ratio between algal groups that was developed for freshwater systems. Although the concept of plankton quotients was severely criticized, the opinion still holds that desmids are generally encountered in nutrient-poor water bodies (Hutchinson 1967, Reynolds 1984). However, Coesel (1975, 1983) pointed out that not all desmid species follow that trend in distribution, so that the indicative significance can better be considered at the species level. Nygaard's Phytoplankton classification for freshwaters is showed in table 6.

Table 6. Nygaard's algal index

Index	Calculation	Oligotrophic	Eutrophic
Myxophycean	Myxophyceae/ Desmids	0.0 to 0.4	0.1 to 0.3
Chlorophycean	Chlorococcales/Desmids	0.0 to 0.7	0.2 to 9.0
Bacillariophycean	Centric/Pinnate Diatoms	0.0 to 0.3	0.0 to 1.7
Euglenophycean	Euglenophyta/ Myxophyceae+Chlorococcales	0.0 to 0.2	0.0 to 1.0
Compound Quotient	Myxophycean +Chlorococcales+Centric+Euglenophyceae Desmids	0.01 to 1.0	1.2 to 2.5

Recently, the Ecological Evaluation Index (EEI) has been proposed by Orfanidis *et al*. (2001). The EEI is a number ranging from 2 to 10, indicating the ecological quality status of transitional and coastal waters in five classes, from high to bad. Marine benthic macrophytes (seaweeds, seagrasses) are used as bio-indicators of ecosystem shifts, from the pristine state with late-successional species (Ecological State Group I, ESG I) to the degraded state with opportunistic species (Ecological State Group II, ESG II). The first group comprises genera with a thick or calcareous thallus, low growth rates and long life cycles (perennials) whereas the second group includes filamentous genera with high growth rates and short life cycles (annuals). Seagrasses were included in the first group, whereas Cyanophyceae and species with a coarsely branched thallus were included in the second group. The EEI quantifies shifts in the structure and function of transitional and coastal waters at different spatial and temporal scales by using non-linear and linear relationships. The evaluation of ecological status into five categories from high to bad includes a comparison of the percentual abundance of ESG I species against ESG II species to which a numerical scoring system is matched (see figure 3 and table 7). The Geographical Intercalibration Groups (GIGs) for Mediterranean Member States has provided an update on the intercalibration exercise including the EEI index as tool index to test.

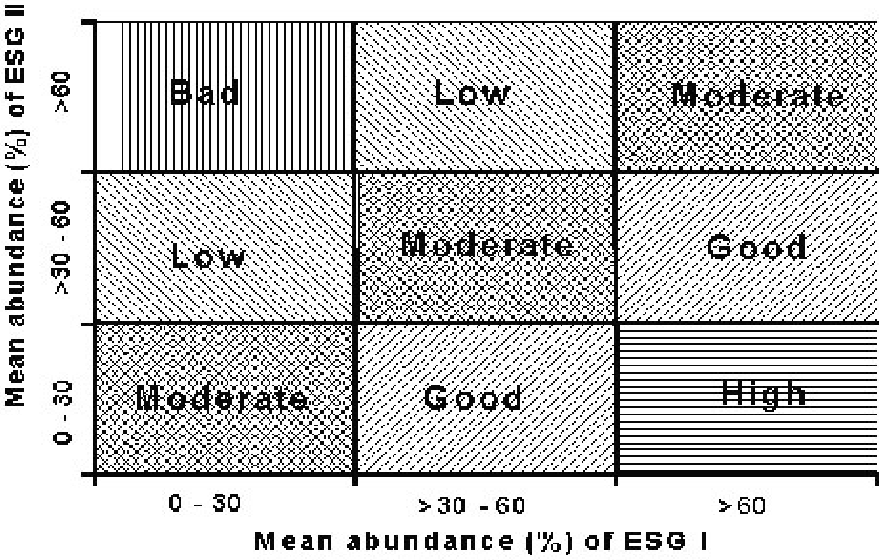

Figure 3. Ecological State Group classification matrix for seaweed and seagrasses. The matrix scores the mean abundance (%) of ESGs to determine the ecological status of transitional and coastal waters (Orfanidis *et al*., 2001).

Table 7. Estimation of EEI and the equivalent ESCs from the abundance of ESGs.

Numerical values of ecological quality classes	Spatial scale weighted Ecological Evaluation Index (EEI)
High = 10	[≤10 - >8] = High
Good = 8	[≤8 - >6] = Good
Moderate = 6	[≤6 - >4] = Moderate
Low = 4	[≤4 - >2] = Low
Bad = 2	2 = Bad

Diatoms/flagellates – shift of functional groups

Changes in ambient nutrient concentrations and their ratios give competitive advantage to some phytoplankton species over the others (Officer and Ryther, 1980, Egge and Aksnes, 1992). Silicate limitation, indicated by decreasing Si: N ratios as a consequence of eutrophication affects the phytoplankton community, potentially by decreasing the relative biomass of diatoms and increasing in the biomass of flagellates, some of which may develop harmful algal blooms (Sommer, 1995, Escaravage *et al.* 1999). It has been observed in the North Sea coastal area, that long-term eutrophication has not only led to increase in phytoplankton biomass (chlorophyll-*a*), but also changes in the phytoplankton community structure towards large algae in the diatom assemblage, and

Phaeocystis pouchetii (Haptophyta) -dominated blooms (Philippart *et al.*, 2000, Cadée and Hegeman 2002). A similar trend has been found from the southern Baltic Sea (Wasmund and Uhlig, 2003), where long-term monitoring data showed decline of diatoms and increase in dinoflagellates during the spring bloom.

Proportion of cyanobacteria

However, in low salinity environments, the relative proportion of cyanobacteria of total phytoplankton biomass can be used as a potential indicator of eutrophication.

Proportion of picoplankton

In oligotrophic conditions when ambient nutrient concentrations are low, the fraction of small-sized phytoplankton (picoplankton: <2 µm-size) algae, that are ubiquitous in aquatic systems often exceeds 50 % of phytoplankton. The share of picoplankton decreases with increasing nutrient concentration both in experimental and natural conditions (Stockner,1988, Kuosa,1990, Thingstad *et al.*, 1998, Agawin *et al.*, 2000, Gotsis-Skretas *et al*,. 2000), and in hypereutrophic environments their proportion of autotrophic biomass is only a few per cent.

Picoplanktonic algae are, however, overlooked in monitoring programmes even if their contribution to the biomass would be easy to estimate, by measuring the share of <2-3 µm chlorophyll-*a* fraction and comparing it to the total chlorophyll. Their biomass is also easy to estimate microscopically (McIsaac and Stockner 1993), which could be used in monitoring.

For what concerns marine coastal and transitional waters, a Joint BSRP/HELCOM (Baltic Sea Regional Project and Helsinki Commission) Coastal Fish Monitoring Workshop 2/2005 proposed a list of indicators for coastal fish community health. Of these, a number of this were ratios such as Cyprinid/Percid ratio (in particular Minnow fish/Perch fish**,** HELCOM 2003), Benthic/Pelagic species ratio for the Eutrophication assessment scheme in the Baltic Sea drafted by the HELCOM EcoQO project. The Danish National Environmental Research Institute (NERI) used a similar scheme in producing their 2003 assessment of the eutrophication status of Danish marine waters (Aerteberg *et al*. 2003).

Coastal fish stocks change due to the multitude of interlinked ecosystem changes connected with eutrophication. Cyprinids seem to be favored by the increasing eutrophication and the ratio between functional groups cyprinid and percid abundances seem to have some promise as a indicator of eutrophication (Appelberg and Ådjers, 2001).

3.3. Specific chemical compounds and biomarkers

3.3.1. Specific chemical compounds

Among the most anthropogenically altered water chemistry variables are the nutrients, i.e. nitrogen (N) and phosphorus (P). These have since the 1960's been associated to degradation of ecosystems, to the process called eutrophication, first recognised in freshwater and most recently in coastal water (Nixon, 1995). Thus, there are some decades worth history of measuring and using these elements as indicators of eutrophication. When considering eutrophication in coastal areas, equally relevant as ambient concentrations N, P and silicate (Si), are their ratios. As nitrate concentrations increase due to anthropogenic loading, systems are moving towards not only higher N: P ratios, but also lower Si:N ratios. Also, other human activities, such as damming of rivers, leads to increasing silicate retention and smaller silicon load to the seas (Humborg *et al.* 2000). During the last decades, N: P ratios have increased dramatically in Dutch coastal waters (deJonge *et al.* 2002), Danish coastal areas (Jorgensen 1996, Kaas *et al.* 1996), and the Black Sea (Shtereva *et al.* 1999).

