EP

United Nations
Environment
Programme

UNEP(DEC)/MED/WG.264/Inf.14
11 May 2005

ENGLISH

MEDITERRANEAN ACTION PLAN

Meeting of the MED POL National Coordinators

Barcelona, Spain, 24 - 27 May 2005

FACT SHEETS ON MARINE POLLUTION INDICATORS

UNEP
Athens, 2005

BIOLOGICAL INDICATORS

MED POL

Introduction

The management of coastal ecosystems requires evaluation of the ecological quality and investigation at the biological community level seems to be appropriate for describing long-term trends in anthropogenic stress. Benthic communities (phytobenthos, zoobenthos) have been used for almost a century as indicators of environmental health and proved to be a useful element in order to describe the ecological status of a given geographical area. These communities which are rich in species that are predominantly stationary and relatively long-lived mirror quite accurately the degree of disturbance and thus are frequently used in EIA studies. A large number of concepts and numerical techniques have been developed for the proper interpretation of data.

The Contracting Parties to Barcelona Convention at their 12th Meeting held in Monaco in November 2001 requested the MED POL Programme "To review and develop a set of marine pollution indicators, in cooperation with Blue Plan, EEA, UNIDO-ICS and other competent bodies and organizations". (UNEP/MAP, 2003a?reference)

Based on Guidelines for the development of Ecological Status and Stress Reduction Indicators (UNEP/MAP 2003b) and relevant Workshop the proposed core set of indicators to assess the state of ecosystem Stress includes the following:

1.Number of exotic species (all taxa) *2.Number of zoobenthic species*	State/Impact
3.Presence/abundance of sensitive/opportunistic zoobenthic species/taxa ***4.Community diversity** (zoobenthos/phytobenthos)*	State State
5.Presence and coverage of benthic macrophytes (sensitive/opportunistic)	State
Biotic index *6a. Ecological evaluation index based on macrophytes (EEI))* *6b. Ecological quality index based on zoobenthos (BENTIX)*	State

The proposed ROAD MAP of UNEP/MAP at short term (2004-2006) includes

- Developing methodology sheets for the set in line with existing sheets developed by related organizations

Case study examples on the use of marine macrophytes and softbottom macroinvertebrates were obtained from a number of countries, the latter particularly in relation to pollution from discharges of sewage effluent, fisheries, dumping and from oil pollution (oil spills). These examples are used in this document to formulate and test potential indicators on the biological quality of Mediterranean coastal waters that would be compatible with the requirements of the Blue Plan, EEA, UNIDO-ICS and other competent bodies and organizations Demonstration indicator factsheets have been produced.

- undertaking a test procedure in a few Mediterranean countries

Zoobenthic indicators were tested with data sets derived from:

a) various geographic areas within the Mediterranean (**Turkey**: Edremit Bay, Izmir bay, Iskenderun Bay), **Syria** (Banias area), **Hellas** many sites but mainly Saronikos, S. Evvoikos, **Italy** (port Augusta), **Spain** (Portman Bay), **Egypt**, **Algeria, Maroco**
b) coastal areas affected by different anthropogenic activities namely **fishing** (S. Evvoikos, Edremit, Izmir); **tourism** (Attiki, Izmir); **sewage** (Saronikos, river (Iskenderun, Banias), **heavy metal pollution** (Portman), **shipping** (Algeria, Izmir)
c) using different methodology (sampler, mesh size, no of replicates, taxonomic effort).

In all cases results are compared with those produced by using the community diversity (H) scale. Zenetos & Simboura, 2001.

A first step towards the interpretation of the impact of human pressures on the benthic communities would be to recognise the different benthic communities in the Mediterranean Sea. Simboura and Zenetos (2002) have revised the main soft bottom community types encountered in the Mediterranean, by adjusting the classical bionomic scheme of biocoenoses described by Peres and Picard (1964) to European typology (see table 1) that is considering both the main environmental (depth, type of substratum) and biotic factors (i.e.phytal cover). Thus, the term "community types" which includes the environmental aspect, is used in a broader sense very similar to that of "habitat".

Table 1: Studied Benthic habitat and community types (biocoenoses) in Mediterranean waters.
Abbreviations used: VTC=Coastal Terrigenous Muds. SFBC= Fine Well-Sorted Sands; SFHN=Upper Fine Sands; SGCF=Coarse Sands and Fine Gravels under the influence of Bottom Currents; SVMC=Calm Water Muddy Sands; AP=Photophilous Algae; DC=Coastal Detritus Bottoms; DE=Muddy Detritus Bottoms

Type of habitat	Peres & Picard, 1964	Description
Sands and muddy sands	DE	Mixed sediment (shallow 30m or deeper 30-100m) muddy detritus bottoms
Muddy sands with phytal cover	AP	In or close to phytal meadows of macroalgae or angiosperms (*Zostera, Posidonia, Caulerpa)*
Shallow muddy sands	SVMC	Muddy sands in protected areas
Shallow muds	VTC	Shallow (sublittoral) muds Sub-community of muddy bottoms with *Amphiura filiformis*
Shallow sands	SFBC, SFHN	Shallow sands (well sorted or very shallow sands)
Deeper coarse sands	SGCF	Coarse sands in high energy environments
Deeper Sands with detritus	DC	Deeper sands with biogenic fragments or Coastal detritic bottoms
	DL	deep circalittoral bottoms or open sea detritus bottom

After consideration of the current status of the EUNIS classification and of habitat mapping proposals with WGMHM, the Bentos Ecology Working Group) (ICES CM 2001) recommended that
.."*The strong temporal variation (related to, for example, changes in climate or human impact) of some benthic habitats and hence benthic communities needs to be considered*

when elaborating the lower levels of the EUNIS habitat classification. Such temporal variation, together with the limitations of performing large-scale mapping studies, suggests that the classification units should not be too specific.

1.Number of exotic species (all taxa) *2.Number of zoobenthic species*	State/Impact
3.Presence/abundance of sensitive/opportunistic zoobenthic species/taxa	State
***4.Community diversity** (zoobenthos/phytobenthos)*	State
5.Presence and coverage of benthic macrophytes (sensitive/opportunistic)	State
Biotic index *6a. Ecological evaluation index based on macrophytes (EEI))* *6b. Ecological quality index based on zoobenthos (BENTIX)*	State

Conclusions/recommendations

Marine macrophytes and macroinvertebrates are widely used for the assessment of transitional and coastal water quality status by countries within the Mediterranean.
However, assessment of ecological status is not unanimous at Mediterranean scale; that is, up-to-date assessments are arbitrary and not derived using common tools.

As expected (different funds assigned to research/monitoring.EIA studies combined with different expertise) the case studies indicate that a directly comparable assessment of the biological quality of coastal water between countries is not yet possible.

Countries should agree into common criteria or metrics for producing assessments that are comparable within countries It is essential to initiate the process to arrive into common criteria for the interpretation of the normative definitions of the high/good and the good/moderate class boundaries. This will not be achievable until countries have developed classification schemes compatible with the requirements of Blue Plan, EEA, UNIDO-ICS or WFD.

It is therefore recommended that data flows be developed between countries and the UNEP/MAP- so that advanced indicators can be developed in line with work at European level. These indicators could then be progressively developed and modified as the information required to achieve better comparability becomes available during the progressive implementation of the WFD.

In accordance with EEA (2004) it is recommended that the following data are collected for as long a time series as possible:

- ✓ *Annually averaged numbers and abundance (coverage or biomass) of marine macrophyte taxa and/or functional groups, collected from each [representative selection of] transitional and coastal waters monitoring station with some measure of sample variability;*

- ✓ *Annually averaged value of the aggregated macrophyte index (diversity or biotic) derived from, for example, number and abundance of taxa and/or functional groups, with some estimate of the statistical error associated with the value;*

- ✓ *Annually averaged numbers of marine macroinvertebrate taxa, abundance collected from each [representative selection of] transitional and coastal waters monitoring station with some measure of sample variability;*

- ✓ *Annually averaged value of the aggregated macroinvertebrate index (diversity or BENTIX) derived from, for example, number of taxa, abundance and taxa sensitivity data, with some estimate of the statistical error associated with the value;*

- ✓ *Disaggregated data on marine macrophytes in terms of measurement of depth limitation and/or density of roots/coverage (as appropriate) from each [representative selection of] transitional and coastal waters monitoring stations with some measure of sample variability;*

- ✓ *The nationally (perhaps periodically) reported classification results (if a classification exists) for all stations assessing marine macroinvertebrates and macrophytes. Ideally this would be in terms of lengths of transitional waters or coastal waters (water bodies) within each class but in many cases would be numbers of stations within each class.*

- ✓ *Disaggregated annual data on aquaculture related introduced species (aliens, deliberate and associated) from transitional and coastal waters monitoring stations It is recommended that the datasets are formulated into indicators in terms of:*

- ✓ *Time series of annual average number of taxa, derived indices, aggregated at a country level and at the transitional and coastal waters type level within a country.*

- ✓ *Proportion of monitoring stations within each country at which there are significant increases, decreases and no changes in quality over time in terms of numbers of taxa, and/or functional groups, derived indices, depth limitation and density of shoots etc. This will require information on the variability of the metrics.*

- ✓ *Summaries of the classifications at national level.*

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Marine Pollution Indicator fact sheet

MED POL

Ecological evaluation index based on zoobenthos (BENTIX)

Key messages

☺ Ecological evaluation of benthic ecosystems across the Mediterranean is feasible by using a simple tool (BENTIX), which is not community type specific or site specific (global application)

😐 BENTIX appears to work well to different stress (sewage, fishing, dumping) but is best applicable to sewage effluent in coastal waters.

☺ Best assessment of EcQS is achieved by combination of BENTIX with H (community diversity) and S (number of species)

Table 1. Classification of EcoQ according to range of the Biotic Index

Pollution Classification	**Bentix**	**Ecological Quality Status (EQS)**	**Physically stressed muds**
Normal/Pristine	4,5 ≤ nBentix < 6	High	4.0 ≤ nBentix
Slightly polluted, transitional	3,5 ≤ nBentix < 4,5	Good	3≤ nBentix < 4
Moderately polluted	2,5 ≤ nBentix < 3,5	Moderate	2,5 ≤nBentix <3
Heavily polluted Azoic	2 ≤ nBentix < 2,5 Azoic	Poor Bad	

Source: modified from Simboura and Zenetos, 2002

Results and assessment

Policy relevance:

The Water Framework Directive (WFD) states that the most important task is the protection of Europe's waters (inland, transitional and coastal waters). Protection of water related ecosystems have high priority in the Directive.

The Green Paper on Common Fisheries Policy (CFP) (Ecosystem approach) resulted in a reform of the CFP so as to ensure sustainable fisheries and healthy marine ecosystems, both in the EU and globally. The impact of trawling on the benthic ecosystems has been recognized and habitat changes caused by fishing are to be taken into account as a tool to measure the effectiveness of policies.
According to the Biodiversity Convention important aims are to restore habitats and natural systems and halt the loss of biodiversity by 2010.

Soft bottom fauna is one of the key groups that are currently used globally for the evaluation and the assessment of the Ecological Status and a key element in the WFD towards its implementation in the transitional and coastal waters of Europe.

Policy context. The WFD requires that all water bodies of each Member State should have at least good quality status by 2015. This implies that 1) all countries should have assessed the EQS of their waters by 2009 and 2) measures should be implemented in order to achieve the environmental objective of good ecological status that is bad, poor and moderate EcoQ statuses should be upgraded to good EcoQ by that time.

In May 2001, a ‘‘common implementation strategy’’ (CIS) was agreed to assist with the implementation of the WFD, with the COAST working group dealing 45with coastal waters. To establish the ecological quality of soft-bottom benthos and to classify coastal water bodies, this group has proposed two marine biotic indices, the AMBI (Borja et al., 2000) and the BENTIX (Simboura and Zenetos, 2002), which are based on the sensitivity/tolerance of benthic fauna to stress gradients mainly caused by organic enrichment pollution, declaring that methods combining composition, abundance and sensitivity are the most promising (Vincent et al., 2002).

In addition to technical measures in the fishery and aquaculture sectors so as to ensure good quality status, sewage treatment plants are among the measures focusing on the improvement of ecological quality of coastal waters in urban areas. Tourism activities (coastal constructions) and shipping are among those anthropogenic pressures considered to pose major threats to coastal biodiversity in the Mediterranean (EEA 1999) and relevant policies (i.e. measures towards prevention of oil spills,) have to be adjusted so as to achieved good quality status, of the on the coastal waters.

Environmental context: (scientific soundness and choice and definition of the indicator)
The impact of pollution on the zoobenthic communities can be summarised as favouring tolerant opportunistic species and displacing sensitive ones thus shifting the balance of the community structure towards a less even and less biologically diverse system. By combining this accumulated information of tolerant and sensitive species, several biological indices have been developed over the last years. A major feature, compared to standard diversity indices, is that biological indices encompass more ecological information than diversity indices. This is because biological indices take into consideration the identity of each separate species included in the sample, not merely the number of individuals per species. There has been a general need for simple new tools for ecological classification, and the development of new biological indicators over the last years has been stimulated in particular through the introduction of the Water Framework Directive.
Classification systems aiming to measure EcoQ status and corresponding trends should be subjected to intercalibration excersise in order to have a common basis of comparison among different countries situated n the same regional Sea. In that sense old and new data sets are valuable in order to test and intercalibrate developing tools and also to highlight trends of improvement of EcoQ status under the impact of operating measures or stress the need of management planning in problematic areas
Assessment

The various tools used as indicators are often adapted to regional requirements and biological particularities. The BENTIX index discriminating among only two ecological groups, is probably more appropriate and convenient for Mediterranean ecosystems with high species richness and diversity. The results obtained are consistent with those obtained using several widely applied methods and parameters, such as species richness and community diversity.

On the other hand the Biotic Coefficient (BC) (Borja et al, 2003) which also provides a simple and clearly defined way to establish the ecological quality of soft-bottom benthos, is descriptive of the ecological quality of a benthic fauna with comparably few species and high densities. (Simboura, 2004)
Temporal trends of BENTIX show an improvement of the EcoQS over the years in areas of operational sewage treatment plants (primary treatment) such as Saronikos gulf (Figure 1).

Classification by using BENTIX appears to be independent of the natural variability (seasonal variations) and of the sample size and methodology (mesh sieve) and as such is advantageous as it reduces the source of uncertainty in the classification. However, BENTIX is effective provided that a) sufficient sampling is involved (unsuccessful with scuba diving and dredges) and b) more than 70-80% is identified to species level and

The indicator species lists presented for the BENTIX is incomplete since a very small number of Crustacea species were included, and it is known that this taxon is generally more sensitive to environmental contaminants and other anthropogenic disturbances than most other components of the infauna, particularly polychaetes. When species lists produced for AMBI and BENTIX are compared, it can be seen that some species are classified into different ecological groups; for example, *Perinereis cultrifera* is classified by the AMBI as species tolerant to excess organic matter enrichment (EGIII), whereas in the case of the BENTIX it is classified as sensitive to disturbance in general (EG1).

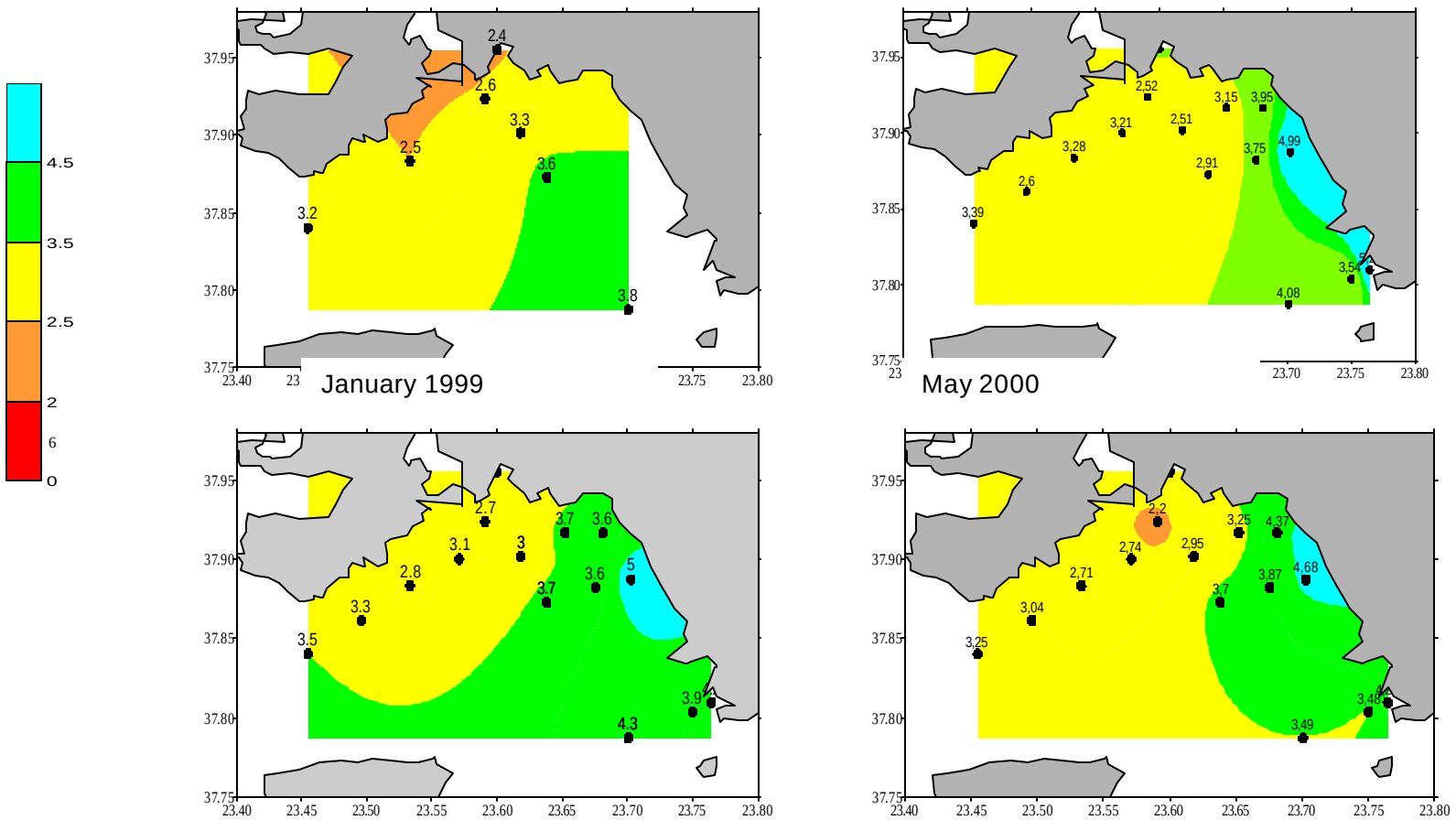

Figure 1: Trends of Ecological quality as defined by the Bentix index in Saronikos gulf benthic communities over the period 1999-2002.
Source: Simboura in EEA 2004

Notes: Saronikos Gulf receiving the sewage effluents of the Metropolitan city of Athens is being monitored since the starting of the Primary Treatment Plant in 1994 in Psittalia. The benthic communities ecological quality status is presented in a sequence of plots following the years 1999 to 2002. As shown in the coloured graphs (colours correspond with the colours of the WFD) the ecological quality is improving with the distance from the sewage outfall. Regarding temporal trends, the "poor" ecological quality zone is recessing from 1999 to the year 2000, replaced by the "moderate" one and the "good" ecological quality class is gradually expanding, taking the place of the "moderate" zone. A reference "high" quality status zone is limited in the more coastal areas not affected by the sewage outfalls. It seems that the overall trend of the EcoQ status is that of improvement throughout the years of active operation of the power plant.

Case studies according to main stress

In the examples that follow the EQS as derived by bentix is always compared with that provided by community diversity H and their compatibility is briefly discussed.

Izmir Bay: Main stressors shipping, urban and industry effluent + agricultural waste.