The OSPAR checklist of parameters for a holistic assessment includes under the causative factors the degree of nutrient enrichment and lists a number of related indicators see table below) that need to be considered when evaluating a marine area for its degree of eutrophication. Also, Ospar has agreed to some qualitative/quantitative criteria for these indicators (see tables 8 and 9).

Table 8. Extract from the Common Procedure for the Identification of the Eutrophication Status of the Maritime Area of the OSPAR Convention – OSPAR 1997 Summary Record - OSPAR 97/15/1.

a.	**the causative factors**
	the degree of nutrient enrichment
•	with regard to inorganic/organic nitrogen
•	with regard to inorganic/organic phosphorus
•	with regard to silicon
	taking account of:
•	sources (differentiating between anthropogenic and natural sources)
•	increased/upward trends in concentration
•	elevated concentrations
•	increased N/P, N/Si, P/Si ratios
•	fluxes and nutrient cycles (including across boundary fluxes, recycling within environmental compartments and riverine, direct and atmospheric inputs)

Table 9.The agreed Harmonised Assessment Criteria and their respective assessment levels of the Comprehensive Procedure (OSPAR, 2001).

Assessment parameters		
Category I	**Degree of Nutrient Enrichment**	
	1	**Riverine total N and total P inputs and direct discharges (RID)** Elevated inputs and/or increased trends (compared with previous years)
	2	**Winter DIN- and/or DIP concentrations** Elevated level(s) (defined as concentration >50 % above salinity related and/or region specific background concentration)
	3	**Increased winter N/P ratio (Redfield N/P = 16)** Elevated cf. Redfield (>25)

3.3.2. Biomarkers

Biomarkers have also been used as indicators of ecosystem health. A biomarker is defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) which can be related to exposure to, or toxic effects of, environmental chemicals (Peakall, 1994).

The ability of various pollutants to mutually affect their toxic actions complicates the risk assessment based solely on environmental levels. Deleterious effects on populations are often difficult to detect in feral organisms since many of these effects tend to manifest only after longer periods of time. When the effect finally becomes clear, the destructive process may have gone beyond the point where it can be reversed by remedial actions or risk reduction. Effects at higher hierarchical levels are always preceded by earlier changes in biological processes, allowing the development of early-warning biomarker signals of effects at later response levels (Bayne *et al.*, 1985). In an environmental context, biomarkers offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990).

The sequential order of responses to pollutant stress within a biological system is visualized in figure 4.

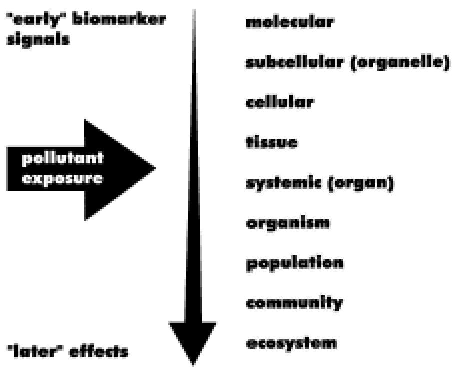

Figure 4. Schematic representation of the sequential order of response to pollutant stress within a biological system. Modified from Bayne *et al.* (1985).

The most compelling reason for using biomarkers is that they can give information on the biological effects of pollutants rather than a mere quantification of their environmental levels. Biomarkers may provide insight into the potential mechanisms of contaminant effects. By screening multiple biomarker responses, important information will be obtained about organism toxicant exposure and stress. A pollutant stress situation normally triggers a cascade of biological responses, each of which may, in theory, serve as a biomarker (McCarthy *et al*., 1991). Above a certain threshold (in pollutant dose or exposure time) the pollutant-responsive biomarker signals deviate from the normal range in an unstressed situation, finally leading to the manifestation of a multiple effect situation at higher hierarchical levels of biological organization (see figure 5).

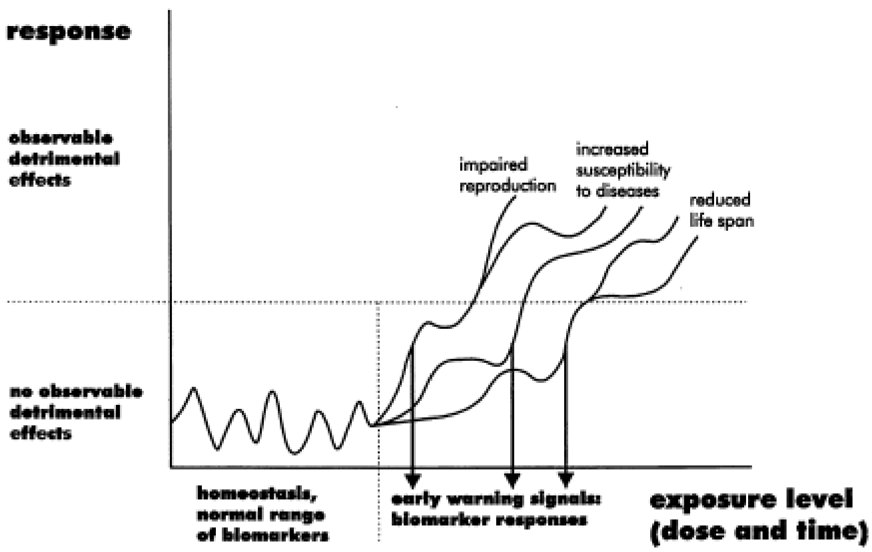

Figure 5. The principal scheme of response in organisms to the detrimental effects of pollutant exposure. Modified from McCarthy et al. (1991).

According to the NRC (1987) and WHO (1993), biomarkers can be subdivided into three classes:

- biomarkers of exposure: covering the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;
- biomarkers of effect: including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease;
- biomarkers of susceptibility: indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure.

However, most of the biomarkers that have been studied in the field are related to the two first categories. The biomarkers, presented below, where chosen for specificity, for robustness and because they are among a limited set a methods proposed by international organizations, including OSPAR and ICES.

3.3.2.1 Biomarkers of exposure

They are not indicative of biological effects but provide sensitive markers of exposure to bioavailable levels of pollutants in the environment.

Mercury in seagrass

One example for Mediterranean marine coastal waters is the use of the seagrass *Posidonia oceanica* as a biological indicator of past and present status of contamination. The capability of *Posidonia oceanica* to concentrate a range of pollutants, such as the organochlorine compounds DDT, lindane and PCB (Chabert *et al*., 1984), certain artificial radionuclides (Florou *et al*., 1985; Calmet *et al*., 1988, 1991), and trace metals (Augier *et al*., 1977; Chabert *et a*l., 1983; Maserti *et al*., 1988; Malea and Haritonidis, 1989; Gnassia-Barelli *et al*., 1991) has been clearly established. In a study conduced by Pergent-Martini (1998), the concentration of mercury was measured in various tissues of *Posidonia oceanica* at three sites presenting distinct degrees of human activity. The accumulation of mercury differed according to the tissue examined and the level of contamination of the site. The use of lepidochronology, a technique for dating the dead sheaths and rhizomes of

Posidonia oceanica, rendered it possible not only to determine the present level of contamination at each site, but also to reconstitute the pattern of change in the degree of contamination of the environment over a period of twenty years.

The bioaccumulation of mercury by the phanerogam is considerable, the concentration factor being estimated for Marseilles-Cortiou at 3200 (the ratio between the mean mercury concentration in the water and that of the living leaves on a wet weight basis, as reported by Joanny *et al*., 1993). This accumulation shows wide variations according to the tissue examined and the degree of human activity at the site. Where mercury contamination is low, accumulation occurs preferentially in the rhizomes, whereas in sites subjected to greater contamination, mercury accumulation is highest in the blades. The concentrations of mercury accumulated in sheaths of *Posidonia oceanica*, are retained in the plant for long periods of time (Calmet *et al*., 1988; Pergent-Martini *et al*., 1992; Pergent-Martini and Pergent, 1995; Pergent-Martini, 1998). Because of this property, it is possible (using only occasional sampling) to determine the patterns of change over time of the mean mercury contamination of the environment.

PAHs metabolites in fish

PAH metabolites are sensitive markers of exposure to bioavailable levels of PAHs in the environment. Metabolite levels in bile can be determined either by analyzing the total level of PAH metabolites as fluorescent aromatic compounds (FAC), or by selecting a single metabolite as a marker for total PAH metabolism. Hydroxypyrene (OH pyrene) has been selected for this purpose because, first of all, relatively high levels of pyrene have been detected in most sediments; secondly, pyrene is biotransformed predominantly into a single, strongly fluorescent metabolite (OH pyrene); and thirdly, the bioavailability of pyrene is relatively high for aquatic organisms (Ariese *et al*., 1993).

Van der Oost *et al*. (2003) summarized in their review the FAC responses for all fish species from 15 laboratory studies and 24 field studies. A significant increase in biliary FAC levels was observed in 93% of the laboratory studies and 79% of the field studies, while strong increases (>500% of control) were observed in 87% and 46% of the laboratory and field studies, respectively. They concluded that levels of biliary PAH metabolites are certainly sensitive biomarkers to assess recent exposure to PAHs. Since PAH exposure cannot be reliably determined by measuring fish tissue levels, this parameter was validated for ERA (Environmental Risk Assessment) processes concerning PAH-contaminated sites.

In 2002 the working Group on Biological Effects of Contaminants (WGBEC) recommended different techniques for biological monitoring programs (Jørgensen *et al*, 2000). The PAH bile metabolites measured in fish assessed the exposure to and the metabolism of PAHs. They proposed a threshold value equal at 2 times the value at the reference site.

Among the 2003 strategies of the OSPAR Commission, the biological effects methods of the Norwegian JAMP program (Green *et al*, 2003) included the measurement of the OH-pyrene in cod (*Gadus morhua*) bile. For the 2003 investigations, 25 cods were sampled at four stations: the inner Oslofjord (30B), Sorfjord (53B) and Sotra-Bomlo (23B) and in the open coastal area outside Lista (15B). The data's showed that no significant temporal trends were found at these stations. The median concentration of OH-pyrene in cod from station 30B was higher than in cod from 15B, 53B and the "reference" station 23B. However, the variability (SD) was higher than the respective medians at stations 15B and 53B. This indicated that some individuals at these two stations had been exposed to PAHs. When considering the whole period (1998-2003), the yearly median concentration at 30B were the highest or next highest compared to the other 3 stations. Furthermore concentrations at 53B were usually higher than 23B. This presumably reflects the general contamination of the two areas (30B and 53B). The location 15B, previously regarded as only diffusely polluted, has an input of PAH which is sufficient to markedly affect fish in the area. The authors suggested to include DNA adduct analyses to clarify whether the cellular repair system of cod is sufficient to protect against damage from PAH radicals.

During the BEEP project (Biological Effects of Environmental Pollution in Marine Coastal Ecosystems), Beliaeff and Bocquene (2004) analyzed the data's generated from 11 laboratories who sample twice a year (2001 and 2002) four sites (France, Italy, Spain and Greece). Fish (*Mullus barbatus*) and mussel (*Mytilus galloprovincialis*) were collected and among other biomarkers EROD activity and PAHs metabolites in bile were measured. Few chemical data were available but 16 PAHs recommended as priority pollutants and seven PCBs (28, 52, 101, 118, 138, 153 and 180) were measured in sediments during one of the cruise. The statistical analysis of the data shows a low efficiency and/or relevance of the selected biomarkers to predict chemical contamination in marine water. The different explanations they proposed are: the influence of confusing parameters (seasons, maturity sex, etc.), the differences in analysis procedure and the inadequate sampling designs.

Note: Generally, the levels of bile metabolites are indicative of short-term exposure (1 week), and therefore, provide information on recent exposure only. It was demonstrated that the PAHs bioavailability can vary markedly in different fish species living in environments similarly contaminated with PAHs.

3.3.2.2 Biomarkers of effect

Biomarkers of effect responses not only indicate chemical exposure, but also predict effects at various levels of biological organization. Their induction are biochemical changes within the organism which often precedes the onset of more serious cellular and physiological changes such as hepatic damage, reproductive toxicity, immunotoxicity etc.

CYP1A responses and EROD activity

Cytochromes P450 is a group of proteins that play a central role in the metabolism of endogenous substrates such as steroid hormones and xenobiotic compounds. CYP 1A (cytochrome P450 1A) are unique in that markedly increased levels as well as variant forms are commonly found in the tissue of animals exposed to different types of inducing compounds. Inducers of environmental importance include PAHs, PCBs and dioxins which effect induction via a cytosolic protein (the so-called Ah receptor). Next to CYP1A protein, a common method to examine the responses of the cytochromes P450 enzymes is to determine its catalytic activity. EROD (ethoxyresorufin O-deethylase) enzyme activity reflects the presence of induced cytochrome P450 1A.

According to the OSPAR Commission (JAMP Guidelines for Contaminant-specific Biological Effects Monitoring), the EROD catalytic enzyme assay is the technique recommended for monitoring CYP 1A activity. Immunoassays for CYP 1A may also be used and may give additional information in cases where contaminant exposure is sufficient to cause inhibition of catalytic activity of CYP 1A. Certain confounding variables, which may affect the enzyme activities, however, will have to be considered when interpreting the responses in these parameters.

Van der Oost *et al.* (2003) summarized in their review the CYP1A and EROD responses for all fish species. A significant increase in CYP1A levels was observed in 91% of the laboratory studies (n=60) and 85% of the field studies (n=48), while strong increases (>500% of control) were observed in 43 and 39% of the laboratory and field studies, respectively. A significant increase in EROD activities was observed in 88% of the laboratory studies (n=137) and 90% of the field studies (n=127), while strong increases (>500% of control) were observed in 69 and 37% of the laboratory and field studies, respectively.

In all fish species considered, hepatic CYP1A protein levels together with EROD activities levels may be used both for the assessment of exposure and as early-warning sign for potentially harmful effects of many organic trace pollutants. They are very sensitive biomarkers of exposure to PAHs and PCBs. Levine and Oris (1999) suggested that CYP1A expression due to exposure to rapidly metabolized substances should preferably be measured in tissues that make direct contact with the environment, such as the gill and intestine. The CYP1A response has been validated for use in ERA (Environmental Risk Assessment) monitoring programs (Bucheli and Fent, 1995), assuming that all potential variables that may affect this parameter are considered in the experimental design.

The scientific Seine-Aval program assesses the usefulness of monitoring programs using a suite of biological measurements in the Seine Bay (Burgeot, 1999). Among the biomarker measured the EROD activity is determined in European flounder fish (*Platichthys flesus L.*) and the results are integrated with AChE activity and DNA adducts results to define a biomarker indicator (Beliaeff and Burgeot, 1999). The biomarker indicator allows to characterize early stress regulation in flounder juvenile according to the contamination of the site by the PAHs and the PCBs.

Table 10. Results of chemical and biological measurements in flounder from the Seine Bay.

Sites	PAHs ng/g sediment	PCBs ng/g sediment	PAHs liver ng/g DW	PCBs liver ng/g DW	Biomaker indicator
La Bouille	7140	ND	371	4120	6.33
Caudebec	1450	ND	217	2734	3.04
Honfleur	2500	ND	166	1902	0.32
Embouchure	950	30	78	5633	1.63

The working Group on Biological Effects of Contaminants (WGBEC) in 2002 recommended different techniques for biological monitoring programs (Jørgensen *et al*, 2000). The EROD activity and the CYP1A protein levels measured planar organic contaminants metabolism in mussel and fish. They proposed a threshold value equal at 2.5 times the value at the reference site.

Among the 2003 strategies of the OSPAR Commission, the biological effects methods of the Norwegian JAMP program (Green *et al*, 2003) included the measurement of the CYP1A protein level and EROD activity in Cod (*Gadus morhua*) liver. For the 2003 investigations, 25 Cods were sampled at three stations: the inner Oslofjord (30B), Sorfjord (53B) and Sotra-Bomlo (23B). The data's showed that no significant temporal trends were found at these three stations. The EROD activities correlated significantly with amount of

CYP1A protein (R^2=0.53, p<0.0001). It has been shown that generally higher activity has been found at the most contaminated stations (Ruus *et al*, 2003). However, median EROD activity at station 53B was lower than at the less contaminated 23B station. At all stations, median EROD activities were lower in 2003 than in 2002. The two extreme PCB concentration fish had moderate hepatic EROD activities. The authors consider that confounding factors or adaptation to continuous exposure led to inconsistent responses for those biomarkers.

During the BEEP project (Biological Effects of Environmental Pollution in Marine Coastal Ecosystems), Beliaeff and Bocquene (2004) analyzed the data's generated from 11 laboratories who sample twice a year (2001 and 2002) four sites (France, Italy, Spain and Greece). Fish (*Mullus barbatus*) and mussel (*Mytilus galloprovincialis*) were collected and among other biomarkers EROD activity and PAHs metabolites in bile were measured. Results are presented in the PAHs metabolites in fish paragraph above.