Table 2: Abiotic and biotic parameters (Indices) along a pollution gradient in Izmir Bay (Turkish Aegean Sea)

station	stress	Depth	substratum	S/0.1m2	BENTIX	H
1	port	10	sand	3	**2,20**	0.80
2	urban+industry	18	sand	3	**2,01**	0.60
3	urban+industry	15	muddy sand	3	**2,06**	0.47
4	urban+industry	12	sand	4	**2,17**	0.81
5	urban+industry	13	muddy sand	13	**2,42**	2.23
6	influence by inner bay	21	muddy sand	33	**2,72**	4.06
7	influence by inner bay	27	mud	22	**2,73**	3.71
8	influence by inner bay	28	sandy mud	19	**2,65**	3.12
9	influence by inner bay	32	muddy sand	21	**3,24**	3.75
10	influence by inner bay	35	muddy sand	24	**3,82**	4.01
11	urban	27	muddy sand	42	**3,29**	4.59
12	ex dumping site	50	mud	28	**3,82**	4.28
13	river	66	mud	20	**3,75**	3.86
14	reference	15	mud	66	**4,35**	5.37
15	ex trawling	16	mud	35	**4,89**	4.60

Data source: A. Dogan (Ege University, Izmir)

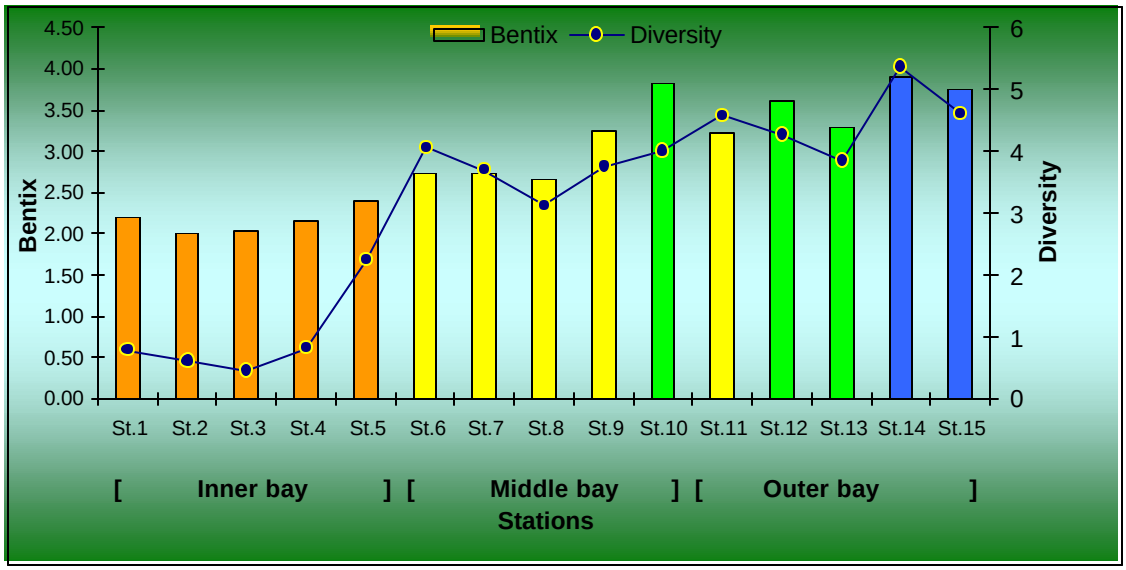

Figure 2. Mean values (over the year) of BENTIX and H along a pollution gradient in Izmir Bay. Colours correspond to EQS classes as defined by BENTIX in accordance with WFD source: Dogan, 2004

Assessment.

Mean values of the BENTIX and H index are 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 and in its turn affects the middle Bay. This gradient is also evident in the chemical parameters of the water column. Based on the faunistic and hydrographical features KOCATAS (1978; 1980) divided Izmir Bay into 3 parts; the inner, middle and outer parts (Fig 2).

Assessment by the two main indicators BENTIX and H coincides in most cases 9 out of 15 (60%). In the innermost sites the actual ecological status could be described as very polluted towards azoic since the area becomes azoic seasonally (no life present in st 1to st 4 during the autumn and summer sampling).

Edremit Bay: Edremit Bay is one of the most important fishery regions of the Aegean Sea. This region supports dense populations of many demersal fishes and is also suitable for trawling (Kocatas & Bilecik, 1992).

Table 3: Abiotic and biotic parameters (Indices) along a pollution gradient in Edremit Bay (Turkish N. Aegean Sea)

	stress	Depth	substratum	S	EQS(nBentix)	EQS H
A5	fishing	30	muddy sand	17	MODER	3.83
C4	fishing	20	sandy mud	28	MODER	4.36
C5	fishing	30	muddy sand	20	GOOD	4.03
D5	fishing	30	muddy sand	9	GOOD	2.75
D4	Reference site	20	sandy mud	13	HIGH	3.5

Data source: S.Albayrak H. Balkis (Istanbul Univ.) and M.E. Çinar (Ege University, Izmir) (Albayrak et al, in press)

Assessment: The According to BENTIX, the EQS in fishing fields of Erdemit Bay appears to be moderate to high. The picture is very different however when community diversity is considered. The assessments of the two indices coincided in two sites but differed in 3 cases. (60%). In all contradicting assessments it is believed that BENTIX "behaved" best i.e. H was insensitive to trawling activities in C4 and oversensitive in D5 -classified it as of poor EQS- and the reference site D4 which it classified as moderate. However, considering the species richness (S) it is believed that the actual status could be somewhere in the middle. There might be a disturbance factor in the area of D4, there might be over fishing or additional pollution source in D5 or C4.

Augusta (Italy): Impacted from a) shipping (StA2: Porto Megarese) and industrial effluent in the coastal areas (mainly chemical and petrochemical)

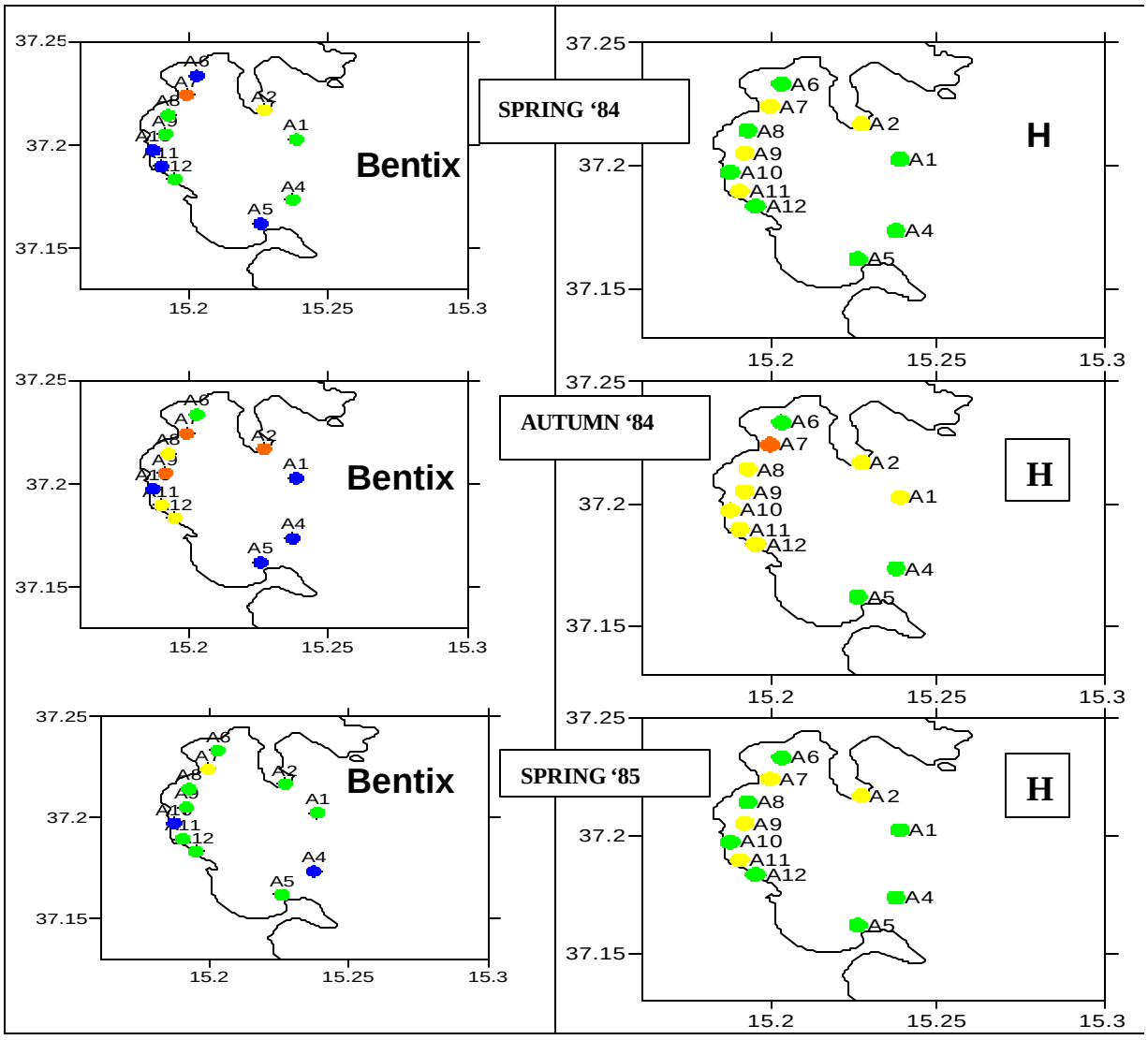

Figure 3: Areal distribution of EQS based on BENTIX and H in port Augusta
Source; *Zenetos et al., 2004 based on data from* Di Geronimo 1990

Assessment Qualitative and quantitative studies based on algae, polychaetes and mollusks have confirmed a degradation of the ecosystem between 1983 and 1985 (Di Geronimo, 1990). In a series of maps showing temporal trends in ecological status as

derived by BENTIX vs H (based on polychaetes+mollusks), mean values of H show no change in the ecological status from 1984 to 1985, whereas BENTIX revealed a degradation of the shallower coastal sites (closer to LBS) and an improvement of the deeper station (A4).

Portman Bay (Spain): main stressor dumping coarse metalliferous waste. A gradient of stations with increasing distance from the discharge point towards a protected area.

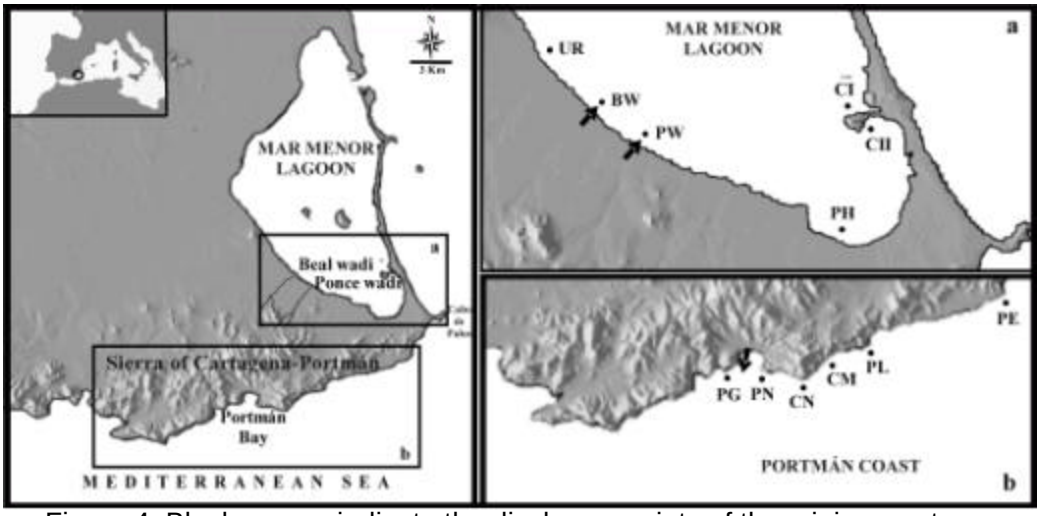

Figure 4: Black arrows indicate the discharge points of the mining wastes

Table 4: Ecological parameters of zoobenthic communities in Portman Bay

	stress	S	H	J	BENTIX
PG	dumping site	7 ± 1	0.80 ± 0.22	0.39 ± 0.08	5.27 ± 0.85
PN		15 ± 3	2.04 ± 0.27	0.72 ± 0.07	3.21 ± 0.71
CN		8 ± 2	1.95 ± 0.15	0.88 ± 0.03	5.38 ± 0.48
CM		18 ± 2	2.12 ± 0.23	0.72 ± 0.07	3.21 ± 0.50
PL		19 ± 4	2.43 ± 0.23	0.82 ± 0.02	3.74 ± 0.27
PE	reference	26 ± 2	2.93 ± 0.14	0.89 ± 0.03	4.21 ± 0.35

Source: Marín-Guirao et al, in press

Assessment

The assessments derived by the bentix and H did not match at all. (100% mismatch). As it appears none of the two indices was efficient in producing interpretable result but the simple parameter number of species.

According to Lazzaro Marin (pers. Commun.) 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. The BENTIX classification obtained for Portman was not correlated with the classification obtained with the toxicity

tests, and only the sediment copper concentration was negatively correlated with the BENTIX results ($p < 0.05$, $r = \_0.30$). (Marín-Guirao *et al*, in press).

It is true that the first list of scores was incomplete but the authors (Simboura and Zenetos) are currently in the progress of updating the list, which is soon to be released in the Internet. One drawback in the effectiveness of using BENTIX could lie in the collection of data. Scuba diving is not the most

Attiki: Internal Tourism, Petalioi: fishing

Touristic development of the coastal zone of E-SE Attiki is associated with the disturbance of the coastal environment. Several summer resorts have recently appeared in the periphery of Attiki, which have replaced the old small villages. Some of these resorts are increasing considerably their population, reaching that of a small town during the summer months, while are inhabited by just a few hundred people in the rest of the year. Consequently disturbance in this area is caused primarily by the organic pollution (wastes of coastal villages, ports etc). The offshore areas of E. Attiki (Petalioi Gulf) are important fishing grounds for bottom trawlers.

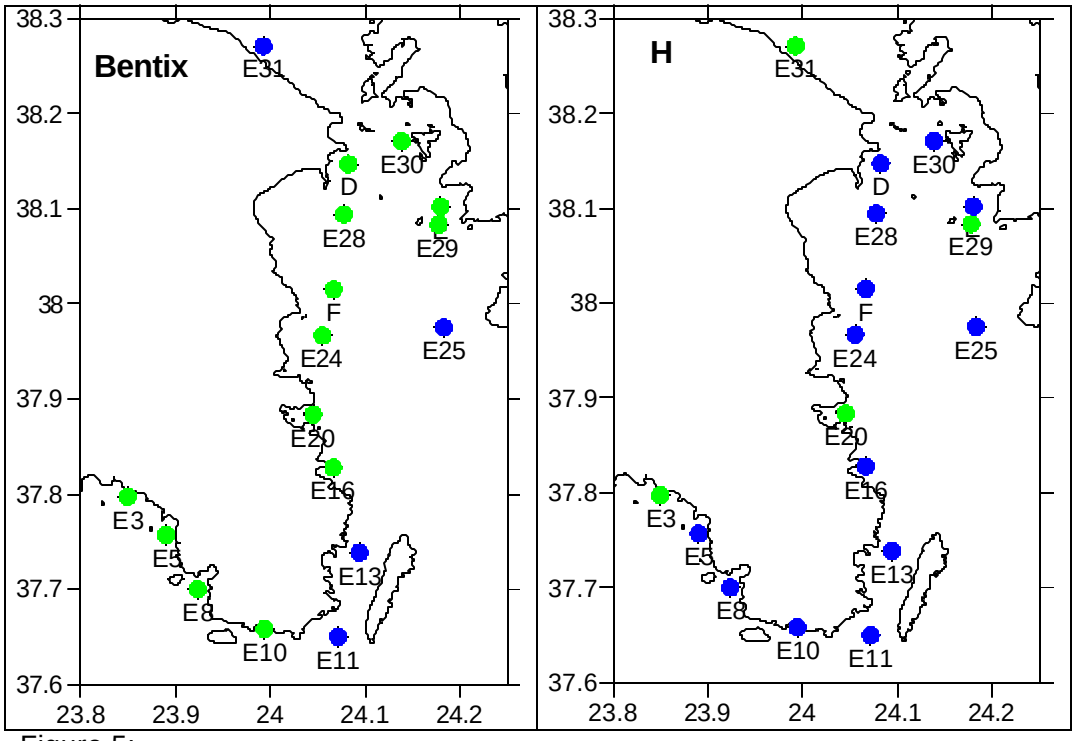

Figure 5:
Source: Zenetos et al, 2004.

Assessment: The ecological quality status of the E, SW coasts of Attiki appears to be good to high. Bentix “”behaves” best in the classification of coasts which are subjected to at least temporary effluent from the establishment of summer resorts. The impact of these temporal LBS are not reflected in the community diversity albeit in cases of intense urbanisation (st E3 :Agia Marina, E20: Porto Rafti).

“Deterioration of water quality, expressed as grade of microbial contamination, measured in the coastal area were characterized by low ecological indices. Thus, the seasonal growth of human pressure appeared to be an important factor for the degradation of the water quality

and also for the decrease of biodiversity in the coastal area of Attiki." (Reizopoulou & Zenetos in press).

Correlation of benthic community indices with number of inhabitants during summer months showed that diversity and evenness decreases with the increase of population of the coastal areas.

Meta data

Dissemination themes: UNEP, EEA;
DPSIR (one value only): **S**
Technical information

Description of data

Definition:

This is a Biotic Coeficient designed (Simboura and Zenetos, 2002) to classify benthic communities to an Ecological Quality Status (EQS) according to the WFD requirements (EEC, 2000). It is based on the initial idea of Glemarec & Hily (1981) modified by Borja et al. (2000) and further modified to become more simple in its use and suit the Mediterranean benthic diversity.

Analyses
The zoobenthic species are classified into two ecological groups and assigned a score according to their degree of sensitivity or tolerance towards pollution. The two ecological groups are:
Group (GS). Species with score GS are sensitive to disturbance in general. Species indifferent to disturbance, always present in low densities with non-significant variations with time are also included in this group. This group corresponds to the k-strategy species, with relatively long life, slow growth and high biomass (Gray, 1979).

Group (GT). This group 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). This group includes opportunistic species (strongly unbalanced situations), pioneers, colonisers, species tolerant to hypoxia, or late successional colonisers (r-strategy: species with short life span, fast growth, early sexual maturation and larvae throughout the year).

Following many calculations, validation and testing with data from Greek ecosystems as well as Mediterranean, an algorithm is developed giving different weight to the presence/abundance of each group:

$$nBentix = \{ 6 \times \%GS) + 2 \times (\% GT\}/100$$

The bentix takes continuous values from 2 to 6, and equals 0 when the area/community is azoic. A classification system appears as a function of Bentix including five levels of ecological quality status (EQS).

Geographical coverage: Izmir Bay, Edremit Bay (Turkey), Augusta port (Italy); Portan Bay, (Spain), Saronikos Gulf, Petalioi Gulf and Attiki coasts (Greece)
Methodology and frequency of data collection
Izmir Bay: data from 15 stations along a pollution gradient from the inner gulf (st1) to outer gulf (st15). Seasonal data (4 sampling cruises) during the period 1995-96. Van Veen grab 0.1m2, Mesh sieve 0.5 mm; **236 species + 63 taxa**, 6165 individuals
Edremit Bay: Data from 5 stations (*3 replicates) collected in October 2002. Van Veen grab 0.1m2, Mesh sieve 0.5 mm
Augusta port (Italy). Seasonal sampling spring '84, autumn; 84, spring 85. Qualitative data including only the groups of polychaetes & mollusks. **89 species** in all
Portan Bay, (Spain). Six stations at depths 10-15m from the mine outlet in Portma´n Bay in the direction of the Marine Reserve of Cabo de Palos-Islas Hormigas. Four replicate

sediment samples were collected in March 2002 by SCUBA divers using a 0.09m2 hand grab and sieved through a 0.5mm mesh bag. **83 species** in all.
Attiki: Internal Tourism + S. Evvoikos: fishing. Data from 17 stations; Sampling in summer1996, Van veen grab: 0.1 m2; Mesh sieve 1mm; **>500 species**; 6.454 individuals
Quality information

Strength and weakness (at data level):
Weakness
a) when >20% of data are not identified to species level
b) in very poor ecosystems with few species
c) when dredge sampling is involved
Reliability, accuracy, robustness, uncertainty (at data level): 2
Overall scoring (give 1 to 3 points: 1=no major problems, 3=major reservations):
Relevancy: 1<see Description of elements for definitions>1
Accuracy: 2
Comparability over time: 1
Comparability over space: 1

Further work required
The development of this type of environmental tool requires the consensus of scientists in the assignation of species to a particular ecological group.