DNA adducts

OSPAR commission recommended a combination of three biomarker techniques to describe the impact of PAH compounds on biota at the biochemical level. The biomarker techniques selected are CYP 1A activity, bulky aromatic-DNA adducts and PAH metabolites in bile. These indicators can be considered as an interconnecting series since planar PAHs are effective in inducing CYP 1A enzyme and some of their members are metabolized to reactive epoxides forming DNA and protein adducts which are linked to mutagenesis and carcinogenesis as well as other potentially important deleterious effects. The suite of biomarkers give a measure of exposure and biochemical effects of which the observation of DNA adducts can be marked as a deleterious effect. DNA adducts are generally determined in the liver, since this is the key organ for biotransformation of xenobiotics. Levels of hepatic DNA adducts may be indicative of cumulative exposure of fish to genotoxic compounds over a longer period of time (several months). The JAMP recommended technique (OSPAR, 2003) for measuring DNA adducts is the P-32 post-labeling technique. The primary reason for using the P-32 technique is its high sensitivity, its requirement for small amounts of DNA and its ability to detect carcinogenic DNA adducts of unknown structure. For interpretation it is important to realize that this technique may also measure adducts from chemicals other than PAHs.

In Van der Oost *et al.* (2003), both laboratory and field studies on DNA adduct formation in fish reviewed by Pfau (1997) were presented. The DNA adduct responses for all fish species from 17 laboratory studies and 30 field studies showed that a significant increase in hepatic DNA adduct levels was observed in 100% of the laboratory studies and 70% of the field studies, while strong increases (>500% of control) were observed in 65% and 30% of the laboratory and field studies, respectively. Due to the strong and consequent responses of hepatic DNA adduct levels to PAHs exposure, this parameter is considered to be an excellent biomarker for the assessment of PAH exposure as well as a sensitive biomarker for the assessment of potentially genotoxic effects. It is important to combine the measurements of DNA adduct and tumor formation to provide insights in the mechanisms involved in chemical carcinogenesis (Maccubbin, 1994).

Hepatic DNA adducts was evaluated one of the most valuable fish biomarkers for ERA purposes.

In her review, Devauchelle (2002) cited Collier *et al*. in Ware (1995) that found a significant positive correlation (r^2=0.97) between PAHs in the sediments and DNA adducts in fish liver (*Opsanus tau*) and Akcha *et al.* (2000) that found a significant positive correlation (r^2=0.9) between B(a)P concentration in the mussel (*Mytilus galloprovincialis*) and the level of 8-oxoGuo which is correlative to DNA adducts.

The scientific Seine-Aval program assess the usefulness of monitoring programmes using a suite of biological measurements in the Seine Bay (Burgeot, 1999). Among the biomarker measured the DNA adducts were determined in European flounder fish (*Platichthys flesus L.*). The results were integrated with EROD and AChE activities to define a biomarker indicator according to Beliaeff and Burgeot (1999) and are presented in the CYP1A/EROD paragraph above.

The working Group on Biological Effects of Contaminants (WGBEC) in 2002 recommended different techniques for biological monitoring programs (Jørgensen *et al.*, 2000). The bulky DNA Adduct measured in fish assessed the genotoxic effects of PAHs and other synthetic organics and is attended to be a sensitive indicator of past and present exposure. They proposed a threshold value equal at 2 times the value at the reference site and/or 20% of change.

3.3.2.3 Recommendations to use biomarkers as indicators of the assessment of thresholds

The OSPAR Commission (OSPAR, 2003) warns that biological effects attributable to PAH are difficult to assess in shellfish, and therefore selected finfish as the principal monitoring species. They recommended that the choice of fish species to be monitored will reflect availability throughout the Maritime Area. Dab (Limanda limanda) is common throughout the North Sea and the Irish Sea but is not generally available in the southern part of the Maritime Area. The dragonet (Callionymus lyra) provides a suitable alternative species. For estuarine monitoring the flounder (Platichthys flesus) is recommended. Other species may be chosen according to particular regional concerns.

Biomarker responses are powerful because they integrate a wide array of environmental, toxicological and ecological factors that control and modulate exposure to, as well as effects of, environmental contaminants. However, these same factors may also complicate interpretation of the significance of the biomarker responses in ways that may not always be anticipated (McCarthy, 1990). Many non-pollution-related variables may have an additional impact on the various enzyme systems. Examples of such 'modifying' factors are the organisms' health, condition, sex, age, nutritional status, metabolic activity, migratory behavior, reproductive and developmental status, and population density, as well as factors like season, ambient temperature, heterogeneity of the environmental pollution, etc. Even if interesting correlation have been found between biomarker and pollutant levels, the relevance of those indicators is mainly to determine the biodisponibility of the contaminants and those measurements should be related to data's on confounding factors and concentration of chemicals. Despite indications that certain biomarker responses are an early warning for adverse effects on the health or fitness of individual organisms, it will be hard to correlate these responses with effects on population, community or ecosystem levels.

Table 11. Summary of potential biomarkers as indicators of the assessment of thresholds.

Indicator/ Index	Mercury in phanerogams	PAHs bile metabolites	CYP1A and EROD	DNA adducts
Easiness to measure	+++	++	++	+
Sensitiveness to pressure	ND	++	+++	++
Predictable response	ND	yes	no	yes
Anticipatory	Yes	yes	yes	yes
Predict changes due to management actions	ND	yes	ND	ND
Integrative	ND	yes	yes	yes
Known response to disturbances , pressures and changes over time	Yes	yes	no	yes
Low variability	ND	yes	no	yes

3.4. Trophic levels

Level 4 applies concentration of entire trophic levels as indicators. An example is Trophic Index TRIX, a linear combination of the logarithm of 4 state variables: Chl-a, %DO, DIN and TP (Vollenweider *et al*., 1998; Giovanardi and Vollenweider, 2004), adopted to characterize the trophic levels of coastal marine areas. Numerically, the index is scaled from 0 to 10, covering a wide range of trophic conditions from oligotrophy to eutrophy. The formulation of the TRIX Index is as follows:

$$TRIX = [Log_{10} (ChA \times aD\%O \times minN \times TP)+k] / m \ (1$$

Each of the four components represents a trophic state variable, to say:

a) factors that are direct expression of productivity:

- *Chl-a* = chlorophyll-a concentration, in μg/l;
- *%DO* = Oxygen as absolute % deviation from saturation;

b) nutritional factors:

- *DIN* = mineral nitrogen also called dissolved inorganic nitrogen (DIN) corresponding to N (as $N\text{-}NO_3+N\text{-}NO_2+N\text{-}NH_4$), in μg/l;
- *TP* = total phosphorus, in μg/l.

The parameters k = 1.5 and m = 12/10 = 1.2 are scale coefficients, introduced to set the lower limit value of the Index and the extension of the related Trophic Scale, from 0 to 10 TRIX units. TRIX point values assign an immediate measurement to the trophic level of coastal waters. Referring to the Italian seas, values exceeding 6 TRIX units are typical of highly productive coastal waters, where the effects of eutrophication determine frequent episodes of anoxia in bottom waters. Values lower than 4 TRIX units are instead associated to scarcely productive coastal waters, while values lower than 3 are usually found in the open sea. Because of the log-transformation of the four original variables, annual distributions of TRIX data over homogeneous coastal zones, are very close to normal kind and show a quite stable variance, with STD around 0.9. The Italian law covering water protection (152/99) classifies marine coastal waters quality using the trophic scale based on TRIX index reported in the table 12.

TRIX was applied successfully in examination, mapping and comparison of trophic states of two European Seas- Adriatic and Black Sea (Doncheva *et al*., 2002). As a principal indicator of coastal water quality contributing to trophic state definition of a particular area is considered the ratio phytobiomass/nutrients (Chl-a√NP) (Innamorati and Giovanardi, 1992).

Table 12. Trophic classification of the coastal water quality (from national Italian law 152/99).