The index based on literature and authors own experience is subjective. Further evaluation is needed with data sets from various Mediterranean areas and validation of results always combined with other metrics and chemical parameters if possible.

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Marine Pollution Indicator fact sheet

MED POL

Use of biotic indices on benthic macrophytes data for ecological evaluation of coastal marine environments in the Mediterranean.

Key Message
☺ Benthic macrophytes are used for ecological quality assessments by most EEA countries.
☹ At present very few Med countries (Spain, Greece) have classification schemes compatible with the WFD.

Macrophytes are generally sensitive to water quality –particularly to turbidity, eutrophication, some chemical residues, but also trawling fisheries and exotic species competition. Thus, several marine macrophytic species have been broadly used as successful phytobenthic indicators, to indicate shifts in the aquatic ecosystem from the pristine state to the degraded state.

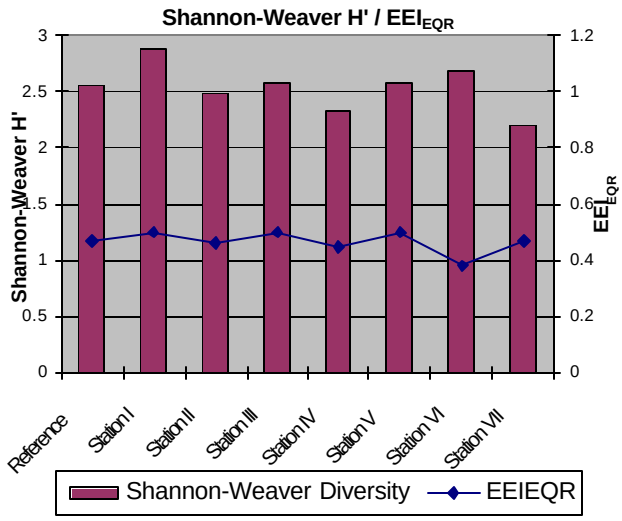
Table 1. *Review of impact of human stress on marine macrophytobenthos (adapted from Orfanidis et al., 2001).*

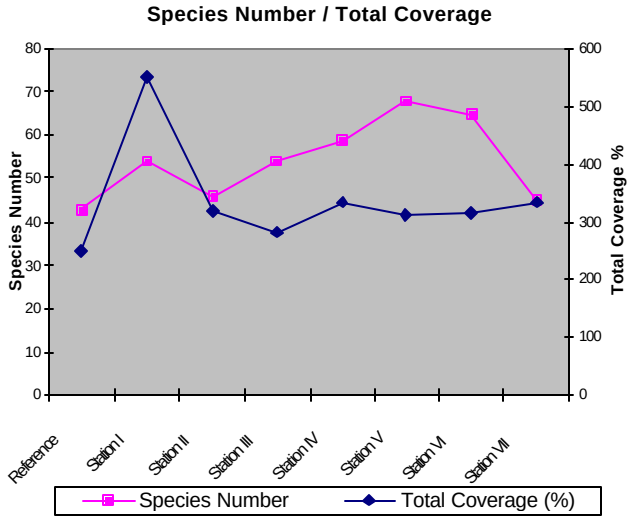
Anthropogenic stress	***Benthic macrophytes***	***Impact***
Eutrophication,	Seaweeds	Dominance of opportunistic species, seaweed blooms, decline of diversity
	Seagrasses	Large scale and regional decline of meadows, dominance of fleshy seaweeds
Organic matter, Siltation	Seaweeds	Light reduction and alteration of hard substrate affects community structure
	Seagrasses	Decline of meadows through reduction of light and accumulation of organic matter in sediment
Heavy metals	Seaweeds	Inhibition of reproduction and development, changes in community structure
	Seagrasses	No direct effect has been observed
Oil spills	Seaweeds	Short term growth reduction in intertidal species
	Seagrasses	No direct effect has been documented
Global warming	Seaweeds	Changes in distribution patterns are expected
	Seagrasses	Changes in distribution patterns are expected
Increase of salinity	Seaweeds	Further expansion in estuarine ecosystems
	Seagrasses	Species displacement, e.g. *Cymodocea* instead of *Ruppia*
Trawling Fishing	Seaweeds	Damage of sublittoral stands
	Seagrasses	Fragmentation - decline of meadows

Case study: Bou-Ismail Gulf (Algerie) .

By its geographical situation, Bou-Ismail Gulf offers an interesting study area. There is a major sewage outfall as long as several secondary outfalls. Local pollution is also observed in some locations presumably caused by an aquarium, a fishfarm and a marine institute. The Reference site is located 18 km west from the major outfall.

Stations	**Reference station**	**Station I** pisciculture	**Station II** Marine Institute	**Station III** Aquarium
	Station IV Local pollution	**Station V** Local pollution	**Station VI** Secondary sewage outfall	**Station VII** Major sewage outfall

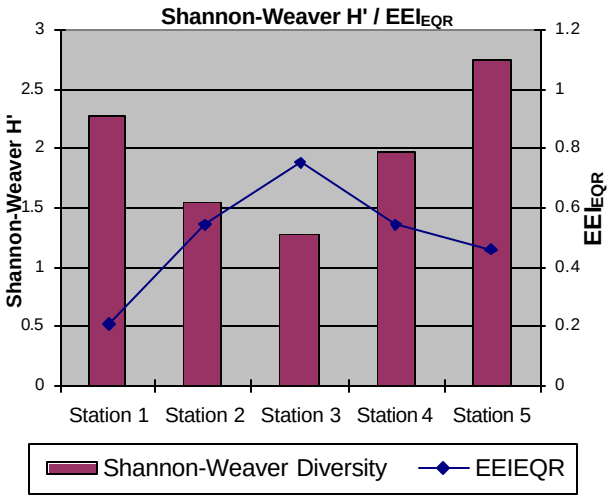


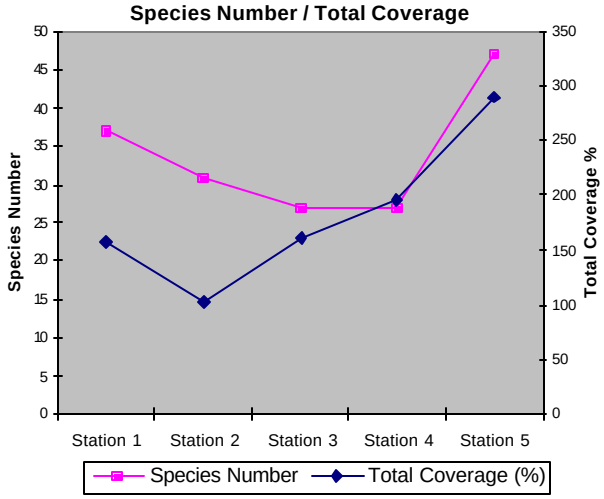


Assessment: All sampling sites present moderate to low ecological status, including the reference site. There is no pollution gradient observed along the coast of Bou-Ismail Gulf, notwithstanding the presence of the major sewage outfall at station VII. As far as the Shannon-Wiener diversity is concerned, only at station VII a significant drop of the index is recorded. In the rest sampling sites, the Shannon-Wiener index presents variability with the highest value at station I

Marseille (France)
There is a sewage outfall in the vicinity of Marseille and as in the case of the Malliakos and Saronikos Gulfs, it presents an eutrophication gradient. However, a wastewater treatment plant has been set up in the area since 1993.

Site	**Marseille (France)**				
Stations	**station 1** sewage outfall	**Station 2**	**station 3**	**station 4**	**station 5** reference



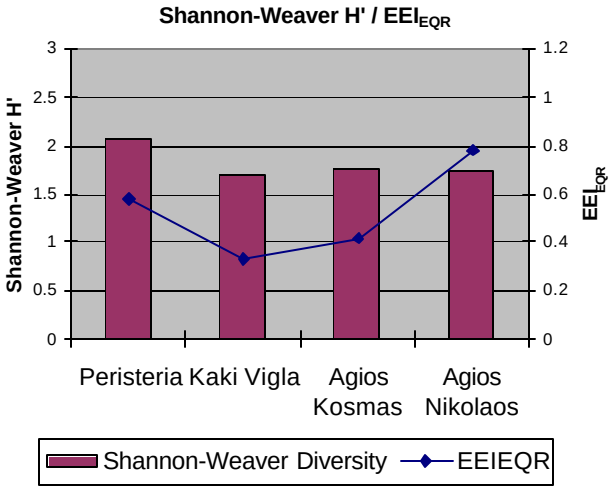


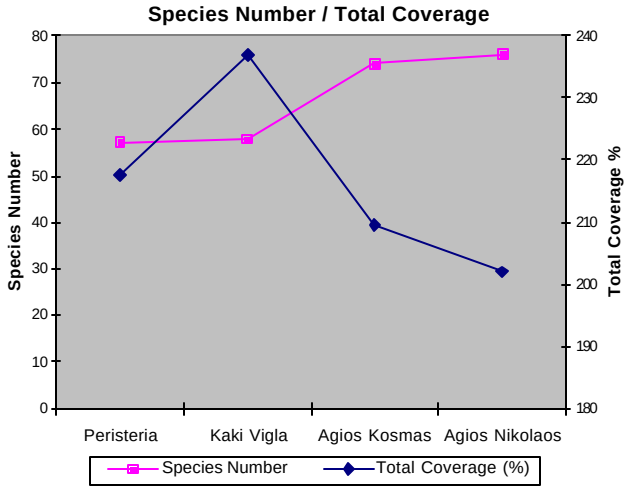
Assessment: Station 1, which is close to the sewage outfall, shows clearly a disturbed environment, presenting bad ecological status. The ecological quality increases towards the outer sampling sites (Stations 2 and 3). However, at station 4 and in the reference site (station 5) the ecological status is moderated. When it comes to the Shannon-Wiener diversity index, high values are recorded at stations 1 and 5 and thus the H' indicator doesn't seem to follow the pollution gradient.

Saronikos (Greece)

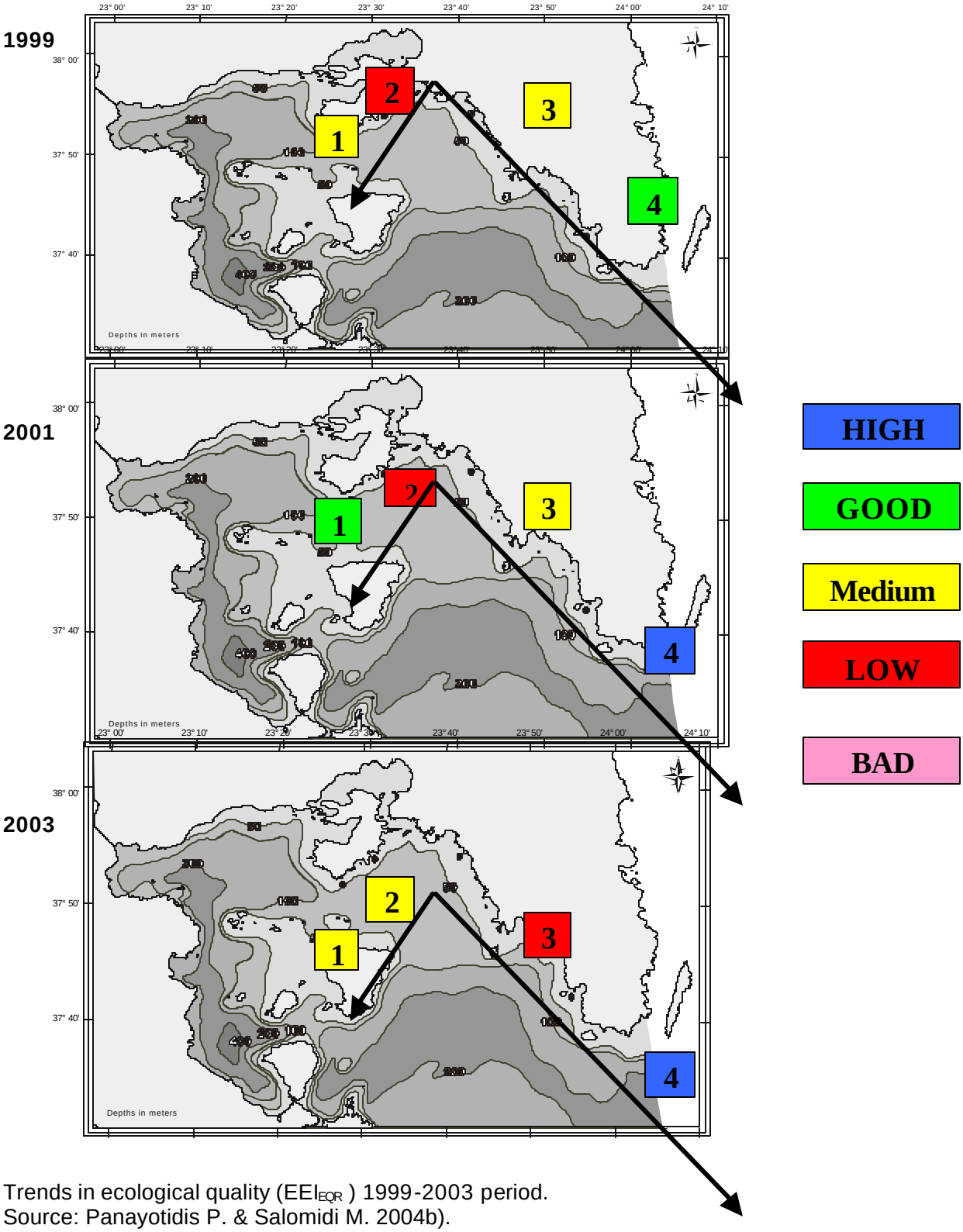
There is a main source of pollution in the Saronikos Gulf caused by the central outfall of urban waste, which is located in the area of Psitallia, between the coasts of Salamina and the port of Pireus. The selected stations were placed on two axes of gradual attenuation of the human impact caused by the central outfall. The sites of Peristeria and Kaki Vigla were on axis starting from the outfall towards the western part of the gulf, while the sites of Agios Kosmas and Agios Nikolaos were on axis starting from the outfall towards the outer part of the gulf. Agios Nikolaos presents minor anthropogenic disturbance and therefore is suitable for RC description. The coastal front of the metropolitan area of Athens and especially the site of Agios Kosmas is hosting the infrastructures for the nautical sports of Athens 2004 Olympic Games

Stations	**Peristeria** Sewage outfall	**Kaki Vigla** Sewage outfall	**Agios Kosmas** Sewage outfall	**Agios Nikolaos** reference





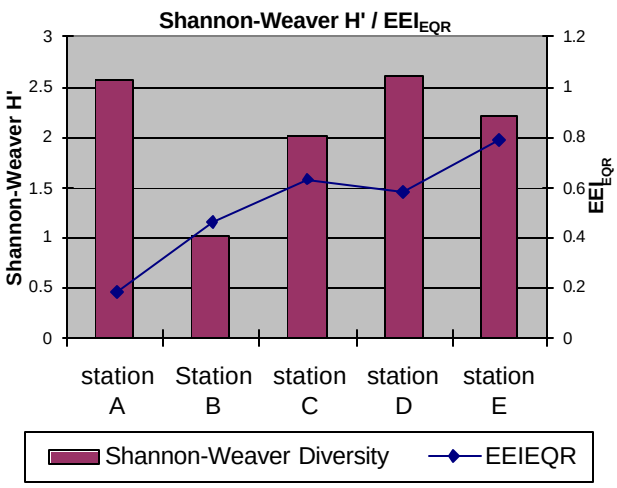
Assessment**: A** pollution gradient is clearly observed: The sampling site close to the central outfall of urban waste of Psitallia (Kaki Vigla) presents low ecological status. The ecological quality increases towards the outer stations (Peristeria and Agios Kosmas) while the reference site presents good-undisturbed conditions. However, the Shannon-Weaver diversity index is about the same among all stations.

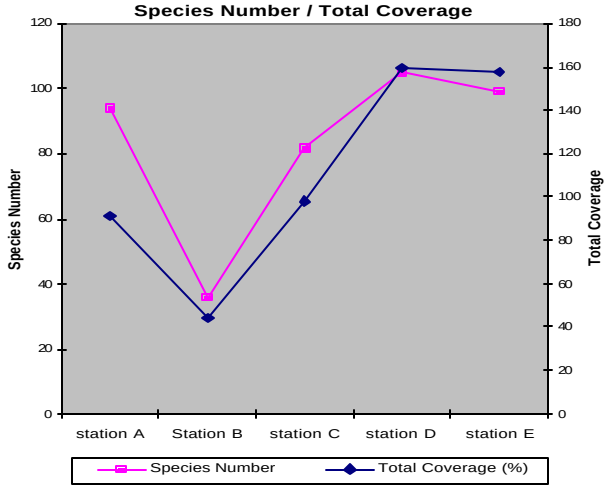


Trends in ecological quality (EEI_{EQR}) 1999-2003 period.
Source: Panayotidis P. & Salomidi M. 2004b).

Malliakos Gulf (Greece) is characterised by some interesting features, as it holds a special biogeographical position (in the central part of the Aegean Sea) and as it presents an eutrophication gradient. The main source of pollution in the area is located in the estuary of Sperchios river. Five sampling locations (sites) were chosen along the north coasts of the Malliakos gulf. These sites are distributed from the estuary of Sperchios river to the Aegean Sea.

Stations	**station A** river mouth	**Station B**	**station C**	**station D**	**station E** reference

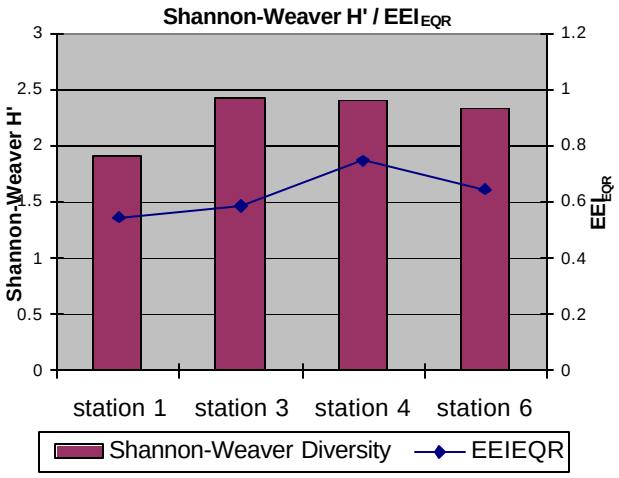


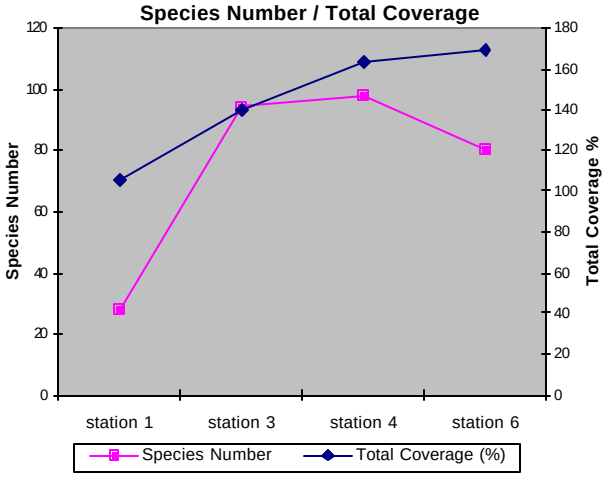


Assessment: Malliakos Gulf represents an excellent example of a eutrophication gradient. Station A, which is close to the main source of pollution in the area (estuary of Sperchios river) presents bad ecological status. The ecological quality rises towards the outer sampling sites (Station B low, Stations C and D moderate). The reference site (Station E) represents good ecological status. As far as the Shannon-Weaver diversity is concerned, only at station B a significant drop of the index is recorded. In the rest sampling sites the Shannon-Weaver index is relatively high and doesn't follow the eutrophication gradient which can be seen with the EQR$_{EEI}$.

Milos Island (Greece) is located in the middle of the Aegean Sea. There is a great interest in the island because of its volcanic origin and the presence of a geothermic field of high enthalpy, which comes out even in littoral areas. In addition, there is an experimental power plant, which exploits the geothermic field, and could affect the marine environment.

Stations	**station 1** geothermic pollution	**station 3** quarry, commercial ships	**station 4** power plant waste outfall	**station 6** reference

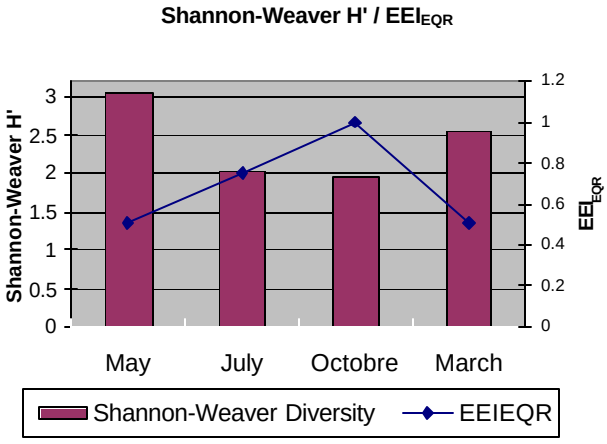


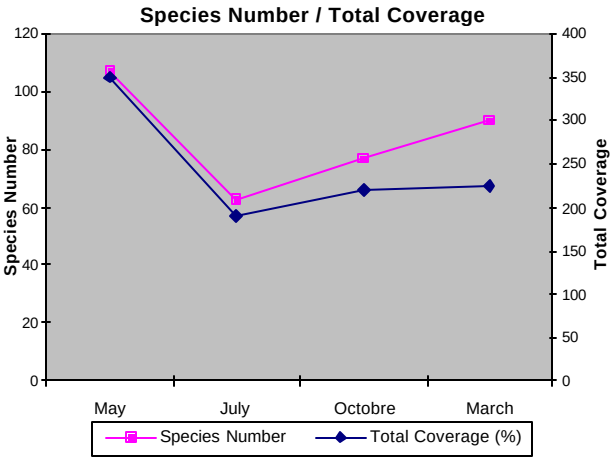


Assessment: Both at sampling sites 1 (geothermic pollution) and 3 (quarry, commercial ships) the ecological status is moderate. The ecological quality is higher at sampling sites 4 and 6 (reference site), revealing good conditions. The Shannon-Weaver diversity index is low only at station 1, while in the rest of the stations is of about the same high levels.

Calancone, Galeria Gulf, **Corse** (France). The site of Calancone is characterised by undisturbed conditions, while there is no pollution recorded in the area. This site belongs to the National Park of Galeria Bay in Corse

Sampling	**Sampling 1** unpolluted conditions	**Sampling 2** unpolluted conditions	**Sampling 3** unpolluted conditions	**Sampling 4** unpolluted conditions





Assessment: Moderate ecological status is observed in May and March, while in July there is good ecological status. Only in October high quality is recorded. On the other hand, the Shannon-Weaver index presents high values in May and March and low ones in October and July.

Assessment

The benthic macrophytes (seagrasses and macroalgae) are a common element of the marine biodiversity along the European co astline, on soft (sand, mud etc) as well as on hard (rocky) substrates.

Some species are considered to be indicators of high ecological quality (Pergent, 1991). On soft substrata typical examples are the seagrasses *Posidonia oceanica* in the Mediterranean and *Zostera marina* in the Atlantic. On hard substrata typical examples are the brown algae *Cystoseira sp.* in the Mediterranean and *Fucus sp.* in the Atlantic.

On the other hand, the massive presence of the green algae *Ulva* sp., *Enteromorpha* spp. and *Cladophora* spp. along the European coastline is considered to be a reliable indicator for nutrient enriched seawater (EEA, 1999). Other green algae are organic mud loving species. A typical example is *Caulepa prolifera* (Meinesz, 1980). Thus, the presence and/or the abundance of some benthic macrophytes could be used for the classification of the Ecological (quality) Status, in the terms of the Water Frame Directive (2000/60/EC), as proposed in the following Figures.

Policy relevance

Marine seagrasses and macroalgae (and in particular their presence) are extensively used in EU environment quality and monitoring projects i.e. Description of the marine “habitat types” in the Habitat Directive 92/43/EEC (e.g. NATURA 2000 codes 1120 and 1170), Conservation of Biological Diversity (CBD), and Mediterranean Action Plan (MAP).

Policy context (relevance of the indicator with reference to specific policy processes).

The European Union has adopted the concept of ecological quality in the Water Framework Directive (2000/60/EC) by using biological communities as Quality Elements (QE) to evaluate the Ecological Status (ES). The key aims are: expanding the scope of water protection to all waters (surface waters and groundwaters) achieving "good status" for all waters by setting deadline water management based on river basins "combined approach" of emission limit values and quality standards getting the prices right and getting the citizen involved more closely streamlining legislation. The innovative approach of the WFD includes the establishment of Reference Conditions (RC) and Ecological Status Class (ESC) boundaries by using indicative parameters or metrics (preferably in numbers) of different QE, e.g. macroalgae (EC, 2000). The ESC is used to describe the degree of human impact on the biological communities in a water body. Five classes of quality (high, good, moderate, low, bad) are foreseen in the WFD, the high class reflecting the RC. Therefore, there is a need for monitoring data and predictive modeling. The marine benthic macrophytes (phytobenthos) are key “quality element” for the classification of marine coastal areas within this Directive.

Environmental context: (scientific soundness and choice and definition of the indicator)

Macroalgae are mainly sessile organisms, they respond directly to the abiotic and biotic aquatic environment and thus they are concerned as one of the QE for the evaluation of the ES of coastal and transitional waters. The other QE for coastal waters are marine angiosperms, benthic invertebrate fau na and phytoplankton. In the upper infralittoral zone of undisturbed Mediterranean locations, macroalgae form well-stratified communities with a clearly defined canopy stratum, dominated by large brown algae, such as *Cystoseira* spp. (Boudouresque, 1969; Giaccone & Bruni, 1972-1973; Ballesteros, 1990; Montesantou & Panayotidis, 2000). The upper infralittoral zone (down to 1.0 m depth) is one of the habitat types described in the “Habitat” Directive 92/43/EEC (NATURA-2000 code 1170). Because the upper infralittoral vegetation could be considered as a well-defined system, easily accessible and able to express the anthropogenic stress, it could be suitable for the first

evaluation of the ES as well as for the future long term monitoring which is foreseen in the WFD.

A good example is water eutrophication. A reliable signal of anthropogenic stress is a shift from pristine to degraded state where opportunistic species dominate. A specific result of increasing eutrophication is the replacement of late succesional, perennial seaweeds (Ecological State Group: ESG I), like *Cystoseira* spp. and *Fucus* spp by opportunistic species (Ecological State Group: ESG II) like *Ulva* spp. and *Enteromorpha* spp. (Harlin 1995; Schramm and Nienhuis, 1996, Schramm, 1999). The relative abundance of ESG I and ESG II can lead to ecological evaluation of transitional and coastal waters.

Data

Case studies were used to demonstrate the usefulness of the indicators:
Bou-Ismail Gulf (Algerie) Yamina Kadari-Meziane, 1994
Marseille (France) Soltan D., Verlaque M., Boudouresque C., Francou P.,2001
Saronikos Gulf (Greece) Panayotidis P. & Salomidi M., 2004b.
Malliakos Gulf (Greece) Chryssovergis, 1995
Milos Island (Greece) Lazaridou E., 1994
Calancone, Galeria Gulf, **Corse** (France) Verlaque M., 1987

Meta data

1. *Description of data:* Indices of Ecological evaluation of transitional and coastal waters based on marine benthic macrophytes.
2. *Geographical coverage*:

Bou-Ismail Gulf (Algerie)
Marseille (France).
Saronikos Gulf (Greece).
Malliakos Gulf (Greece)
Milos Island (Greece)
Calancone, Galeria Gulf, **Corse** (France).

3. *Temporal coverage*: depending on area see Annexes
4. Methodology and frequency of data collection: per case study
5. Methodology of data manipulation, including making 'early estimates':

Methodology

All samplings of all studies were carried out in the infralittoral zone and on rocky substrate. The sampling was destructive on a quadrate 20 cm x 20 cm, which is considered to be the minimal sampling area in the case of the Mediterranean infralittoral communities (Dhont & Coppejans, 1977; Boudouresque & Belsher, 1979). In all cases the quantitative study was performed according to the methodology proposed by Boudouresque (1971) and developed by Verlaque (1987). Each sample was sorted carefully, and the surface covered by each species in vertical projection was quantified as % of coverage of the sampling quadrat (1% sampling surface=4cm^2). Total coverage usually exceeded 100% due to the presence of different layers in the vegetation (canopy, bushy layer, crusts and epiphytes).

Data manipulation

Biotic indices

The use of biotic indices has been well developed for fresh-water environments since the beginning of the previous century with the classic work of Kolkwitz & Marsson (1908). However, in the salt-water environments the institution of a major and reliable biotic indicator of general use is difficult because of many variable environmental factors (Spathari, 2004).

The two most important factors for the coastal environments include the hydrodynamic impact and salinity, which can be very changeable (Pergent 1991, Spellerberg 1991). Today there are several biotic indices which are used for marine ecosystems and pollution impact (Gonor & Kemp 1978, Levine 1984, Norton et al., 1996) but the credibility of some of those has been judged, e.g. the Shannon-Weaver diversity index (Cairns et al. 1993; Cao et al. 1996; Burel et al. 1998; Lydy et al. 2000; Gray 2000; Rice 2000). In this study several biotic indicators based on coverage and/or presence/absence of macroalgae data are used as tools for a rapid evaluation of coastal environments in the Mediterranean:

- **Species Number**

The most common measurement of a community structure is the number of species (Magurran 1998, Gaston 1996). However, this measurement represents a low-accuracy evaluation of real taxonomic diversity (of the real number species which actually live in the area) and this inaccuracy depends on the sampling intension (Gaston 1996, Griffiths 1997). In this study the species number was calculated based on the total species number of all samples for each station.

- **Total Coverage**

Coverage is the surface percentage which each species takes up if it was alone in the community, and it is calculated based on the vertical projection of each oneon the sampling surface. The total coverage can be found after the sum of the coverage of each species in the community. In most cases total coverage is given as a percentage (%) of the sampling surface. As mentioned above, the total coverage usually exceeded 100% due to the presence of different layers in the vegetation (canopy, bushy layer, crusts and epiphytes).

- **The Ecological Evaluation Index (EEI).**

The EEI is an original biotic index based on the concept of morphological and functional groups (Littler & Littler, 1980, 1984). The species found in various samples are divided in two Ecological State Groups (ESG). In the ESG I were grouped the thick leathery, the articulate upright calcareous and k-selected species. In the ESG II were grouped the foliose, the filamentous and the coarsely branched upright species. Most of them are r-selected species.

The EEI was calculated according to Orfanidis et al. (2001, 2003), described also on the site: www.fishri.gr (Laboratories / Marine Ecology / Water Quality). Each sampling site was classified in one of the five ESC after cross-comparison of the coverage value of the ESG I and the ESG II (Table 2) on a matrix (Figure 1). A numerical scoring system was used to express the category of ESC to a number (bad=2, low=4, moderate=6, good=8, high=10). According to the REFCOND CIS, (2003) document, the biological parameters should be expressed as a numerical value between 0 (bad ES) and 1 (high ES). For each ecological quality metric in a given sampling site, this range results from the ratio of the observed value versus the value of the same metric under reference conditions (Ecological Quality Ratio-EQR). The principal of EQR for the case of the EEI could be applied following the formula (Panayotidis *et al*., 2004):

EEI_{EQR}=1,25x(EEI value/RCvalue)-0,25, where RCvalue=10.

Table 2: Functional characteristics and growth strategies of marine benthic macrophytes (after Orfanidis et al., 2001).

Longevity (Succession)	*Growth Strategies sensu Grime 2001*	*Sampled Genera*	*Ecological State Group*
Annuals (Opportunistic)	Ruderal	*Ulva, Enteromorpha, Scytosiphon* (erect phase), *Dictyota*	II
Annuals (Opportunistic)	Ruderal	*Cyanophyceae, Chaetomorpha, Cladophora, Polysiphonia, Ceramium, Spyridia*	II
Annuals (Mid-successional)	Stress-tolerant-Ruderal or Stress-tolerant-Competitors	*Acanthophora, Caulerpa, Chordaria, Gracilaria, Laurencia, Liagora*	II
Perennials (Late-successional)	Competitors	*Cystoseira, Chondrus, Fucus, Laminaria, Padina, Sargassum, Udotea*	I
Perennials (Late-successional)	Competitors	*Amphiroa, Corallina, Galaxaura, Halimeda, Jania*	I
Perennials (Late-successional)	Competitors	*Hydrolithon, Lithothamnion, Peyssonnelia, Porolithon*	I
Perennials (Pioneers to late-successional)	Stress-tolerant	*Cymodocea, Posidonia, Ruppia*	I

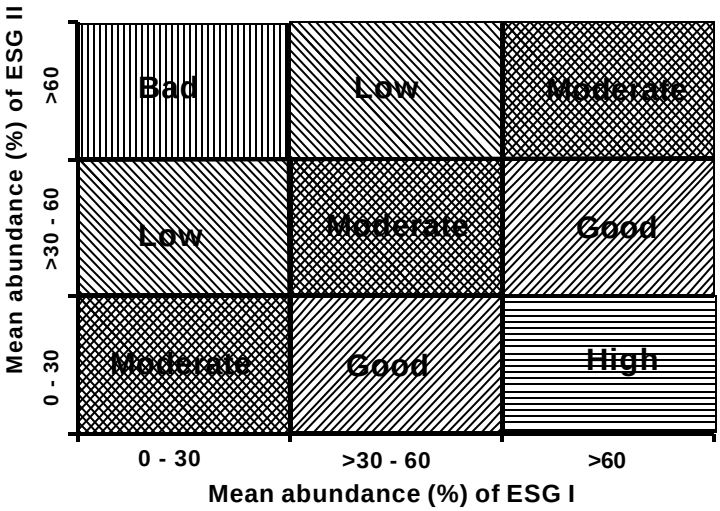


Figure 1. Matrix proposed by Orfanidis et al. (2001) for the evaluation of the ESC, according the classification induced in Directive 2000/60/EC.

The calculation of diversity indices was based on coverage measurements, a methodology adapted to the macroalgae by Boudouresque (1971) and Frontier (1983). In addition, species number and total coverage were also calculated for each sample. All calculations were performed using the Primer v. 5.2 software package except for the EEI and the EEI_{EQR}.

6. Strength and weakness (at data level):

Advantages: Widely applicable (at European scale). Identification could be limited to the functional groups

Limitations: Difficult taxonomy (if identification has to reach the specific level). Different sampling methodology in the case of hard and soft bottom. Different approaches for the estimation of the abundance (eg surface for the seagrasses, linear for the seaweeds)

7. Reliability, accuracy, robustness, uncertainty (at data level):

Ecological Evaluation Index (EEI), is an indicator requiring a relatively small sampling set, a taxonomic effort limited to the abundant species and a simple statistical treatment, while on the other hand it seems capable to distinguish more clearly the variation of the ecological quality among coastal environments (Panayotidis et al., 2004). However, it should also be mentioned that according to Orfanidis *et al.* (2001), the EEI is calculated based on absolute coverage. Nevertheless, benthic communities of marine macroalgae consist of many layers, and this is why total coverage can be much higher than 100%, even reaching nearly 500% in some cases. Therefore, in these cases the EEI could be calculated based not only on absolute coverage but also on relative coverage, giving the "Relative EEI" and the "Relative EEI_{EQR}". Thus, the use of Relative EEI and Relative EEI_{EQR} can give better results when it comes to larger coverages.

8. Overall scoring (give 1 to 3 points: 1=no major problems, 3=major reservations): see comment on data

Relevancy: 1
Accuracy: 1
Comparability over time: 1
Comparability over space: 2

Further work required
This study aims to show how data of the benthic macroalgal community could contribute to the evaluation of the ES of a marine environment. Therefore, several biotic indices based on the composition and abundance of the hard bottom macroalgae, are used as tools for the rapid classification of a water body type. Obviously, the result of this assessment cannot be the final ES evaluation, because this latter is the resultant of both biological and physical-chemical quality.

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Site	**BOU-ISMAIL (Algerie)**			
Stations	**Reference station**	**Station I** Fish farm	**Station II** Marine Institute	**Station III** Aquarium
Sampling Periods	1/1989 9/1989	1/1989 9/1989	1/1989 9/1989	1/1989 9/1989

Site	**BOU-ISMAIL (Algerie)**			
Stations	**Station IV** Local pollution	**Station V** Local pollution	**Station VI** Secondary sewage outfall	**Station VII** Major sewage outfall
Sampling Periods	1/1989 9/1989	1/1989 9/1989	1/1989 9/1989	1/1989 9/1989

Site	**Marseille (France)**				
Stations	**station 1** sewage outfall	**Station 2**	**station 3**	**station 4**	**station 5** reference
Sampling Periods	Winter 1995 Summer 1996	Winter 1995 Summer 1996	Winter 1995 Summer 1996	Winter 1995 Summer 1996	Winter 1995 Summer 1996

Site	**Saronikos Bay (Greece)**			
Stations	**Peristeria** Sewage outfall	**Kaki Vigla** Sewage outfall	**Agios Kosmas** Sewage outfall	**Agios Nikolaos** reference
Sampling Periods	Aug-1998 Mar-1999 Jun-1999 Jun-2001 Sep-2001 Mar-2002 Sep-2002 Mar-2003 Jun-2003	Aug-1998 Mar-1999 Jun-1999 Jun-2001 Sep-2001 Mar-2002 Sep-2002 Mar-2003 Jun-2003	Aug-1998 Mar-1999 Jun-1999 Jun-2001 Sep-2001 Mar-2002 Sep-2002 Mar-2003 Jun-2003	Aug-1998 Mar-1999 Jun-1999 Jun-2001 Sep-2001 Mar-2002 Sep-2002 Mar-2003 Jun-2003

Site	Malliakos Bay (Greece)				
Stations	**station A** river mouth	**Station B**	**station C**	**station D**	**station E** reference
Sampling Periods	3/1992 9/1992 2/1993 7/1993	3/1992 9/1992 2/1993 7/1993	3/1992 9/1992 2/1993 7/1993	3/1992 9/1992 2/1993 7/1993	3/1992 9/1992 2/1993 7/1993

Site	Milos Island (Greece)			
Stations	**station 1** geothermic pollution	**station 3** quarry, commercial ships	**station 4** power plant waste outfall	**station 6** reference
Sampling Periods	- - - Summer 1989 Autumn 1989 Winter 1990 Spring 1990 Summer 1990	Autumn 1988 Winter 1989 Spring 1989 Summer 1989 Autumn 1989 Winter 1990 Spring 1990 Summer 1990	Autumn 1988 Winter 1989 Spring 1989 Summer 1989 Autumn 1989 Winter 1990 Spring 1990 Summer 1990	Autumn 1988 - - Summer 1989 - Winter 1990 Spring 1990 Summer 1990

Site	Calancone (France)			
Sampling	**Sampling 1** unpolluted conditions	**Sampling 2** unpolluted conditions	**Sampling 3** unpolluted conditions	**Sampling 4** unpolluted conditions
Sampling Periods	05/1985	07/1982	10/1982	03/1985

UNEP

Marine Pollution Indicator fact sheet

MAP

MED POL

Number and Abundance of Exotic Species (Zoobenthos, Phytobenthos, Zooplankton, Phytoplankton, Fish)

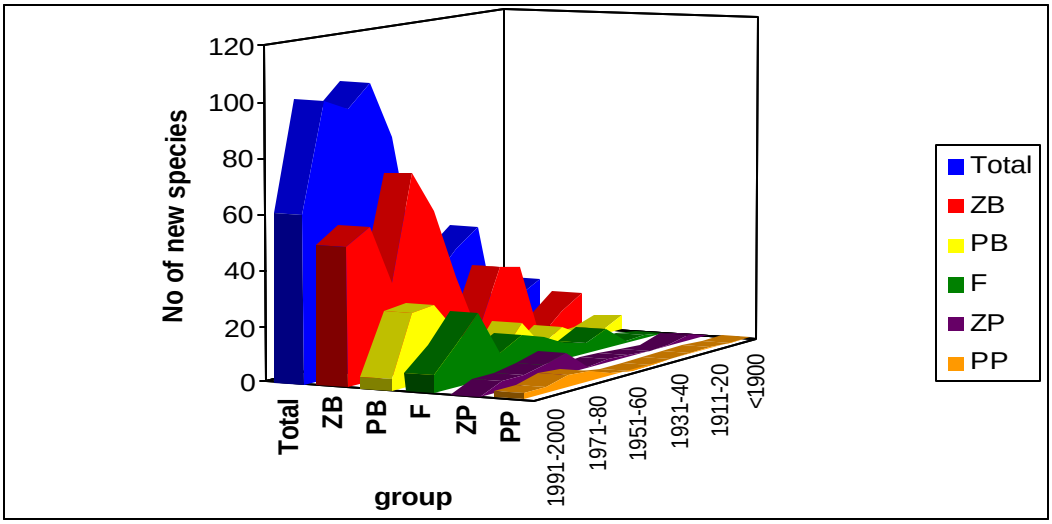
Key messages

☹Over 600 marine exotic species have been recorded in the Mediterranean Sea.