Trophic scale	Water quality
2-4	low trophic level; good water transparency; absence of coloured waters; absence of oxygen deficiency in bottom waters;
4-5	medium trophic level; occasional water turbidity; occasional anomalous coloration of waters; occasional hypoxia in benthic waters;
5-6	High trophic level; bad water transparency; anomalous coloration of waters; hypoxia and occasional anoxia in benthic waters; suffering level at benthic ecosystem level;
6-8	very high trophic level; high water turbidity; diffuse and persistent anomalies in colour of waters; diffuse and persistent hypoxia/anoxia in benthic waters; extensive benthic organism- kills may also occur; alteration/simplification of benthic community; economic damages to tourism, aquaculture, fishing;

The "efficiency" concept (Efficiency Coefficient), better defined as ratio between biotic and abiotic components, gives rise to a complex series of interpretative problems; however, it assumes a consistent meaning as discriminating function among coastal systems. The Efficiency Coefficient is defined as:

$$\text{Eff. Coeff.} = \text{Log10}\left([\text{Chl-a} \times \%\text{DO}]/[\text{DIN} \times \text{TP}]\right)$$

to say as the log of the ratio between the two aggregated main components of TRIX. Numerically, values are usually negative, ranging in our analyses from –4.48 (recorded in the Ionian Sea) to 0.45 (NW Adriatic Sea). The GIGs for Mediterranean Member States has provided an update on the intercalibration exercise including the TRIX index as tool index to test. Further in the REBECCA project, the TRIX is an index to test and validate within Member States.

The Mediterranean coastal waters in Catalonia (NW Mediterranean) are under a monitoring phytoplankton programme since 1990 conduced by the Institut de Ciències del Mar (CSIC) and Agència Catalana de l'Aigua, Generalitat de Catalunya (ACA). The parameters used are:

- Harmful Phytoplankton species (composition and abundance);
- Chl-*a* (biomass).

Based on previous knowledge (validation of their database) and expert judgment they have established levels for mean Chl-*a* and frequency and concentrations of harmful algal species (HA) (see table 13).

Table 13. Established levels for mean Chl a and frequency and concentrations of harmful algal species (HA) (Vila and Masó, 2005).

Status	Chl a- mean	Freq. HA (%)
High	$X \leq 1$	Freq. (%) ≤ 2
Good	$1 < x \leq 2$	$2 <$ Freq. (%) ≤ 15
Moderate	$2 < x \leq 4$	$15 <$ Freq. (%) ≤ 25
Poor	$4 < x \leq 6$	$25 <$ Freq. (%) ≤ 70
Bad	$X > 6$	Freq. (%) > 70

NOTE: these values refer to non-confined water areas, neither aquaculture areas. They refer to beaches and rocky areas.

Harmful algal species considered here are grouped as potentially PSP-producing species (*Alexandrium catenella, A. minutum*), DSP-producing species (*Dinophysis acuta, Dinophysis caudata, Dinophysis fortii, Dinophysis* cf. *ovum, Dinophysis hastata, Dinophysis rotundata, Dinophysis sacculus, Dinophysis tripos, Dinophysis* spp. and *Prorocentrum rhathymum*), *Ostreopsis* spp., bloom-forming and fish-killing species (*A. taylori, Dictyocha fibula,, D. speculum*, *Fibrocapsa japonica, Gonyaulax polygramma, Gymnodinium sp, G. pulchellum, Gymnodinium/Gyrodinium impudicum, Gyrodinium corsicum, Lingulodinium polyedrum*, *Noctiluca scintillans, Pratjetella medusoides, Prorocentrum minimum, Pseudo-nitzschia* spp.). The threshold level in which the frequency of HA has been considered is detailed in table 14. Each water body has been classified considering the worse score between mean Chlorophyll-a concentration and the frequency of blooms. The GIGs for Mediterranean Member States has provided an update on the intercalibration exercise including the Catalan Phytoplankton index as tool index to test.

Table 14. Frequency of HA's threshold level (Vila and. Masó, 2005).

PSP producing-species>10.000 cells/L
DSP producing-species>1.000 cells/L
Ostreopsis >5.000 cells/L
Bloom >250.000 cells/L

The following ratios could be also proposed as trophic level indicators: total phytoplankton biomass to total zooplankton biomass ratio (PhB/ZB), which is a measure of the grazing pressure. The typical value is 10:1 (Odum, 1985). It was found that ratio could exceed by a factor of 3 for the coastal Black Sea sites and more than 10 times in the eutrophic lakes along the coast (Moncheva *et al*., 2002). Bacillariophyceae to Copepoda biomass ratio (Bac/Cop) and Dinophyceae to Copepoda biomass ratio (Din/Cop), reflecting the selective grazing, also differs significantly between eutrophic and non eutrophic sites.

Species are thought to adopt one of three life history strategies for survival in stable and unstable environments: (i) r-selection; (ii) K-selection; or (iii) bet-hedging (Smayda and Reynolds, 2001). Smayda and Reynolds (2001) in the phytoplankton life-form concept recognize the occurrence of invasive, small- to intermediate-sized colonist species (C-strategists) which often predominate in chemically-disturbed water bodies. Representative C-strategists species are *Gymnodinium*, *Heterocapsa*, *Prorocentrum* and *Scrippsiella* spp., *Alexandrium minutum*, *Heterosigma akashiwo*, *Phaeocystis pouchetii*. Typically invasive, colonist species of small cell size (ca. 103 μ) are r-selected species. Stable environments are characterized by species with a K-strategy, while fluctuating environments are characterized by species with an r-strategy. A comparison of Mediterranean (Aegean Sea) and Black Sea phytoplankton communities based on such approach is presented in Moncheva *et al*. (2001).

3.5. Rates

Level 5 uses process rates as indicators. An index obtained from oxygen fluxes is the Trophic Oxygen Status Index (TOSI). The TOSI is derived from the Benthic Trophic Status Index (BTSI) proposed by Rizzo *et al*. (1996), and basically represents the net potential metabolism. The index results from the relationship between net maximum productivity (NP), measured at saturating light, and dark respiration (DR). The index was developed to provide a simple portrayal of oxygen processing over time and space for shallow aquatic systems and has two modes: a categorical classification and a graphical representation (table 15). The categorical classification of the index from autotrophy to heterotropy provides a rapid assessment of the potential oxygen balance and thus evidences critical situations in the lagoonal metabolism. In the graphical representation of the TOSI, three pieces of information are given: the categorical TOSI, the magnitude of flux for both NP and DR and the time line of the fluxes.

Where flushing is slow, the TOSI reflects dissolved oxygen dynamics since the NP:|DR| ratio clearly correlates with both the maximum oxygen concentration (MOC) and the daily quantity of oxygen remaining in the water column (RO). However, TOSI is less well related to MOC and RO in open systems in which oxygen concentrations are dominated by physical factors. The graphical representation of TOSI seems also suitable to represent the degree of intrasystem disturbance that is related to the excess of primary production and changes in oxygen availability. It can also discriminate among different photoautotrophic conditions, including

- hyperautotrophy, as an abnormal oxygen production with respect to the biomass build up, and;
- dystrophy, as the subsequent abnormal oxygen deficit which causes prolonged anoxia and the onset of anaerobic metabolism.

Overall, the index provides a tool for rapid assessment of system metabolism and potentially its consequences.

Table 15. A qualification of the categories proposed by Rizzo et al. (1996) with the addition of hyperautotrophy and dystrophy.

Category	Condition	System qualification		
		Rate (g O_2 m^{-2} h^{-1})	BP/BD	Timing
Dystrophy	DR = NP < 0	1–10	H/La	S
Total heterotrophy	DR = NP < 0	<1	L/La	C
Net heterotrophy	DR < NP ≤ 0	<1	L/R	C
Net autotrophy	0 < NP ≤ |DR|	<1	L/R	C
Total autotrophy	0 < |DR| < NP	<1	L/R	C
Hyperautotrophy	0 < |DR| ≪ NP	1–10	H/La	S

NP and DR as in the text; BP = biomass peak; BD = biodegradability; L = relatively low; H = high; R = refractory; La = labile; C = NP and DR peaks are coincident; S = DR peak follows the NP peak.