☹ The rate of introduction of exotic species in the Mediterranean Sea has peaked in the 1970-1980 period, and since then has remained stable or even continuing at an increasing rate for most groups and especially for zoobenthos (mollusca).

☹An average of one introduction every four weeks has been estimated over the past five years.

Rate of introductions across the Mediterranean



Notes: PP= phytoplankton, PB= phytobenthos, ZP= zooplankton, ZB= zoobenthos, F= fish.
Sources: compiled by A. Zenetos

Results and assessment

Policy relevance: target or objective for the indicator

Much has been written about the phaenomna collectively names "tropicalization of the Mediterranean"that have introduced changes in the biodiversity and biogeography of the area.Significant changes in the Adriatic physical conditions have been recorded that may have favured the establishement of thermophilic species. According to Bello et al, (2004), the tropicalization of the Adriatic is confirmed by the occurrence and establishment of three tropical species- namely the toxic dinoflagelates (microalgae) *Ostreopsis lenticularis, Coolia monotis & Protocentrum mexicanum*

Polluted or physically-degraded environments are prone to invasion more than pristine sites. A recent study of macrofouling organisms discovered that many more species were found in a polluted than in a nonpolluted marina, and that the cosmopolitan serpulid worm *Hydroides elegans* that comprised 65% of the population in the polluted marina was only infrequently found in the nonpolluted marina (Kocak et al., 1999). The mariculture introductions are mostly restricted to lagoonar or estuarine habitats, and the vessel-transported exotics to polluted harbours (Zibrowius, 1992) - environments known for their low biodiversity. **Therefore, the response of exotics to pollution monitoring makes them good candidates for assessing EcoQs**.

To facilitates EU policies towards monitoring the introduction of exotic species in European waters. EU targets to ensure that the deliberate introduction into the wild of any species which is not native to their territory is regulated so as not to prejudice natural habitats within their natural range or the wild native fauna and flora

Policy context
At European level, the Bern Convention in 1979 provides that "*each contracting party undertakes ... to strictly control the introduction of non-native species*" The EC Directive on the Conservation of Natural Habitats and of Wild Fauna and Flora requires Member States to "*ensure that the deliberate introduction into the wild of any species which is not native to their territory is regulated so as not to prejudice natural habitats within their natural range or the wild native fauna and flora and, if they consider necessary, prohibit such introduction*" (article 22(b)).
Furthermore EU Directives legislate for the protection of the ecosystem against the adverse effects of aquaculture-related introduced organisms (Directive on the Deliberate release in to the environment of Genetically Modified Organisms (GMOs) (90/220/EEC) and Environmental Impact Assessment (EIA) Directive and its amendment (85/337/EEC & 97/11/EC)).
Environmental context: (scientific soundness and choice and definition of the indicator) I

Exotic species (numbers, mode and rate of introduction and establishment success) are those species which do not belong to the native fauna and have arrived recently i.e. within historical times i.e. any species intentionally or accidentally transported and released by man into an environment outside its native geographical range of habitat. They represent a growing problem due to the unexpected and harmful impacts they cause to the environment (biodiversity changes), economy (e.g. fouling organisms) and human health (exotic Harmful Algal Blooms introduced via shipping and/or Aquaculture).

Assessment

According to a compiled list of Mediterranean exotics which is based on records reported up to September 2004, 611 species appear to have been introduced in the Mediterranean. Zoobenthos is by far the dominant group (60% of all invaders) followed by phytobenthos (18%) and fish 16%).

The introduction of exotics is a dynamic non-stop process with new species reported each day. The phenomenon which has peaked in the 1970-80 period (total of 103 records) continues at a steady rate (95 and 100 species in the next two decades) not equally for all groups. It is characteristic that in the 21st century 58 new species have been reported in the Mediterranean, 24 of them recorded within 2004 which reveals the difficulties in keeping records up to date as well as calling for continuous research on this issue.

It is argued that exotics have increased the biodiversity of the Eastern Mediterranean. Today, 12% (68 out of 569) of the benthic biota of Israeli coasts are of Erythrean origin (Fishelson, 2000). According to an updated checklist of the macroage of Thau lagoon (France) it was estimated that introduced species made up the 23% of the total flora (Verlague 2001).

The alterations of marine ecosystems due to new introductions have been poorly studied in most areas. There only few well documented cases such as that of *Caulerpa racemosa.* Biodiversity changes such as the dominance of certain species exhibiting invasive character at the expense of others has been often reported but not quantified. Typical examples are the rapid decreases in the Israeli coast of populations of the sea star *Asterina gibbosa* , the prawn *Melicertus kerathurus*, and the jelly fish *Rhizostoma pulmo*, as those of *Asterina burtoni*, *Marsupenaeus* (=*Penaeus*) *japonicus* and *Rhopilema pulmo* increased in numbers, and fish populations of red mullet (*Mullus barbatus*) and hake (*Merluccius merluccius*) that have been forced to migrate to deeper waters by the exotics *Upeneus moluccensis* and *Saurida undosquamis*, respectively (Galil & Zenetos 2002).

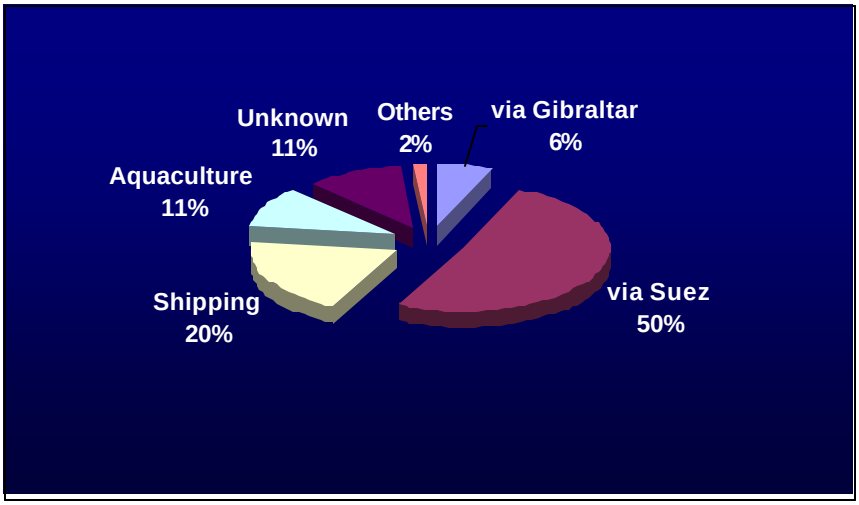
It is far more difficult to document the invasion of exotic meiofaunal elements into the Mediterranean Sea, as early records are significantly more scarce. However, benthic foraminifera have good preservation potential and may be present in large numbers, tending to leave behind a superior record of their presence over time, in comparison with macrofaunal elements. A recent, extensive study on benthic foraminifera from the shallow continental shelf along the SE Mediterranean (Hyams, 2001) indicates that nearly 20% of the local foraminifera species are suspected to be of an exotic origin. Our ability to make this estimation may in part be attributed to recent publication of the Atlas of Recent Foraminifera of the Gulf of Aqaba (Hottinger et al., 1993) and modern compilations of Mediterranean species (Yanko et al., 1998), which enable comparison of the benthic foraminifera assemblages in both regions.

Sub indicators Mode of Introduction

Key message

☹ Via Suez migration and shipping constitute the major ways for the introduction of new species in the Mediterranean followed by aquaculture (deliberate and unintentional) and those cases where the mode remains undetermined

Vectors of introduction of exotic species in the Mediterranean November 2004



Sources: compiled by A. Zenetos

Assessment for the sub-indicator

The high number of exotics in the Mediterranean Sea has been attributed to human activities, e.g. sea faring, commercial and tourism activities over centuries, to the presence of numerous habitats susceptible to invasions (lagoons, estuaries, marinas) and to the recent expansion of aquaculture (Verlaque, 2001). The opening of the Suez Canal (19th century) has led to the introduction of hundreds of Lessepsian immigrants (Por 1978).

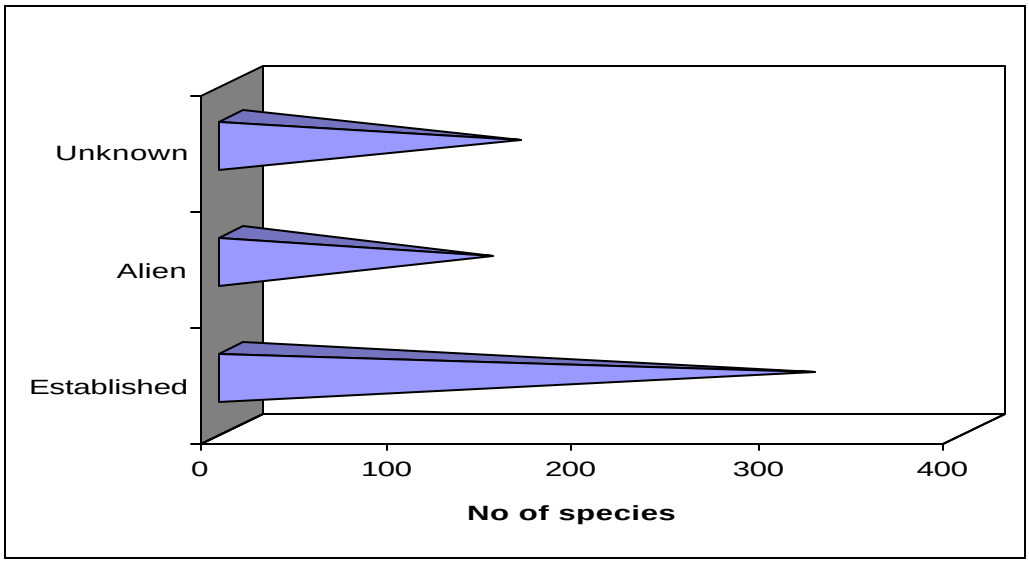
Transportation via the Suez Canal and shipping appear to be the major vectors of introduction (accounting for 50% and 20%, respectively); however, a significant part remains unaccounted for (11 %). Aquaculture is the third most important means of introduction (11%) with unintentionally introduced species being more numerous compared with those introduced intentionally.

Although the origin of the newcomers is well known, the mode of transportation is not always clear for many species. For example of the 45 exotic taxa in Thau lagoon, the majority may originate from the Pacific region, having arrived either directly (Lessepsian migration) or via other aquaculture sites. A highly probable vector of macroalge introductions is the transfer of oysters, which appear at present, to be the main vector of macrophyte introductions in the Mediterranean Sea, surpassing the Suez Canal (Verlaque, 2001).

However it appears that introduction mode differs with exotic group studied. Thus Lessepsian migration is documented or suspected for the great majority of zoobenthic species (56 %) while for some a two mode introduction is assumed. According to Gofas & Zenetos (2003), [based on data up to 2001] 115 out of 143 non-indigenous molluscs in the Mediterranean are of Indo-Pacific origin and are most likely to have spread by their own means through the Suez Canal (Lessepsian immigrants).

Penetration via the Suez Canal is also the prevalent mode of introduction for fish and decapod crustaceans (Golani et al., 2002; Galil et al., 2002).

Success on Introduced Species in the Mediterranean



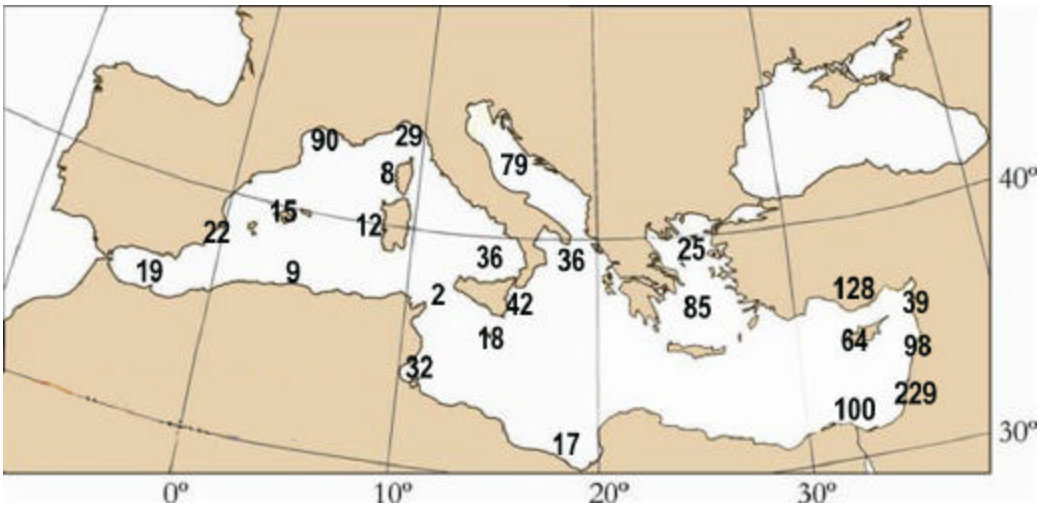
Notes: Established species are the species with self maintained populations or with many records including cryptogenic species; alien, species with sporadic recordings in place and times
Sources: compiled by A. Zenetos

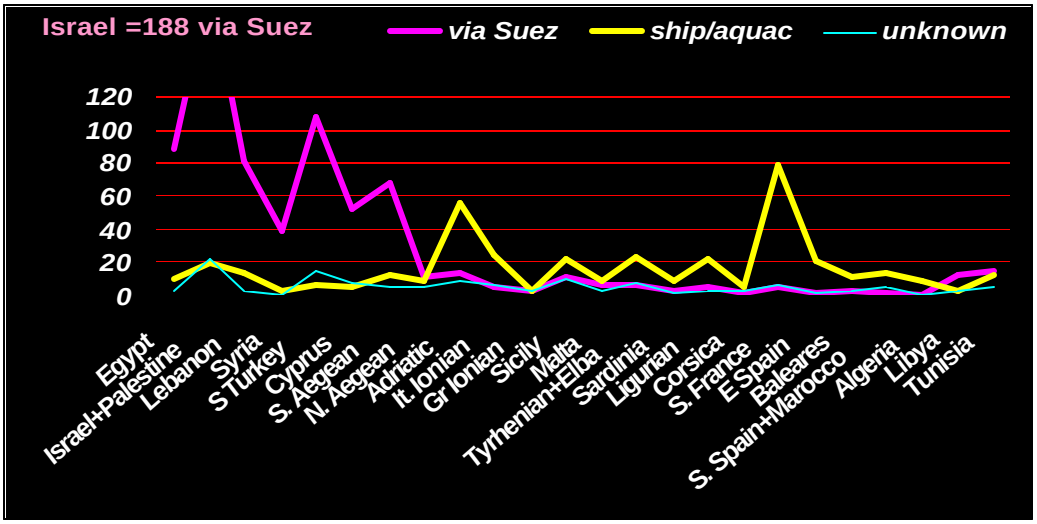
Assessment for the sub-indicator

More than 50% of the introduced species appear to have been established in one or more geographic areas, 23% have been registered as aliens; however no data exist for 25% of the species. As new information comes into light the geographic coverage of the species expands from the so-called Lessepsian Province (Por, 1978) to the western Mediterranean.

The story of establishment success requires further scientific research. A major hindrance lies in the fact that no data could be found on the establishment success in the North Africa coasts (with the exception of Tunisia). Furthermore, in many cases the establishment success remains unknown, particularly among the more recent introductions. Further research will inevitably lead to revision of our figures.

Sub indicators Distribution of exotics across the Mediterranean





Assessment for the sub-indicator

The phaenomenon of introduction of exotic species is very intensive in the eastern Mediterranean but apparent across the western basin, although the vector of introduction is different. Whereas in the eastern Mediterranean penetration via the Suez Canal is the main mode of introduction, in the western Mediterranean shipping and/or aquaculture are responsible for the great majority of the exotic species. Lagoonal ecosystems in the Northern Adriatic and S. France (with 56 and 79 exotic species respectively, mostly via aquaculture) are considered hot spot areas for exotics.
The Lessepsian immigrants extend their distribution in the Western Mediterranean surpassing the so-called Lessepsian Province. In the calculations of figure x reservations are raised on the mode of introduction of the following species per area.

Tyrrhenian: *Cerithium scabridum, Caulerpa racemosa*
Corsica: *Acanthophora nayadiformis* (PB)macroalge) unkn (Ribera, Less (Wallentinus);
French coasts: *Sarconema filiforme, Caulerpa racemosa*; *Gracilaria disticha*

Sicily: *Asparagopsis taxiformis?* Less? /Un*, Brachidontes* Less/ship*?; Caulerpa racemosa, Cerithium scabridum*
Ligurian: *Cladophora cf patentiramosa & Lophocladia lallemandii* (Less/shipping)
Baleares: *Asterina burtoni, Caulerpa racemosa*

Data Data have been compiled from a wide variety of sources from existing databases and supplemented by bibliographical research. Entries range from species-specific papers to museum collections and web sites, dating from 1969 to 2004. The backbone of our review has been based on CIESM series of atlases [Golani et al. (2002), Galil et al. (2002), Zenetos et al. (2003)] and numerous works covering the Mediterranean such as Por (1978), Zibrowius (1992), Ribera & Boudouresque (1995), Athanasiadis (2002), Ribera Siguan (2002), Verlaque, (2001); Zibrowius & Bitar (2003) and finally the work by Wallentinus (2002) covering marine algae in European aquatic environments. The CIESM atlases series on main exotic taxa, accessible through the Internet, provide the means to distinguish among similar species. However, the CIESM atlases do not cover all groups

Meta data

Web presentation information

Abstract / description / teaser (max 3 lines): Introduced species in marine and coastal waters can pose a threat to marine and coastal ecosystems.
Policy issue / question (max one line): What are the risks from potential hazardous introductions of marine species in Mediterranean coastal waters with respect to the consequences on ecosystems and the sustainable use of coastal resources?
dissemination themes: UNEP, EEA; ICES/IMO/IOC WGBOSV; CIESM
DPSIR (one value only): **P**

Technical information

Main data source: Updated check list of paper by Streftaris, N., Zenetos, A. and E. Papathanassiou, 2004 Globalisation in marine ecosystems - The story of non indigenous marine species across European Seas. OMBAR, in press
Mapping the exotics distribution across the Mediterranean original info compiled for UNEP/MAP

Description of data: Data has been compiled from a wide variety of sources from existing data bases and supplemented by bibliographical research. Key data source containing the full data set:

Definition: Number of extra-Mediterranean faunal and floral marine species (also known as non-indigenous introduced, invasive, alien or non-native species) that have been unintentionally introduced or invaded and established reproducing populations and/or imported species that are subsequently living in the wild.

Exotic species have been grouped into six broad categories covering all relevant phyla: phytoplankton (PP), phytobenthos (PB), zooplankton (ZP), zoobenthos (ZB), fish (F). Cryptogenic species (species with no definite evidence of their native or introduced status according to Carlton (1996) and species whose probable introduction has occurred prior to 1800 i.e. has not been witnessed) have been included in our compiled list.

Vectors of introduction: where the means of transportation are investigated, namely shipping (fouling and ballast water), aquaculture (intentional and unintentional; intentional releases and stocking), via Suez Canal, via Gibraltar, and other modes (e.g. escapees, ornamental etc.). When more than one mode is argued (as is often the case), then all modes are computed. Thus the number of vectors is higher than the number of organisms transported.

Rate of introduction: where the chronological trend of introductions is presented in ten-year intervals per group

The year of introduction (or first report when the former is missing), the place of origin and recipient site, and the means of transportation have been recorded where possible. In the graphs and tables that follow each of the seas is treated separately, i.e. introduced species in more than one sea have been recorded in each of them. Non-certain recordings (reports cited followed with question marks in relevant sources) have been treated as positive. Care has been taken to ensure that the nomenclature problems encountered (e.g. the same species recorded in different regions, lists, or data banks with different names, i.e. synonyms), have not resulted in multiple separate recordings.

Geographical coverage: Mediterranean coasts (marine and brackish waters):
Temporal coverage: <1900 - 2004
Methodology and frequency of data collection: data collected at ad hoc basis when available on data bases and relevant publications
Methodology of data manipulation, including making 'early estimates': Introduced species have been grouped into six broad categories covering all relevant phyla: phytoplankton (PP), phytobenthos (PB), zooplankton (ZP), zoobenthos (ZB), fish (F), and protozoa (P). Cryptogenic species have been included in our compiled list.

The year of introduction (or first report when the former is missing), the place of origin and recipient site, the means of transportation and establishment success, have been recorded where possible. When more than one mode of introduction is argued (that is more than often the case), then all modes are computed. Although establishment success of a given species may differ between geographic areas and this is reflected in the per subarea "picture", for the Mediterranean 'picture' it counted positively if the species is established in at least one subarea. Non certain recordings (reports cited followed with question marks in relevant sources) have been treated as positive ones and have been counted as certain ones. Care has been taken so that the nomenclature problems encountered, as the same species have been recorded in different regions, and/ or list, data banks with different names (i.e. synonyms), have not resulted in multiple separate recordings.

Quality information

11Strength and weakness (at data level): see below
12. Reliability, accuracy, robustness, uncertainty (at data level): Care must be exerted on reviewing the relevant papers and data bases as the studies of exotics and thereafter the derived records are fragmentary and sporadic, based mostly on shear scientific interest. The validity of some sources maybe questionable and the synthesis of such records may lead to somewhat biased results.

More specific: In many cases the mode of introduction is unknown or assumed. Furthermore a number of species appears to have been introduced through different, multiple ways. The computation of assumed introductions and of all modes may lead to a degree of uncertainty.

Establishment success requires serious further scientific research, which could lead to the redrawing of our figures. The drawback persists as for too many new introductions the establishment success remains yet undetermined.
The time span between the first finding and publication time may range from 1 to many years

Overall scoring (give 1 to 3 points: 1=no major problems, 3=major reservations):
Relevancy: 1<see Description of elements for definitions>
Accuracy: 2
Comparability over time: 2
Comparability over space: 3

Further work required Heightened public awareness and increasing political interest across Europe on the effects of introduced microorganisms in aquatic ecosystems and on human health, have stimulated a number of research projects (Globally: GloBallast Programme,; EU funded: ALIENS, HAB,MARTOB, STRATEGY, and National Risk assessments in many countries (e.g Italy, Spain, Slovenia, and Trilateral IT-SLO-HR) are currently in progress.

Update of the basic table serving as database for EEA, from on-going research projects and developing databases. Link to European network on Invasive Marine species.

(ERNAIS: http ://www.zin.ru/projects/invasions/gaas/ernais_m.htm).
National and/or EU funds should be channelled towards monitoring of ports, lagoons and coastal mariculture sites for exotic species so as to fill the gaps in unexplored geographical areas and follow trends in hot spots Since the issue of exotics encompasses also many socio-economic as well as scientific (environmental, biological and biodiversity) aspects, a legislative framework must come into force and be rigorously applied to safeguard European seas from invasion by harmful species.

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Marine Pollution Indicator fact sheet

MED POL

Community diversity (H) (zoobenthos, phytobenthos)

Key messages

☺ In Mediterranean coastal Waters, Shannon-Wiener diversity based on the zoobenthic community structure can lead into one of 5 ecological classes (bad-poor-moderate-good-high).

😐 For phytobenthos (hard subsrata mostly) Shannon-Wiener diversity should be only considered as accessory tool for the ecological evaluation of a coastal environment

Table 1. Ecological quality assessment in the Mediterranean Sea using the Shannon-Wiener diversity index (H) on macrozoobenthos.

EQS	Zenetos & Simboura, 2001	Simboura & Zenetos, 2002	description
bad:	0<H=1.5	0<H=1.5	azoic to very highly polluted
poor:	1.5<H=3 :	1.5<H=3 :	highly polluted
moderate:	3<H=4:	3<H=4	moderately polluted
good:	4<H= 4.6	4<H=5	transitional zones
high:	H>4.6	H>5	reference sites

Source: EEA, 2004

Results and assessment

Policy relevance: target or objective for the indicator same as for BENTIX

Policy context as for BENTIX

Environmental context: (scientific soundness and choice and definition of the indicator) The number of species and their relative abundance can be combined into an index that shows a closer relation to other properties of the community and environment than would number of species alone. The Shannon-Wiener diversity index, developed from the information theory, has been widely used and tested in various environments. Although it reflects changes in the dominance pattern, it has been argued that it is no more sensitive than the total abundance and biomass patterns in detecting the effect of pollution and is more time-consuming. When evaluating H, one should take into account separately its two components together with the faunistic data, in order to detect extreme abundance of opportunists indicating disturbance. There are some cases where the diversity is significantly high, even higher than normal, whereas the community is disturbed. The ecotone point is a transitional zone between two succesional stages after which the community returns to normal. The community at the ecotone point consists of species from both adjacent environments (enriched and less enriched). After the ecotone point the community often reaches a maximum in the number of species, probably due to the presence of both sensitive species recolonising community and tolerant species, while abundance declines to a steady state level usually found in normal communities. Thus diversity may become higher than that of the normal communities (Pearson and Rosenber, 1978; Bellan, 1985).

The continued popularity of this index, generally relates to how deeply entrenched the index has become in the literature rather than belief in biological relevance of the index. (Miles & Price, 2004). Examining data from Belfast Lough, Northern Ireland, Breen (2001) demonstrated significant differences in the index if the wrong log base is used

Assessment

In Mediterranean coastal Waters, based on the community diversity index, 5 classes of community health are arbitrarily divided applying mostly to muddy sands or sandy muds marine benthic habitats (Table 2). The limits of these classes are somewhat arbitrary, and they are based on long experience of the authors and data series on community diversity index H mostly from Greek areas. However, it is further supported by literature in other Mediterranean areas. Effects of stressors such as oil spills, dumping, fishing (trawling) and sewage effluent are always mirrored in the community structure and therefore in the values of community diversity.

Correlation of benthic community indices with number of inhabitants during summer months showed that diversity and evenness decreases with the increase of population of the coastal areas. (Reizopoulou & Zenetos in press).

However, only a few examples at Mediterranean scale are presented below; more examples are given in combination with the BENTIX biotic index.

The validity of the Shannon-Wiener index in the case of hard substrate communities (assessments based on phytobenthos) is argued, because sedentary organisms are not easily enumerated. Consequently, species variety and community diversity values can only be compared if the same sampling methodology has been followed, including same level of taxonomic expertise (Panayotidis *et al.*,2004a). Thus, Shannon-Wiener diversity perhaps should be considered as accessory tool for the ecological evaluation of a coastal environment (Panayotidis *et al.*,2004b).

Subindicator: Community diversity according to community type

Table 2: Range of Community diversity (H) according to sampler (0.05. 0.1, 0.2, 0.5m^2) and community type (modified from Simboura and Zenetos, 2002).

Community type	**H min (disturbed to polluted)**	**H max (undisturbed)**
Midlittoral sands	0.57-1.31 (Thermaikos)	1.12-1.40 (Strymonikos)
Deltas	0.85/0.2m^2 (Evros)	3.74/0.2m^2 (Strymonikos)
Muddy sands	3.5/0.1m^2 (Saronikos, Izmir)	5.67/0.1m^2 (Petalioi)
Muddy sands with phytal cover	3.5/0.1m^2 (Izmir, Turkey)	5.21/0.1m^2 (Ionian)
Sandy muds	1.99/0.1m^2 (Saronikos)	4.94/0.1m^2 (Pagassitikos
Shallow muds	3.17/0.1m^2 (Maliakos)	4.97/0.1m^2 (Strymonikos) 4.5 / 0.1 m^2.(Algeria)
Deeper muds	2.36/0.1m^2 (N Evvoikos)	4.04/0.1m^2 (S. Evvoikos)
Shallow Sands (SFBC)	1.82/0.5m^2 (Marseille) 1.56 (Maroc)	5.16/0.5(Milos isl./Kyclades) 4.42 (Maroc)
Deeper Sands with detritus	2.87/0.1m^2 (Ionian)	5.22/0.1m^2 (Ionian)
Deeper Coarse sands	3.74/0.1m^2 (Ionian)	6.06 /0.1m^2 (Strymonikos)
Shallow muddy sands	2.35/0.05m^2 (Geras)	5.23/0.05m^2 (Oropos)
Coralligenous	4.84/0,1m^2 (Chalkis)	5.16/0.1m^2 (Ionian)

Assessment: Shannon-Wiener Diversity, is one of most commonly used indicators in the assessment of pollution in marine benthic communities worldwide. However, the use and interpretation of this index has been subjected to long debate (Clarke & Warwick, 1994). Sampling methodology, sample size and identification procedure influences the value of Shannon-Weaver index. This index is also habitat type dependent, which means that different ranges of values or classification schemes should apply for different habitat types.

Case study: dumping coarse metalliferous waste

	PORTMAN ALGERIA		LARCO N EVVOIKOS coarse mine waste	
	stress	**H**	**stress**	**H**
PG	**dumping site**	0.80 ± 0.22	**dumping site**	3,76
PN		2.04 ± 0.27		
CN		1.95 ± 0.15		
CM		2.12 ± 0.23		
PL		2.43 ± 0.23		
PE	**reference**	2.93 ± 0.14	**reference**	5

Source: Marín-Guirao et al, in press

Assessment: Not usefull alone in case of Portman where it classifies as bad the polluted site but as polluted even the reference site (marine protected area). Reason: Sampling by scuba diving. Grab samples are recommended.

Case study: Algeria ports

	depth	H min	H max	source
Port de Djendjen	4-19m	1.21	5.87	Grimes & Queraini, 2001a
Port of Jijel (1986)	3-15m	3.3	3.5	Grimes & Queraini, 2001b
>> (1997)		1.3	4.5	Grimes & Queraini, 2001b

Assessment: Bad to moderate status.

Meta data

Web presentation information

dissemination themes: UNEP, EEA;
DPSIR (one value only): **S**
Technical information

Main data source:

Description of data
Geographical coverage: Mediterranean coasts

Temporal coverage: Mean values over seasonal sampling is considered.
Methodology and frequency of data collection:
Grab samples, standard size (0.1m2), mesh sieve 0.1mm.
Marocco: Oued Laou: 20 stations at 5-10m depths, April 2003, Dredge samples, mesh sieve 1mm **(**Bayed et al., 2004) All community parameters estimated.
Algerian coasts (bay of Algeria, Bou Ismail, gulf of Jijel) Van Veen grab 0.1m^2, mesh sieve 1mm. Quantitative samples from November 1984 to July 1986. 548 species. All community parameters estimated (Bakalem, 2001)

Portan Bay, (Spain). Six stations at depths 10-15m from the mine outlet in Portma´n Bay in the direction of the Marine Reserve of Cabo de Palos-Islas Hormigas. Four replicate sediment samples were collected in March 2002 by SCUBA divers using a 0.09m^2 hand grab and sieved through a 0.5mm mesh bag. **83 species** in all.

Methodology of data manipulation

Definition: Definition: Diversity as calculated using the Shannon-Wiener formula (*H*) (Shannon and Weaver 1963):

$$H = -\sum_{i}^{S} P_i \log_2 P_i$$

where $P_i = n_i / N$ (n_i the number of individuals of the *i*th species and *N* the total number of individuals) and *s* the total number of species. High diversity values normally are correlated with high numbers of species and indicate beneficial environmental conditions.

Analyses.. The values of community diversity are influenced by sample size, sampling methodology and identification procedures. Consequently, species diversity values can only be compared if the same sampling methodology has been followed, with equal efforts of taxonomic scrutiny. Only under these conditions of quality assurance, trends or changes in species diversity can be investigated, such as has been done for experimental plots by Simboura and Zenetos (2002) in the Mediterranean Sea.

Quality information

Strength and weakness (at data level):see below
The values of community diversity are influenced by sample size, sampling methodology and identification procedures. Consequently, species diversity values can only be compared if the same sampling methodology has been followed, with equal efforts of taxonomic scrutiny. Only under these conditions of quality assurance, trends or changes in species diversity can be investigated. If Shannon-Wiener is to be used for comparison of historic data, it is essential to know the base that the index has been calculated in (log base 2 should be used). Examining data from Belfast Lough, Northern Ireland, Breen (2001) demonstrated significant differences in the index if the wrong log base is used

Reliability, accuracy, robustness, uncertainty (at data level):
Overall scoring (give 1 to 3 points: 1=no major problems, 3=major reservations):
Relevancy: 1<see Description of elements for definitions>
Accuracy: 1
Comparability over time: 1
Comparability over space: 1

Further work required

Define 5 EQS with data sets from more areas across the Mediterranean. More raw data of macrozoobenthos along pollution gradient is needed.

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Marine Pollution Indicator fact sheet

MED POL

Number of benthic species (S) (zoobenthos)

Key messages

☺The number of benthic species (S) encountered in a well-defined community type. Can be a reliable measure of environmental stress.

😐Reference values (range of values) of S for "normal/undisturbed" communities should be developed.

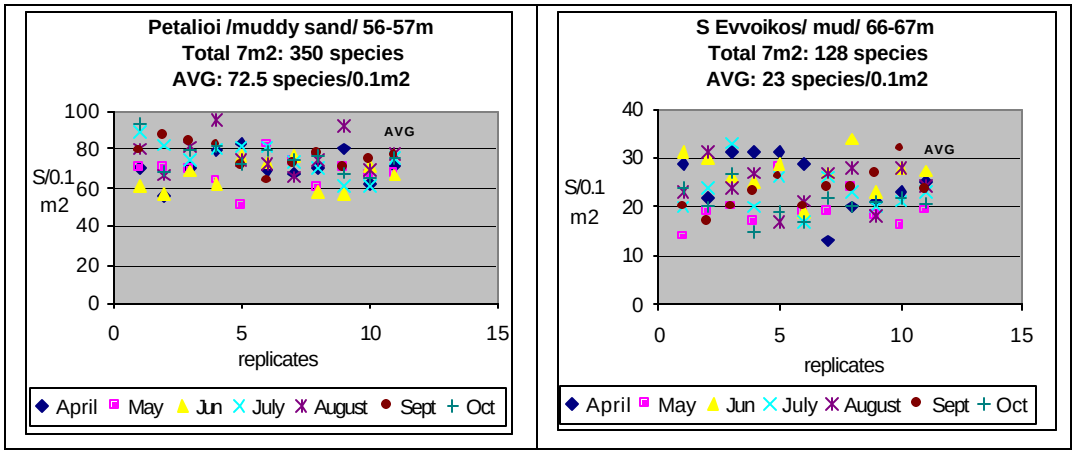

Figure 1: Variation in species number per sampling unit (0.1m^2) and monthly average (0.1m^2) in different habitats (depth, sediment type).
Data from TRIBE project (NCMR 1997)

Table 1: Ranges of species number per biotope/community type

Community type	Peres & Picard, 1964	S /0.1m2 min	S/0.1m2 max
Sands and muddy sands	DE	41	62
Muddy sands with phytal cover		28	100
Shallow muddy sands	SVMC		82
Shallow muds	VTC	18	32
Shallow sands	SFBC, SFHN	28	45
Deeper coarse sands	SGCF	20	124
Deeper sands with detritus	DC	19	82

Source: compiled by A. Zenetos from various sources

Policy relevance: target or objective for the indicator. The most fundamental meaning of biodiversity probably lies in the concept of species richness (May, 1995), that is the number of species occurring in a site, region or ecosystem. Sustainable biodiversity, and moreover halting biodiversity loss are key objectives for developing further S as an indicator of EQS.
Policy context
Anthropogenic activities may cause direct loss and degradation of biodiversity Among the sensitive habitats of the Mediterranean some are occurring in the mediolittoral zone which suffers most from such activities (UNEP RAC/SPA. 1997) and others deeper in the infra and circa-littoral zones (see Table1). The CBD, WFD, CFP are legal frameworks in need of EQS assessment related with various coastal activities.

Environmental context: (scientific soundness and choice and definition of the indicator)
Anthropogenic activities may lead to the temporal or permanent elimination of some species from a given biotope. The number of taxa (S), is generally cited in environmental benthic reports. It has been shown to be useful in detecting typical gradient effects (e.g. Pearson & Rosenberg 1978). Decrease in number of taxa attributed to anthropogenic impact has been also cited in cases of fishing (trawling), dumping, oil pollution etc

For example preliminary results of the differences in areas intensively exploited by trawlers and areas not trawled due to wrecks or other obstacles in NW Mediterranean (Sanchez & Demestre, 2004) have revealed significant changes in the number of species, 56 in the fished zone and 63 in the unfished.

Although S has been shown to be of use in disturbance evaluation along pollution gradients its role in evaluating water quality for the purposes of the WFD is questionable. For example, Breen (2001) investigating the S values determined for the 1993 UK National Marine Monitoring Program (NMMP), showed that the expected patterns for estuaries (high Abundance and low Species number) and coastal sites (low Abundance and high Species number) were not found. The statistics have been shown prone to error as variation is induced by sample size, sieve mesh size, number of replicates and skill of the taxonomist (Breen 2001.

However, investigation of species variety along Greek coasts has shown to be directly related environmental stress (Zenetos & Simboura, 2001; EEA, 2002)

Assessment: The number of species in a benthic community varies greatly with depth and sediment type. A typical trend exhibited within the Mediterranean is a significant decrease in species number with depth. Sediment type is the second most significant factor influencing the species variety in a given biotope. In the two examples presented in Figures 1 it is depicted that different communities (benthic assemblages in certain sediment type/depth) hold different species numbers**.** In the graph, it is clear that species number per sampling unit is not dependent on season. On comparing the species number of a sampling unit with that of the average of either 10 samples (AVG S in figure) or of 70 samples (AVG/0.1m^2 in the legend of figure), **it appears that species number of a given unit (sediment surface area), can be an accurate measure of the state of environmental**. Scattered EIA (Environmental Impact Assessments) across the Mediterranean (see case studies below- have demonstrated the decrease in number of taxa along a pollution gradient but unfortunately there is still no scale to classify a given community to a certain EQS.

Case study: oil spill accident
An example of the effectiveness of S in ecological quality assessment is presented in Table 2 and Figure 2. The reduction in species variety is directly correlated to the stressor. The data is from an impact study following an oil spill in an Aegean gulf. (Table 2). The trend is the same whether calculated on smaller sampling area (mean values) or bigger (pooled samples) (Figure 2).