3.6. Composite indicators

Level 6 covers composite indicators to assess whether an ecosystem is at an early stage of development or has reached maturity (Odum, 1986). An example of composite indicator for the Mediterranean marine coastal and transitional waters is the POMI Index (Posidonia oceanica Multivariate Index), a multivariate method to assess ecological status of Catalan coastal waters (NE Spain) (Romero *et al.*, 2005). The POMI is based on a number of physiological, morphological, and structural descriptors of *Posidonia oceanica* and its ecosystem, combined into a variable using PCA. The method seems to provide reliable results, although use is relatively costly, given the high number of variables to measure. The descriptors used by the POMI are:

- *Individual level*
  - Morphometric measures
    - Shoot surface
    - Percent of necrosis in leaves

- Physiological measures
  - Nitrogen content in leaves and rhizomes
  - Epiphyte nitrogen content
  - Phosphorus content in leaves and rhizomes
  - Total non-structural carbohydrates in rhizomes
  - Nitrogen isotopic ratio (d15N) in leaves and rhizomes
  - Sulfur isotopic ratio (d 34S) in rhizomes
  - Trace metals content in rhizomes: Fe, Zn, Mn, Cu, Ni, Pb, Cd, Cr.
- *Population level*
  - Meadow cover
  - Shoot density
  - Dominance of rhizome growth type

Two additional descriptors are currently being tested:

- Kinetics of depth limit
- Shoot burial

Most of the descriptors appear to provide pertinent information about the vitality of the meadow and more generally about the quality of the environment. Some of them provide data about the disturbances in a more specific way, and even allow identification of direct and indirect causes of temporal and spatial changes (table 16). In Catalunya, 22 extensive meadow sites, representative of most of the ca. 500 km of coastline, were selected and sampled in October-November 2003 for application of POMI. The GIGs for Mediterranean Member States has provided an update on the intercalibration exercise including the P.O.M.I. index as tool index to test.

Another example of composite bioindicator is the Eelgrass, linked with its depth limits of eelgrass (Krause-Jensen *et al*., 2005). The depth limit of eelgrass, defined as the greatest depth at which eelgrass grows, is generally regarded as a useful bioindicator, because depth limits respond predictably to eutrophication, being largely regulated by light availability. The clearer the waters, the deeper eelgrass and other seagrasses grow (Duarte, 1991; Nielsen *et al*., 2002). Krause-Jensen *et al*. (2005), in a ongoing study, analyze how the depth limit of eelgrass in Danish coastal waters can be used as a bioindicator of water quality under the WFD.

Table 16. Directs and secondary impacts on Posidonia oceanica descriptors and their time of answers (Pergent-Martini *et al.*, 2005).

Directs and secondary impacts on *Posidonia oceanica* descriptors and their time of answers

	Level of information	Direct impact	Secondary impact	Time of answer	
				In case of improvement	In case of deterioration
Density	Population	Water transparency	Anchoring Nutrients concentration	Annual	Annual
Lower depth limit	Population	Water transparency	Trawling Water movement Sedimentary dynamics Nutrients concentration	Decades	Annual
Upper depth limit	Population	Coastal development Sedimentary dynamics	Water movement	Decades	Monthly to annual
Epiphytic coverage	Individual	Nutrients concentration	Herbivory pressure Water transparency Water movement	Monthly	Monthly
Matte structure	Population	Sedimentary dynamics Water movement	Anchoring Trawling	Decades	Monthly to annual
Leaf biometry	Individual	Nutrients concentration Water transparency	Water movement Herbivory pressure	Monthly to annual	Monthly to annual
Bottom cover	Population	Sedimentary dynamics Water transparency Trawling	Water movement Anchoring	Annual to decades	Monthly to annual
Species associated to the meadow	Population	Herbivory pressure Competition Invasive species	Water movement Chemical inputs Organic matter concentration Nutrients concentration	Annual	Monthly to annual
Datation measurement	Individual	Sedimentary dynamics Water transparency	Herbivory pressure	Annual	Annual
Biochemical and chemical composition	Tissue	Nutrients concentration Water transparency Chemical inputs	Sedimentary dynamics Organic matter concentration Invasive species Competition	Weekly to monthly	Daily to weekly
Contamination	Tissue	Chemical inputs		Monthly	Weekly to monthly

Structural parameters such total abundance and biomass are considered as indicators to level 6. For example, indicators for the capacity of the system to produce and sustain organic matter are Chlorophyll-a and phytoplankton biomass (WFD, 2000). Zooplankton biomass may be used as indicators of trophic condition. There is recent focus on the use of biomass size spectra as an indicator of zooplankton response to changes in ecosystems. The slope of the size spectra appears to vary with hydrologic conditions, including nutrient inputs, thus may serve as a tool to assess the efficacy of nutrient reduction efforts. The zooplankton size spectra may be combined with phytoplankton and fish size spectra in order to create a whole ecosystem based indicator. The health of the populations typically is expressed as a number of individuals (per area) or biomass, reflecting possible stress from anthropogenic sources. The shift in phytoplankton species that has attracted the most interest is the abundance shift (blooms) from diatoms to other non-motile species and flagellates (a *functional group* shift). Functional groups and their shifts are of interest because of significant differences in their physiology and ecological impacts. There is special interest in the diatom:flagellate ratio as a potential indicator of eutrophication, as the global increase in harmful microalgal blooms (HABs) is primarily a flagellate-species phenomenon (Moncheva and Krastev, 1997). It has been hypothesized that the diatom:flagellate ratio should decrease with increasing nutrient enrichment, and consequently might serve as an indicator of eutrophication status. Such ratio is normally used as an indicator of the taxonomic structure of phytoplankton communities; the typical spring-summer value reported for an undisturbed system is 10:1. There is some supporting evidence of such value in Kastela Bay (Adriatic Sea) and Varna Bay (Black Sea), where a progressive, long-term increase in anthropogenic nutrient has been accompanied by a 10-fold decrease in the ratio of diatom to flagellate abundance (Marasovic and Pucher-Petkovic, 1991; Moncheva and Krastev, 1997). The primary nutrient expected to regulate the shift in functional groups from diatoms to flagellates is silica, which is required by diatoms but not by other microalgal groups exclusive of silicoflagellates (Officer and Ryther, 1980). The anthropogenic enrichment of N and P has led to long-term declines in the ratios of Si:N and Si:P, potentially favouring non-diatom blooms in such impacted regions (Granéli *et al*., 1990). Mescocosm experiments have led to suggest that there is a threshold of approximately 2 μM Si, below which "*diatoms, as a group, are outcompeted by the `flagellate group'*" (Egge *et al*., 1992). The merit of the Si ratio and threshold concepts as eutrophication switches that result in species shifts and altered community abundance is still under investigation. However, it is clear that the species-specific responses to these proposed Si effects are under multifactorial control rather than simple linear responses. For example, Sommer's experiments (1994) have shown that diatoms became dominant at Si:N ratios >25:1, while flagellates were superior competitors at lower ratios. The ratio of the biomass of the common crustaceans Cladocera:Copepoda is also an indicator for changes in size spectrum and quality of phytoplankton (Kamburska *et al*., 2003). Generally, the percentage share of Cladocera biomass increased in waters with higher trophic levels and indicates a significant nutrient loading. The ratio is also anindicatore for meteorologial/climatological variability (Vuorinen *et al.*, 1998). Thus significant decrease of the copepoda/cladocera biomass ratio is the result of decreasing salinity due to the long-lasting freshwater run-off and reduced salt-water intrusions from the North Sea.

Diversity indices

These quantify and simplify the species richness and diversity of ecosystems into a single number that can be used to assess the state of the community (Washington 1984).

Different types of diversity indices for illustrating the community structure have been developed (Pielou 1975, Washington 1984), for these to be useful in eutrophication-related phytoplankton classification, must be (Tsirtsis and Karydis 1998)

- sensitive to changes in phytoplankton community structure
- robust to the requirements of statistical analyses
- efficient to distinguish different eutrophic conditions

Measuring diversity is one of the mostly used methods for assessing environmental disturbances, so that some indices are based on diversity values. The hypothesis is that large portion of native species richness is required to maximize ecosystem stability and function (Schwartz *et al*., 2000). GESAMP (1995) has published a list of biological indicators, which are more applicable to long-term series for measuring the biological response to the evolution of anthropogenic eutrophication. Recently, several diversity indices have been applied and tested demonstrating their usefulness for detecting and evaluation (PRIMER 5, 2001). The most common used diversity index is the index of Shannon-Weaver (1963), also calculated as a classical measure of stability (% of dominance increase under stress), and applied for pelagic and benthic assemblages. The ratio of the number of exotic (aliens) to endemic species by taxonomic groups (Ei=exotic/endemic×100) is a measure for the extinction of local species (Zaitzev and Ozturk, 2001; Moncheva and Kamburska, 2002; Kamburska and Moncheva, 2003).