Table 2: Mid term effects of an oil spill on the macrozoobenthic species variety
Source: NCMR, 2001

Indicator of impact	Before the oil spill (reference values)	1 month after	4 months after	8 months after
S (number of species) At the site of the accident (S. Evvoikos G.) (32m depth)	23 /0,1m2	8 / 0,05 m2	16 / 0,05 m2	17 / 0,05 m2

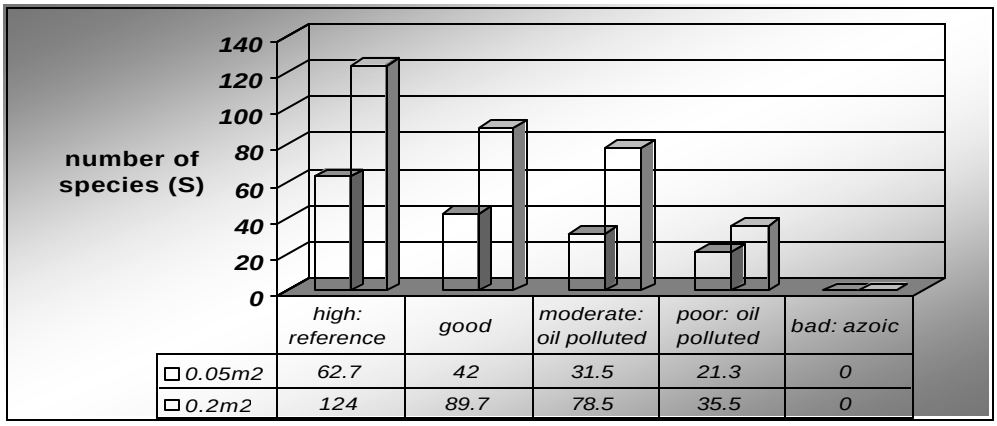

Figure 2: Response of species number to a oil pollution gradient in a given community type (shallow muddy sands). Source (NCMR, 2001).

Case study: Effects of dumping (Portman: SE Spain; N. Evvoikos : Aegean Sea)

Table 3: Definition and range of species number along a gradient as a result of dumping. Data source: Portman Bay (SE Spain): Martin et al in press,; N. Evvoikos: NCMR, 1998)

Portman Alboran Sea			N. Evvoikos : Aegean Sea	
	stress	S	stress	S
PG	dumping site	7 ± 1	dumping site	30 (0,2 m2)
PN		15 ± 3		
CN		8 ± 2		
CM		18 ± 2		
PL		19 ± 4		
PE	reference	26 ± 2	reference	70 (0,2 m2)

Assessment: The decrease in number of species from the reference (26 species) towards the dumping site (7 species) appears to be the most reliable indicator in Portman Bay. For more details see also fact sheets on H and BENTIX.

Case study: Effects of sewage: Izmir Bay

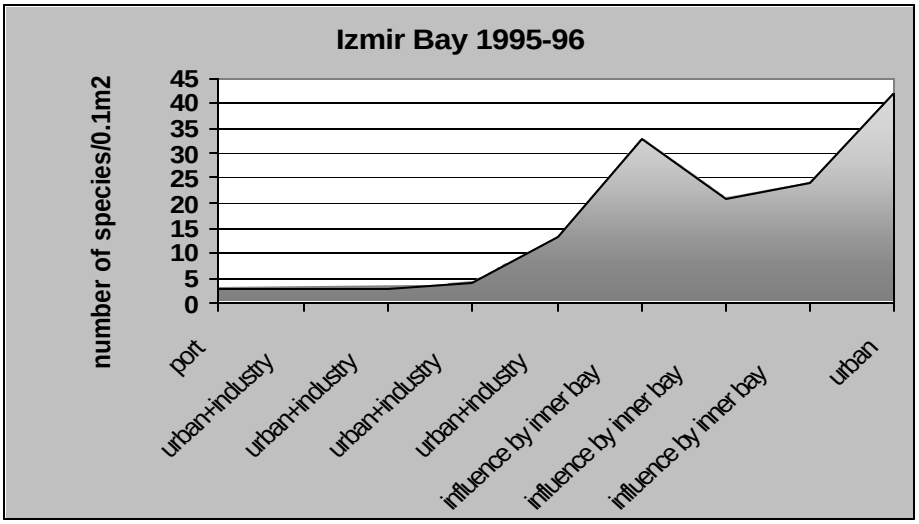

Figure 3. Trends in species number with increasing distance from LBS in Izmir Bay. Source: Dogan, pers. Commun.

Assessment:
A decrease in number of species is evident from the inner to the middle Bay. Results refer only to sandy/muddy biotopes/community types. For more details see also fact sheets on BENTIX
Data Source: Dogan, pers. communication

Case study Fishing with bottom trawlers.
The mid-term impact of intensive (experimental) trawling on the benthic ecosystem was studied in Petalioi Gulfs (Aegean Sea) an area open to trawlers 7 months a year.

Monitoring was carried out for a 6 -month period, during which the area was closed to trawling so that natural recovery could be detected.

Table 4: Total (S) and mean ($S \cdot m^{-2}$) of species number of the infaunal and epifaunal components recorded throughout the study period. (source: NCMR, 1997a)

SANDY SUBSTRATUM				
	Control site (E)		Experimental site (TR)	
	S	$S \cdot m^{-2}$	S	$S \cdot m^{-2}$
Infauna	342	239	302	170
Epifauna	61	42	48	19
Totals	**403**	**281**	**350**	**188**

Table 5: Petalioi Gulf: Aegean Sea

	stress	Depth	substratum	S/0.1 m^2
E24	trawling	59	muddy sand	65
E29	trawling	43	muddy sand	55
E30	trawling	43	muddy sand	77
D	trawling	35-54	muddy sand	76
E	trawling	65-69	muddy sand	79
F	trawling	48-64	muddy sand	79
E13	Ref. Site	47	muddy sand	96

Source: NCMR, 1997a, b

Assessment:
In the sandy area, the experimental trawling resulted in a decrease of species (Table 4). An overall decrease due to trawling by about 20% was calculated (188 species per m2 in the experimentally trawled area vs 281 in the control area).(NCMR, 1997a). When the areal distribution is considered (Table 5) the disturbance due to trawling appears to be about the same. 65-79 species per sampling unit (0.1m^2) vs 96 species in the reference site.

Meta data

Web presentation information

1. Abstract / description / teaser (max 3 lines): **Trends in species variety may be used as an indicator of disturbance.**
2. Policy issue / question (max one line): **Is biodiversity declining/improving?**
3. Dissemination themes: UNEP, EEA
4. DPSIR (one value only): **S**

Technical information

Description of data:

Definition: The number of benthic species encountered in a well-defined community type. By species full taxonomic work is preferable but can be applied even if species are coded as sp1, sp2, sp3….
Analyses.
Methodology of data manipulation,
Simple calculations of means per standard sampling unit (minimum unit is preferable)
Geographical coverage:
Overall assessment based on scant data across the Mediterranean monitoring studies I;e. Saronikos or impact studies, Izmir, from various sources
Case studies from Izmir Bay, Edremit Bay (Turkey), Portan Bay, (Spain), Petalioi Gulf and South Evvoikos Gulf.
Methodology and frequency of data collection
Izmir Bay. data from 9 stations with sandy, muddy sand substratum along a pollution gradient from the inner gulf to outer middle bay. Seasonal data (4 sampling cruises) during the period 1995-96. Van Veen grab 0.1m2, Mesh sieve 0.5 mm
Portan Bay, (Spain). Six stations at depths 10-15m from the mine outlet in Portma´n Bay in the direction of the Marine Reserve of Cabo de Palos-Islas Hormigas. Four replicate sediment samples were collected in March 2002by SCUBA divers using a 0.09m2 hand grab and sieved through a 0.5mm mesh bag. **83 species** in all.

S. Evvoikos TRIBE: 1996-97
S. Evvoikos Aegean. A gradient of coastal stations at an increasing distance for aa accident: Eurobulker oil spill 2001.

Van Veen o.1m2, mesh sieve 0.1mm, 5 replicates. Sampling 1, 4, and 8 months after an oil spill.

Methodology
Methodology of data manipulation,

Number of species should apply
to a well defined sampling unit (standard 0.1m^2)
to samples collected with the same gear (standard grab 0.1m^2, mesh sieve 0.5mm or 0.1mm)
at the same community type (depth range and sediment type). For description of community types see Table in Annex
If identification is being done at the same taxonomic level (4 major groups: Polychaeta, Mollusca, Crustacea, Echinodermata, or all groups)
Quality information

Strength and weakness (at data level):
Strength. Applicable for soft substrata in coastal areas regardless of sample size (mean per standard sampling unit is required).
Limitations: Definition of S should apply
to a well defined sampling unit (standard 0.1m^2)
to samples collected with the same gear (standard grab 0.1m^2, mesh sieve 0.5mm)
at the same community type (depth range and sediment type).
If identification is being done at the same taxonomic level (4 major groups or all groups)

Reliability, accuracy, robustness, uncertainty (at data level):
The species level is required. Genus or upper level of identification is inaccurate.

Care must be exerted on reviewing the relevant papers as the studies may be based on experts in different groups i.e. polychaeta only, molluscan only or at different taxonomic level

Overall scoring (give 1 to 3 points: 1=no major problems, 3=major reservations):
Relevancy: 1<see Description of elements for definitions>
Accuracy: 1
Comparability over time: 1
Comparability over space: 1

Further work required.
Reference values (range of values) of S for “normal/undisturbed” communities should be developed for all different community types (biotopes) to be used for quality assessment studies in disturbed ecosystems. Deviation from reference values will then be indicative for the degree of environmental stress. Subsequently a scale for the 5 ecological quality classes should be developed per community type

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UNEP Marine Pollution Indicator fact sheet MAP

MED POL

Presence/abundance of sensitive/opportunistic zoobenthic species/taxa

Key messages

☺ The presence of sensitive taxa is a reliable measure of ecosystem health.

☹The dominance of tolerant species/taxa is proportionate to the degree of disturbance.

Table 1. Amphipod species present in **fine sands** under different environmental stress (after Bakalem, 2001) (r): rare underlined the dominant species.

Fine sands Reference site	**- Urban pollution**	**Urban+industrial pollution**
Ampelisca brevicornis	*Urothoe poseidonis*	*Pariambus typicus f. armata*
Ampelisca spinipes	*Urothoe brevicornis*	*Atylus swammerdami*
Ampelisca sarsi	**Urothoe grimaldii**	*Ampelisca spinipes*
Ampelisca diadema	*Ampelisca brevicornis*	*Ampelisca sarsi*
Lembos spiniventris	*Lembos spiniventris (r)*	
Urothoe poseidonis	*Ampelisca diadema (r)*	
Urothoe brevicornis	*Ampelisca sarsi (r)*	
	Siphonoecetes dellavallei (r)	
	Lembos angularis (r)	
	Phtisica marina (r)	

Table 2: Species groups according to EQ in **hard substata** (from Bellan-Santini, 1980)

Pure to very pure	**Intermediate**	**More or less polluted**
Hyale	*Amphithoe ramondi*	*Caprella acutifrons*
Elasmopus pocillimanus	*Stenothoe tergestina*	*Podocerus variegatus*
Caprella liparotensis		*Jassa falcata*

Case study: Fine Sands: Oued Laou: Maroc

Table 3. Trend in macrobenthic parameters (including percentage of contribution of tolerant species) along a pollution gradient.(data source: Bayed et al., 2004)

Depth	*S*	H		Dominance
5.1 m,	*28*	1.56	*Ampelisca brevicornis*	78%
5.7m	12	2.04	*Ampelisca brevicornis*	62
5	11	2.25	*Ampelisca brevicornis*	48
5	19	3.81	*Ampelisca brevicornis*	20
10.2	27	4.22	*Ampelisca brevicornis*	13

Assessment: Percentage of prevalence of *Ampelisca brevicornis* (a species tolerant to pollution) is increasing (table 3) as community structure (H) decreases, that is the worst the ecological quality is.

Case study. Fish farming and benthic opportunistic species (SE Turkey)

Dense aquaculture activities in the coastal zone have resulted in decreasing the quality of the sea water and bottom sediments creating many problems to human health, tourism and to the farmers.(Ergen et al., 2004)

Table 4: Community data under cages (A), at the vicinity of cages (B) and at a control site (data source: Ergen et al., 2004)

Date	Station	Site	Depth	Biotope	S	N.	Dominant species
7/96	3	A	12	Muddy sand	30	572	*Protodorvillea kefersteini*
11/99	6	>>	31	Muddy sand	30	*70*	*Lumbrineris gracilis*
11/02	1	>>	50	mud	24	*34*	*Capitella capitata*
2/03	2	>>	25	*Posidonia*	64	*234*	*Paralacydonia paradoxa*
11/99	6	B	31	Muddy sand	15	*20*	*Cirriformia sp*
2/03	2	>>	45	Mud	56	*334*	*Paralacydonia paradoxa*
11/99	6	C	31	Muddy sand	26	*51*	*Nematonereis unicornis*
11/02	1	>>	35	Sandy mud	52	*209*	*Piromis eruca*
2/03	2	>>	45	Sand	44	*440*	*Cirrophorus branchiatus*

Assessment: Work in the Turkish Aegean Sea established that cage farming affect the distribution of the polychaete assemblages eliminating sensitive species and favouring opportunistic species (table 4). Under cages dense populations of *Capitella capitata* 30 ind./m^2 *Protodorvillea kefersteni* 3060 ind./m^2 *Nereis zonata* 30 ind./m^2 and *Lumbrneris gracilis* 180 ind./m^2 prevailed.

Results and assessment

Policy relevance: target or objective for the indicator: Keep biodiversity high by giving sensitive species such an environment that they have the chance to survive

Policy context .The use of indicator organisms for the detection of anthropogenic change appears attractive to environmental managers, to the extent now that the term indicator generally implies a link to pollution studies (Wilson 1994). The WFD specifies the use of 'disturbance sensitive taxa' and 'taxa indicative of pollution' in its definition of ecological status of the benthic invertebrate fauna for both transitional and coastal waters.

Environmental context: (scientific soundness and choice and definition of the indicator) Indicator species of disturbance has been employed for a long time, particularly species that indicate severe disturbance like the polychaete *Capitella capitata* . At the other end of the scale we find species that are particularly sensitive to disturbance, indicative of only minor anthropogenic impact. By combining this accumulated information of tolerant and sensitive species, several biological indices have been developed over the last years

Assessment Several scientists have tried different methods to classify the sensitity or tolerance of benthic organisms to various degrees of disturbance. Summary of some published work dealing with sensitive and tolerant benthic species is presented in Rosenberg et al (2004). It is clear that quality assessments, based on literature, are mostly subjective.

(Table 5). Rosenberg et al (2004) determined tolerance values to environmental stress in an objective analyses for benthic species a long the Swedish coasts.

In the Mediterranean, benthic axa indicative of environmental disturbance have been reviewed by Orfanidis et al. (2001) [phytobenthos] and by Borja et al (2000), & Simboura and Zenetos (2002) [zoobenthos] who have compiled preliminary lists. These species may be present in more than one community types. However, species density is different among community types and reference levels and EcoQOs should be formulated for every species for each community type separately. The mean densities could be seen as highest estimates of the reference levels. Reference levels are formulated for minimal human pressure. Reducing human pressure should therefore decrease the densities of these opportunistic species.

At a higher taxonomic level it has been established that some taxa such as **echinoderms** and **amphipods** are sensitive to environmental stress. An inverse relationship was found between the richness of the Amphipod population and the degree of pollution in hard substrata (Bellan-Santini, 1980). **Polychaete** species belonging to families like Spionididae, Capitellidae and Cirratulidae are considered good candidates for this indicator. The usage of polychaetes in assessing impact from river inputs and river discharges has been demonstrated by many workers and recently by Cardell et al. (1999).

Table 5. Published work dealing with sensitive and tolerant benthic species (from Rosenberg et al, 2004)

Authors	**Sensitive/tolerant species**	**Habitat assessment**	**Remarks**
Reish (1955)	Own data	5 zones identified	Subjective
Pearson & Rosenberg (1978	Literature data	4 successional zones	subjective
Gray & Pearson (1982)	Log-normal distributions	No	objective
Grall & Glemarec (1997)	Literature, own experience	5 ecological groups	subjective
Borja et al (2003)	Literature, own experience	7 classes	subjective
Simboura & Zenetos (2002)	Literature, own experience	5 quality status groups	subjective
Weisberg et al (1997)	Probably literature data	17 variables used	Subjective / objective
Rygg (2002)	Diversity index	Indicator species index	objective
Rosenberg et al, 2004	Diversity index	Faunal quality assessment	objective

Subindicator: Abundance/Coverage of key species

Rapid assessment techniques (e.g. rapid ecological assessment or side-scan for landscape diversity) and in particular specific surveys of species considered as "key-species" for marine biodiversity are gaining increased attention. *Among the species of a region, "key-species" are those that contribute to the architectural, trophic and functional complexity of a marine ecosystem*. Those which according to the BIOMARE (10.2001 workshop) have been cited as directly related to known stressors, are tabulated in Table 6. To these we must add the endemic sponge species *Petrobiona massiliana* and *Ircinia cheuvreuxi* which consist important biotopes for many endobiotic, epibiotic, symbiotic organisms and can attain large

densities (up to 409 ind./dm3: Rutzler, 1976). Air borne photography for example is a fast way to define surface versus potential surface of coverage of key phanerogams or sponges.

Table 6: species cited as "key-species" for the Mediterranean region BIOMARE (updated 10/01)

	Species	Type (Rare, endemic, keystone, threatened, biogenic building, emblematic)	Known stressors
SPONGES	*Spongia spp.*	**Commercial, endemic, threatened**	**Fishing, climate change**
	Asbestopluma hypogea	**Endemic**	**Global change**
	Oopsacas minuta	**Endemic**	**Global change**
CNIDARIANS	*Cladocora caespitosa*	**Builder**	**Climate change**
	Corallium rubrum	**Commercial, endemic**	**Fishing, climate change**
	Eunicella spp.	**Keystone**	**Climate change**
	Paramuricea clavata	**Keystone, endemic**	**Climate change, fishing, diving, shipping, anchoring**
ECHINODERMS	*Centrostephanus longispinus*	**Threatened**	**Climate change**
CRUSTACEA	*Scyllarides latus*	**Threatened, commercial**	**Fishing**
MOLLUSCA	*Lithophaga lithophaga*	**Threatened**	**Loss of habitat, fishing**
	Patella ferruginea	**Threatened**	**Loss of habitat, tourism**
	Pinna nobilis	**Threatened**	**Loss of habitat**

Case study: Mortality of key-species of sponge s in north Tunisia

Table 7: Density of gorgonians (colonies/m2)
source: Ben Mustafa & A.El Abed, 2001

	Cape Bon	E Zebra & Zembretta	Zone of Sidi Daoud	SE Zebretta	W Zebretta	Tabarka
Eunicella singularis	8 to 10	17 to 25	50	40	35	70
Eunicella cavolinii	2 to 3	3 to 5	25	15	absent	absent

Assessment: The phenomenon of massive mortality of marine invertebrates is not rare in the Mediterranean. Mortality of sponges has been reported in NW Mediterranean by Perez et al. (2000) as well as by other workers. However, the degradation of a rich in biodiversity ecosystem such as that of the marine park of the isles of Zebra (Tunisia) is clearly evidenced by the distribution (measured as population density of sponges: Key species). It is assumed that the degradation is related to the presence of *Caulerpa racemosa* and *Caulerpa taxifolia.*

Meta data

Web presentation information

Abstract / description / teaser (max 3 lines):
Policy issue / question (max one line):
dissemination themes: UNEP, EEA
DPSIR (one value only): **S**
Technical information

Main data source
Description of data

Definition:: The presence of indicator species mentioned ether as sensitive (fragile, and slowly reproducing) or opportunistic (small, short-lived, species). The presence can be expressed either as absolute density per m^2 or as relative abundance in percentage. This parameter/indicator is also used to calculate other community indices and highlight changes in species diversity

Analyses
Geographical coverage: case studies from Mediterranean coastal waters

Temporal coverage: scant data from case studies
Methodology and frequency of data collection

SE Turkey: Benthic samples collected with a grab sampler/ dredge and by scuba diving from 6 stations under cages (A), at the vicinity of cages (B) and at a control site. Samples were sieved through a 0.5mm mesh. 256 species, 2795 individuals identified to species level.(data source: Ergen et al., 2004)

Algerian coasts (bay of Algeria , Bou Ismail, gulf of Jijel) Van Veen grab 0.1m^2, mesh sieve 1mm. Quantitative samples from November 1984 to July 1986. 548 species but emphasis on Amphipoda. (Bakalem, 2001)

French coasts : Review paper by Bellan-Santini, 1980

Marocco: Oued Laou: 20 stations at 5-10m depths, April 2003, Dredge samples, mesh sieve 1mm **(**Bayed et al., 2004) All community parameters estimated.(Bayed et al., 2004)

Tunisia: 50 stations in north Tunisia by autonomous scaphander (70 observations) at depths down to 62m. (Ben Mustafa & A.El Abed, 2001)

Methodology of data manipulation
Because densities of sensitive (vulnerable) species are community dependent, reference levels will vary among indicator species and community. The mean densities could however be regarded as lower estimates of the reference levels. Suggestions for sensitive indicator species on phyto and zoobenthic communities are given in Orfanidis et al. (2001) and Simboura and Zenetos (2002). The EUNIS biotope classification programme promises a valuable basis for evaluation of indicators according to community type (Connor, 2000).