To level 6 could be referred energetic category indicators such as Production vs. Respiration (P/R): in classical ecology, the trophic state of a system is determined by the equilibrium between the oxygen producing autotrophic metabolism and the oxygen consuming heterotrophic metabolism. Therefore, the P/R diagram is a means of summarizing the trophic state, autotrophic-heterotrophic state, of an aquatic system during its evolution in time and space. , Autotrophic-heterotrophic diagrams (P/R diagrams) show, on the y-axis, the intensity of the metabolic production of oxygen or carbon in the system (P, algal photosynthesis) and on the x-axis, that of the processes of oxygen consumption or of organic matter degradation (R, respiration of bacteria, zooplankton and the benthos as well as of phytoplankton). The systems at equilibrium have an oxygen concentration at saturation level and fall on the diagonal, with increasing distance from the origin as the biological activity rises. Predominantly autotrophic systems, net producers of oxygen, lie above the diagonal (P/R >1), while the systems dominated by heterotrophic processes, net consumers of oxygen, fall below the diagonal (P/R <1). That indicator was applied for river watersheds in order to assess the ecological functioning. Theoretically, an increase in community respiration should be the first early-warning sign of stress since repairing damage caused by the disturbance requires diverting energy from growth and reproduction to maintenance. Net primary production compared to the sum respiration by heterotrophs is used at the scale of river watersheds to determine their functional ecological equilibrium. Autotrophy is evaluated by a P/R > 1, with possible symptoms of eutrophication, when P reaches 1mg C/m^2/day. On the contrary P/R <1 reveals heterotophy of the system, and an organic pollution at R from 1mg C/m^2/day.

Finally, IFREMER developed a classification scheme for French Mediterranean lagoons (Souchu *et al.*, 2000). The scheme is based on the identification of physical, chemical and biological potential indicators potential of eutrophication in the various compartments of the lagoon ecosystem (benthic, phytoplankton, macrophytes, macrofauna, sediments and water). It allows the classification of a lagoon into five eutrophication levels, formalized by five different colours: blue, signifying no eutrophication, to red, signifying high eutrophication. Results of the exercise allow immediate identification of the compartment in which degradation has developed. Table 17 presents a typical layout of results from such procedure. The final evaluation of the general ecosystem‘s state is equal to the one of the compartment with the lowest classification results. The diagnosis tool consists of five quality categories, defined using the same colour scheme from the Water Framework Directive (WFD), as in the legend of table 17. Boundaries for the various components were determined by taking of account the states of the lagoons obtained starting from the diagnosis of eutrophication (table 18). Further details on the classification procedures for each of the compartments included in te scheme can be found in Souchu *et al*. (2000).

3.7. Holistic indicators

Under this level are the holistic indicators such as resistence , resilience , buffer capacity, biodiversity, all forms of diversity, size and connectivity of the ecological network, turn over rate of carbon, nitrogen and energy.

The buffer capacity, defined as:

$$\beta = \frac{1}{\delta(\text{state variable}) / \delta(\text{forcing function})}$$

is another quantity that has been used when studying suitable indicator for inland and marine water ecosystems. The buffer capacity consists of a set of values, rather than one value, depending on which internal variable and forcing function we are using; it is also related to the parametric sensitivity of the ecosystem.

Table 17. General Results Table (Souchu *et al.*, 2000).

SEDIMENTS	
PHOSPHOROUS in the sediments	
PHYTOPLANKTON	
MACROPHYTES	
PROLIFERATIONS	
BIOLOGICAL POTENTIAL MACROBENTHOS	
GENERAL EUTROPHICATION STATUS	

Legend:
VERY MOD SUFF

Table 18. Selected variables for characterisation of eutrophication status.

Phytoplankton	Macrophytes	Macrobenthos	Sediment	Water column
Nr. Cells < 2 µm	Climax species biomass	Species richness	Organic matter	Dissolved oxygen
Nr. Cells > 2 µm	Species diversity	Population density	Total nitrogen	Turbidity
			Total phosphorous	SRP
				N-NO2
				N-NO3
				N-NH4
				Chlorophyll a
				Chlorophyll a/phaeo
				Total nitrogen
				Total phosphorous

3.8. Thermodynamic indicators

Level 8 indicators are thermodynamic variables able to enclose all ecosystem characteristics, e.g. exergy, specific exergy, emergy, entropy production, etc.

3.8.1. Exergy and Specific exergy

Exergy is defined as the amount of work a system can perform when it is brought to thermodynamic equilibrium with its environment or reference state, the latter defined as the primordial inorganic soup present on Earth some 4 billion years ago (Jørgensen, 1997). The exergy (*Ex*) of a system cannot be measured, but may be computed for each system component by multiplying its concentration, c_i, measured in terms of its average standing biomass, with its genetic information content, W_i, using conversion factors:

$$\frac{Ex}{RT} = \sum_{i=1}^{n} W_i \cdot c_i \qquad (1)$$

where R is the ideal gas constant, T the temperature.

Exergy has been presented as a goal function in ecosystems development, i.e. ecosystems evolve towards a state of higher exergy (Jørgensen, 1997). It also expresses the energy expended in the organization and construction of living organisms, by accounting for the genetic information accumulated within organisms (the higher the organization of an organism, the higher its exergy).

Related to the exergy concept, Jørgensen (1997) introduced the concept of specific (or structural) exergy, which is the exergy calculated relatively to the total biomass:

$$\frac{Ex_{st}}{RT} = \sum W_i \frac{c_i}{c_t} \qquad (2)$$

where c_t is the total biomass concentration, which is the sum of all c_i including inorganic matter available to growth of biomass. Specific exergy express the dominance of the higher organisms because, per unit biomass, they carry more information, that is, they have higher *W*-values. A very eutrophic ecosystem will have a very high value of exergy due to the large biomass, but the specific exergy will be low, as the biomass will be dominated by species with low W-values. The combination of the exergy and specific exergy indexes usually gives a more satisfactory description of the health of an ecosystem than the exergy index alone, because it consider diversity and life conditions for higher organisms.

The combination of exergy, specific exergy and buffer capacities has been used as an ecological indicator for lakes (Xu *et al.*, 1999) and coastal areas (Jørgensen, 2000). The results of these studies show that high exergy, high specific exergy and high buffer capacities are indicative of a healthy ecosystem. However, it seems that there are other parameters that could also be considered as indicators of ecosystem health, and which are not correlated with those values; hence, use of these three indicators only is probably not sufficient for complete characterization of an ecosystem. For example, Jørgensen (2000) shows that exergy or specific exergy are not correlated with diversity (defined in this case as the number of state variables of the model) or with complexity (defined as the product of the number of state variables times the connectivity in the model). Replacing activities by concentrations, under the assumption that there is no change in pressure and temperature between both systems, it is possible to calculate the exergy of the living system and that of the same system in the form of an inorganic soup without life, biological structure, information or organic molecules, as:

$$Ex = RT\sum_{i=0}^{n} c_i \ln\left(\frac{c_i}{c_i^0}\right) \tag{3}$$

where c_i is the concentration of the i-th component expressed in a suitable unit, c_i^0 is the concentration of the i-th component at thermodynamic equilibrium and n is the number of components ($i=0$ stands for inorganic compounds). To calculate the c_i^0, which is in general a very small number, one can use the probability P_i to find the i-th component at thermodynamic equilibrium (Jørgensen, 1997):

$$P_i = \frac{c_i^0}{\sum_{i=0}^{n} c_i^0} \tag{4}$$

As the inorganic component, $i=0$, will be the dominant one at equilibrium, we may assume that

$$P_i \approx \frac{c_i^0}{c_0^0} \tag{5}$$

For the case of detritus (dead organic matter, $i=1$) this can be found from thermodynamics:

$$\mu_1 = \mu_1^0 + RT\ln\left(\frac{c_1}{c_1^0}\right) \tag{6}$$

The difference between the chemical potentials $\mu_1 - \mu_1^0$ is a known quantity for detritus (mixture of carbohydrates, fats and proteins), and hence, we can write:

$$c_1^0 = c_1 \exp[-(\mu_1 - \mu_1^0)/RT] \tag{7}$$

Substituting Eq. (7) into Eq. (5), we get, for i=1:

$$P_1 \approx \frac{c_1}{c_0^0}\exp[-(\mu_1 - \mu_1^0)/RT] \tag{8}$$

For the biological components, Jørgensen *et al.* (1995) suggested an approximate procedure to calculate the exergy for organisms that overcomes the problem of defining reference states for different components under different conditions. Based on the assumption of a common reference state (detritus or dead organic matter), they defined the exergy in terms of the probability P_i as:

$$P_i = P_1 \cdot P_{i,a}; \quad \text{(for } i \geq 2\text{; 0 covers inorganic compounds and 1 detritus)} \tag{9}$$

where P_1 is the probability of i for producing organic matter (detritus) and $P_{i,a}$ is the probability to obtain the information embodied in the genes. Living organisms use 20 different amino acids and each gene determines the sequence of about 700 amino acids, and, hence

$$P_{i,a} = 20^{-700 \cdot g} \tag{10}$$

where g is the number of genes.

By combining Eq. (6) and (8) we can write:

$$Ex \approx RT \sum_{i=0}^{n} c_i \ln\left(\frac{c_i}{P_i \cdot c_0^0}\right) \tag{11}$$

Assuming that the inorganic component, i=0, may be omitted because negligible, and that: $P_i << c_i$, $P_i << P_0$, $1/P_i >> c_i$ and $1/P_i >> c_i/c_0^0$ (Jørgensen, 1997), we obtain:

$$Ex \approx -RT \sum_{i=1}^{n} c_i \ln(P_i) \tag{12}$$

Combining Eq. (12) with Eqs. (8) and (9) the following expression is obtained:

$$\frac{Ex}{RT} \approx (\mu_1 - \mu_1^0) \sum_{i=1}^{n} \frac{c_i}{RT} - \sum_{i=2}^{n} c_i \ln(P_{i,a}) \tag{13}$$

It has been found that for detritus the free energy released is about 18.7 kJ/g organic matter. Assuming an average molecular weight of 10^5 g/mol, T=300 K and R=8.314 J/(mol·K), we obtain:

$$\frac{Ex_1}{RT} \approx 7.5 \cdot 10^5 c_1 \tag{14}$$

where c_i is the detritus concentration expressed in g/l.

Assuming that a typical monocellular phytoplankton cell has 850 genes (Li and Grau, 1991), then:

$$-\ln(P_{phy,a}) = -\ln(20^{-700 \cdot 850}) = 1.78 \cdot 10^6 \tag{15}$$

and hence,

$$\frac{Ex_{phy}}{RT} \approx (7.5 \cdot 10^5 + 1.78 \cdot 10^6) c_{phy} \tag{16}$$

For pluricellular organisms it is necessary to include not only the number of genes, but the number of cells of the organism, i.e. $P_{i,a} = 20^{-700 \cdot g} / n_{cells}$. Assuming that zooplankton has 10^5 cells and 10000 genes (Cavalier-Smith, 1985; Levin, 1994) then:

$$-\ln(P_{zoo,a}) = -\ln\left(\frac{20^{-700 \cdot 10000}}{10^5}\right) = 2.097 \cdot 10^7 \tag{17}$$

and hence,

$$\frac{Ex_{zoo}}{RT} \approx (7.5 \cdot 10^5 + 2.097 \cdot 10^7) c_{zoo} \tag{18}$$

Then the exergy value may be found as the concentration of various components c_i multiplied by a weighting factor W_i, which reflects the exergy that the various ecosystem components possess due to their chemical energy and to the information embodied in their DNA:

$$\frac{Ex}{RT} = \sum_{i=1}^{n} W_i \cdot c_i \tag{19}$$

By dividing by $7.5 \cdot 10^5$ we can convert all values to g detritus exergy equivalents l^{-1}. In this case, for example the exergy of an ecosystem containing detritus (D), phytoplankton (P) and zooplankton (Z), could be expressed as: $Ex/RT=D+3.4 \cdot P+29 \cdot Z$.

As explained above, Jørgensen *et al*. (1995) proposed, for the estimation of the information content, W_i, to take into account the number of genes. Currently, data on the total number of genes for most organisms are not available, which makes this variable difficult to calculate. Consequently, the genetic information content must be estimated, and this introduces a source of uncertainty into the exergy calculations. Often the values listed did not include a taxon being considered in this ecosystem, resulting in additional uncertainty in their estimation. To overcome such uncertainty, Fonseca *et al*. (2000) have suggested a more operational approach by using the haploid nuclear DNA contents of organisms (C-values) to use nuclear DNA contents of organisms. The nuclear DNA content replaces the term '$700 \cdot g$' in the evaluation of W_i in the exergy calculations. In this work, genetic information content has been estimated using values from Jørgensen (2000) with the exception of shellfish for which we have used the method from Fonseca *et al*. (2000). Estimates of nuclear DNA contents in picograms (pg) were taken from the report by Fonseca *et al*. (2000), which correspond to a certain number of base-pairs (bp) in the DNA (1pg = $0.98 \cdot 10^9$ bp). The term C^* denotes the conversion to base-pairs. C^* is divided by 2 to give the number of nucleotides in one polynucleotide chain (one DNA strand), represented by C^{**}. Under the assumption that for each adjacent triplet of nucleotides in non-repetitive DNA corresponds a transcribed RNA-signal, this number is then divided again by 3 to give the number of nucleotide triplets (maximum coding capacity), represented by C^{***}. C^{***} is the value, which replaces the term '$700*g$' in Eq. (10), becoming: $\ln 20^{C^{***}}$. Table 19 indicates the values used for the calculation of the genetic information content, in brackets there are some of the values provided by Fonseca *et al.* (2000).

Table 15. Example of parameters used to evaluate the genetic information content, from Jørgensen (2000).

Ecosystem component	Number of information genes	Conversion factor (W_i)
Detritus	0	1
Bacteria	600	2.7 (2)
Flagellates	850	3.4 (25)
Diatoms	850	3.4
Micro-zooplankton	10000	29.0
Meso-zooplankton	15000	43.0
Ulva sp.	2000 [(1)]	6.6
Seagrass	10000[(1)]	29.0
Shellfish (Bivalves)	-	287[(2-3)]

Coffaro *et al*. (1997), [(2)]Marques *et al*., (1997), [(3)]Fonseca *et al*, (2000)

The method used by Fonseca *et al*. (2000) is considered to be a more accurate estimate, as it accounts for inconsistencies in the genome size-structural complexity, central hypothesis of the concept of ecosystem exergy. However, the two methodologies seem to give very similar results in the exergy calculations.

Concerning the detection of thresholds, it is easy to see that exergy and specific exergy will give valuable information. For example, considering the case of ecosystem shift from benthic to planktonic as in S3-WP2, in which nutrient load favors the growth of fast primary producers that reduces the light availability for benthic vegetation, it is possible to see that such a change will produce a reduction of specific exergy, as seagrasses (e.g. *Zostera noltii*) have an information content (W_i) of about 29 whereas *Ulva* sp. Has a value of about 6.6. Furthermore, specific exergy has proved to be able to detect changes from normal conditions to hypoxia in coastal lagoons (Zaldivar *et al.*, 2005) subject to shellfish farming and macroalgae blooms.

4. CONCLUSIONS

Template for initiation of the process of selection of indicators and for summarizing the information on selected indicators

Suggested indicator name	**Pressure(s)**	**Hierarchy**	**Key ecosystem characteristics**	**Indicator Purposes**	**References[9]**

		Organi	Species	Populat	Ecosyst	Landsc	Composition[6]	Structure[7]	Function[8]	Early warning	condition	Trend	Diagnose	
'average taxonomic distinctness'	Nutrient loading													Mouillot *et al.* 2005
'variation in taxonomic distinctness'	Nutrient loading													
EROD CYP1A	Organic chemical								X	X				Van de oost *et al*, 2003
DNA adduct	PAH								X	X				Van de oost *et al*, 2003
PAH metabolites	PAH								X	X				Van de oost *et al*, 2003
AchE	pesticides								X	X				Beliaeff, Bocquene (2004).
Metallothioneins (MTs)	metals								X	X				Van de oost *et al*, 2003

[1] Examples of indicators for this component are physical lesions and deformations, and parasite load.
[2] Examples of indicators for this component are range size, presence and frequency of occurrence.
[3] Examples of indicators for this component are number of populations, age or size structure and dispersal behaviour.
[4] Examples of indicators for this component are species richness, species evenness number of trophic levels.
[5] Examples of indicators for this component are spatial distribution of communities and persistence of habitats.
[6] Examples of indicators of ecosystem composition integrity are presence, abundance, frequency, cover, biomass, richness, evenness, diversity, presence and proportion of indicator species.
[7] Examples of indicators of ecosystem structure integrity are dispersal, range, population structure morphological variability, substrate and soil conditions, slope, aspect, living and dead biomass, patch size, patch and distribution.
[8] Examples of indicators of ecosystem function are demography, population changes, life history, acclimatisation, biomass, decomposition, herbivory, parasitism, predation and rates of nutrient cycling.
[9] Note that this table is only for the purposes of initiating the process of selection of indicators and summarising that information. However, each indicator purposed should be accompanied by more detailed information on how it is calculated, how it relates to anthropogenic pressure, ecosystem where it has applied (e.g. coastal lagoons), and its envisaged applicability in the Thresholds IP. It should also include references to the available literature.

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