Quality information

Strength and weakness (at data level):see below
The presence of sensitive taxonomic units (species/genera/taxa) is community dependent, reference levels will vary among indicator species and community. Therefore absence from a community type does not necessarily imply disturbance. The mean densities could however be regarded as lower estimates of the reference levels. Disadvantages: up-to-date unable to distinguish the 5 classes.(bad to high).

Reliability, accuracy, robustness, uncertainty (at data level):
Overall scoring (give 1 to 3 points: 1=no major problems, 3=major reservations):
Relevancy: 1<see Description of elements for definitions>
Accuracy: 2
Comparability over time: 2
Comparability over space: 2

Further work required

- ✓ Reference levels to be established from pristine communities (human pressure minimum to non-existing).
- ✓ An agreed list with sensitive taxa characterizing different communities within the Mediterranean with reference levels needs to be constructed.
- ✓ Reference levels and scales for EcoQs should be established for indicator species for each benthic community.

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Marine Pollution Indicator fact sheet

MED POL

Presence and Coverage of Benthic Macrophytes (Sensitive and/or Opportunistic)

Key messages

☺Presence of Sensitive Benthic Macrophytes is indicative of good ecological quality

☺ Depth limit and density of roots are successfully employed in assessing ecological quality status/changes

😐 Presence of opportunistic benthic Macrophytes (such as some newly introduced macroalgae) may be indicative of environmental degradation.

Benthic macrophytes (marine Angiosperms and macroalgae) are a common biological element along the Mediterranean coastline. The Cystoceira settlements together with the Posidonia meadows are the main carriers of biodiversity in the infralittoral zone. Since these settlements are best developed between the surface and 10 m depth, they are often exposed to marine pollution. The most typical example is that of *Posidonia oceanica* , which is included in Key species for the Mediterranean region (Table 1) and its population is therefore monitored as "*Populations of Key species including protected ones*".

Table 1: Species of seagrasses and seaweeds cited as "key-species" for the Mediterranean region BIOMARE (updated 10/01)

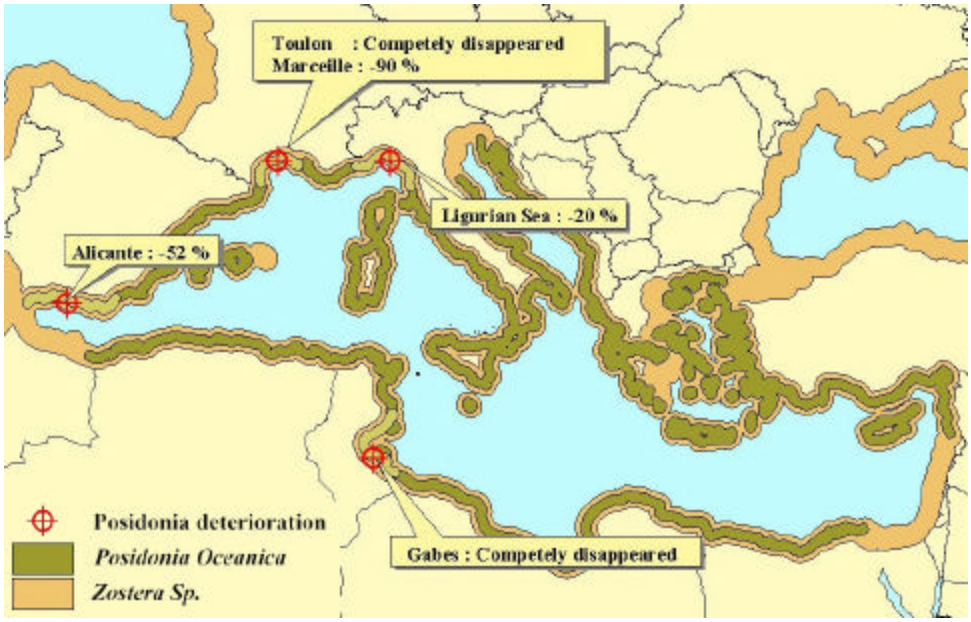
Species	Type (rare, endemic, keystone, threatened, biogenic building, emblematic)	Known stressors
Marine Angiosperms (seagrasses)		
Posidonia oceanica	**Endemic, keystone**	**Eutrophication, pollution, turbidity, invasive species etc.**
Ruppia maritima	**Threatened**	**Eutrophication, pollution, turbidity, etc.**
Zostera noltii	**Threatened**	**Eutrophication, pollution, turbidity, etc.**
Cymodocea nodosa	**Keystone**	**Eutrophication, pollution, turbidity, etc.**
Macroalgae (seaweeds)		
Cystoseira stricta	**Endemic, keystone**	**Eutrophication, pollution, turbidity, etc.**
Cystoseira mediterranea	**Endemic, keystone**	**Eutrophication, pollution, turbidity, etc.**

Table 2: Mediterranean countries where benthic macrophytes are studied

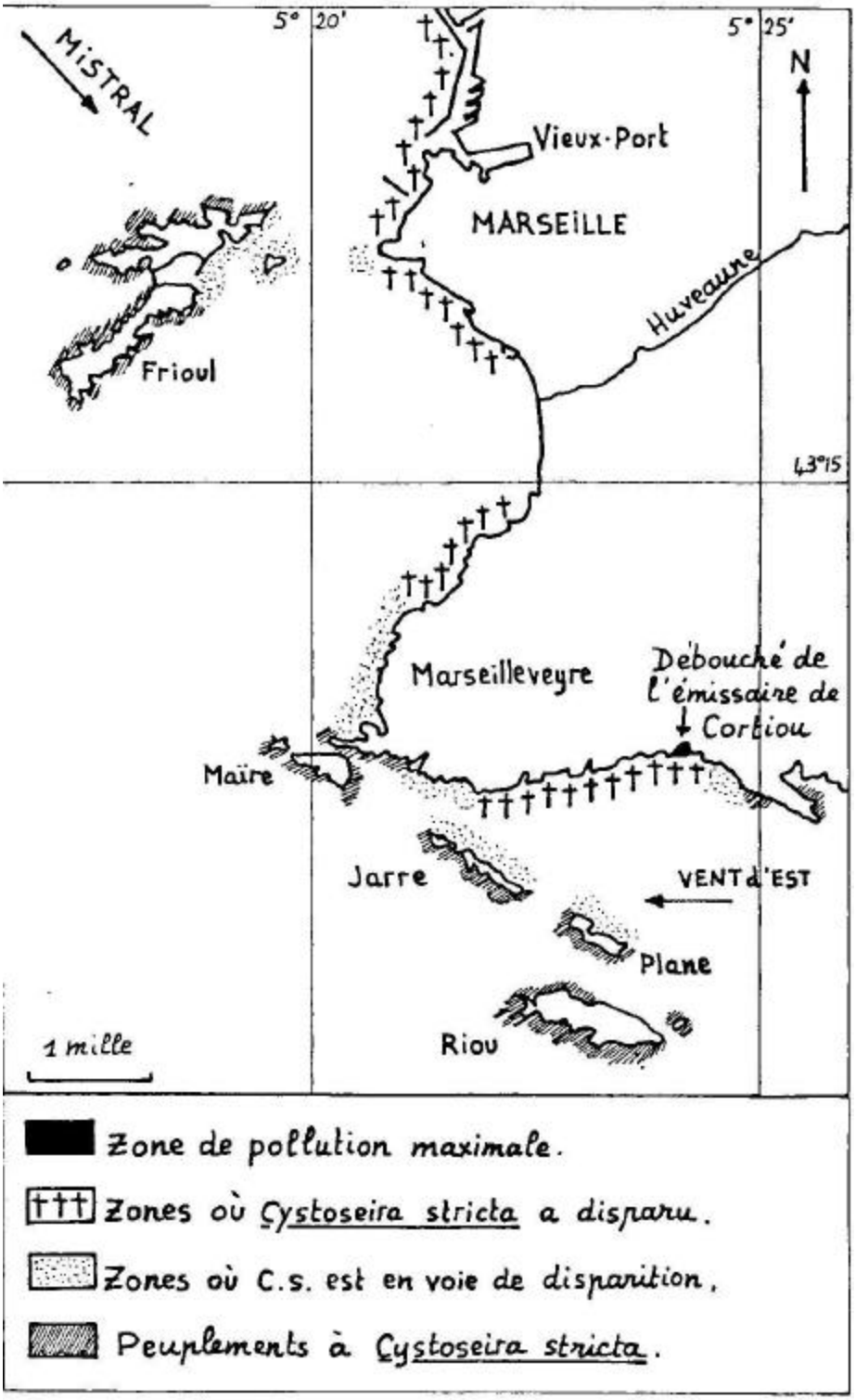
	National monitoring	Regional monitoring	Specific area/experts
France	No	Yes	Parc National de Port-Cross: Prof. Ch. F. Boudouresque Boudouresque@mailhost.com.univ-mrs.fr Parc regional de Corse Prof. G. Pergent
Greece	No	Yes	Thermaikos Gulf: Prof. Haritonidis haritoni@bio.auth.gr Saronikos Gulf: Dr. Panayotidis ppanag@ncmr.gr N. Aegean coasts & lagoons: Dr. S. Orfanidis,sorfanid@otenet.gr
Italy	No	Yes	Tyrrhenean & Ligurian Sea: Prof. Cinelli Cinelli@discat.unipi.it Sicily: Prof. G.Giaccone giaccone@mbox.dipbot.unict.it Prof. G. Furnari g.furnari@mbox.dipbot.unict.it Prof. M. Cormaci cormaci@mbox.dipbot.unict.it
Spain	No (?)	Yes	Prof. A. Santolaria gvbsadea@lg.ehu.es Dr. E. Ballesteros, kike@ceab.csic.es Catalunia: Dr. E. Ballesteros
Turkey			
Cyprus			
Syria			
Lebanon			
Egypt			Youssef Haim- youssefhalim@hotmail.com Dept. of Oceanograhy, Faculty f Science Univ. Alexandria, Egypt
Croatia			Boris Antolic: antolic@izor.hr Institute of Oceanography & Fisheries Split, Croatia

Source: compiled by P. Panayotidis UNEP/MAP

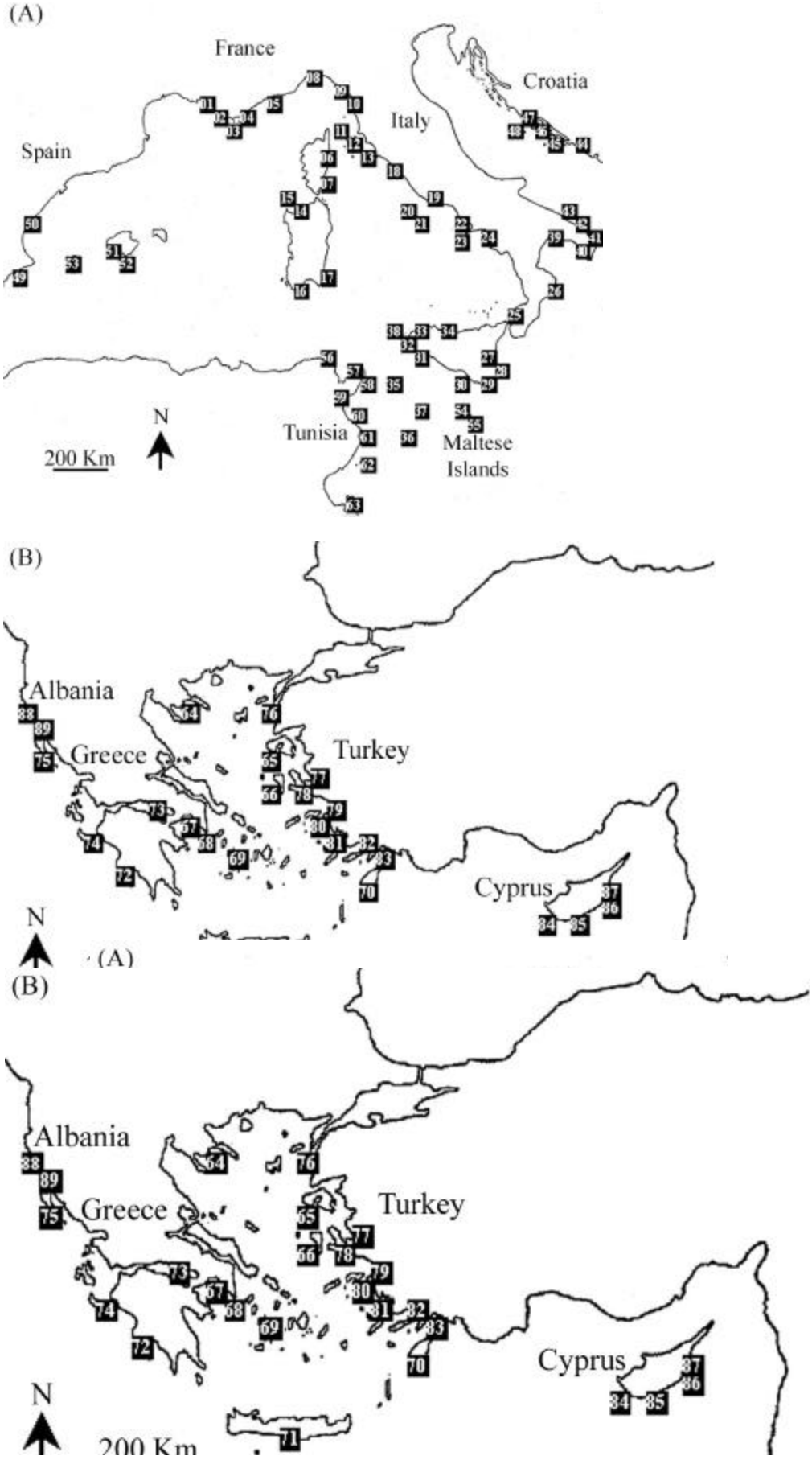
Distribution of the marine Angiosperm *Posidonia oceanica* and *Zostera* in the Mediterranean
Source: EEA, 2004



Distribution of *Cystoseira* in Marseilles area **(Belsher, 1977)**



Case study: Distribution of *Caulerpa racemosa* Source: Piazzi et. al. (2004)



Reports on *Caulerpa racemosa* invasion

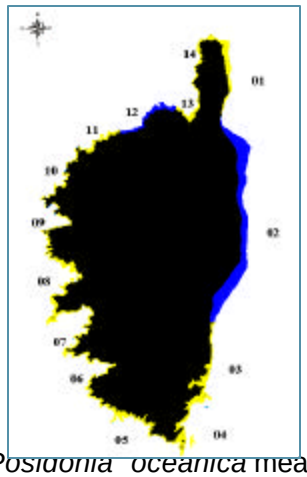
Recorded for the first time in the Mediterranean in the early 1990s in Libya (Nizamuddin, 1991), the invasive *C. racemosa* appeared during the same period in different parts of the basin (Alongi *et al*., 1993; Panayotidis & Montesanto, 1994; Piazzi *et al*., 1994). The species showed traits of invasiveness right from its first phases of spread (Piazzi *et al*., 1997a). Thirteen years later, nearly the whole Mediterranean basin is colonized and the Canary Islands have just been reached (Verlaque *et al*., 2004).
Although highly invasive, *C. racemosa* has not been the subject of large-scale research projects to describe its expansion. Several studies have been carried out on the ecology of this species, but its spread was not quantified at a Mediterranean level.
Piazzi et. al. (in press) reports that *Caulerpa racemosa* has been recorded along the coasts of 11 nations, developing on all kind of substrata, both in polluted and in unpolluted areas, between 0 and 70 m depth. The length of coastline affected by the invasion of *C. racemosa* in Spain, France, Italy and Croatia ranged from 700 to 750 km at the end of 2003.

Aranda (2004) reports the presence of *Caulerpa racemosa* in Valencia area (Spanish coast). In 1999, about 3 Km^2 of the bottom surface were occupied by *Caulerpa racemosa* at Castellon. In 2000, the species was found in Alicante and a cartography carried out in 2002 showed that 10 Km^2 of the bottom surface were occupied, corresponding at 18 Km of coast line. In 2002, the species was also found at Sagundo (Valencia) and in 2003 at Tabarca (Marine Park) where only 3.000 m^2 of the bottom surface were occupied.

Subindicator: Depth limits of meadows

Case study from Corsica

Depth	Status
> 35 m	High/good
25-35 m	Moderate
15-25 m	Poor
< 15m	Bad

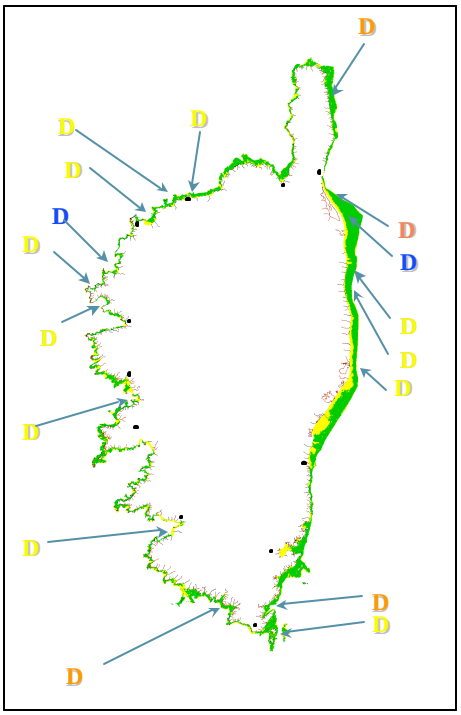


Notes: Scheme based on the lower depth limits of *Posidonia oceanica* meadows

Source: *By permission of F. Bruchon (Seine Normady Water Agency) and G. Pergent (University of Corsica), France*

Subindicator: Density (D): number of *P. oceanica* shoots per m²

depth	Density / Interpretation						
2	¬	646	«	758	«	982	
4	¬	470	«	582	«	806	
6	¬	367	«	479	«	703	®
8	¬	294	«	406	«	630	®
10	¬	237	«	349	«	573	®
12	¬	191	«	303	«	527	®
14	¬	151	«	263	«	487	®
16	¬	117	«	229	«	453	®
18	¬	88	«	200	«	424	®
20	¬	61	«	173	«	397	®
22	¬	37	«	149	«	373	®
24	¬	14	«	126	«	350	®
26			«	106	«	330	®
28			«	87	«	311	®
30			¬	70	«	294	®
32			¬	53	«	277	®
34			¬	38	«	262	®
36			¬	23	«	247	®
38			¬	10	«	234	®
40			¬		«	221	®



Results and assessment

Policy relevance

Marine Angiosperms (seagrasses) and macroalgae are key elements for the application of the "habitat" Directive (92/43/EEC). They are used for the description of the habitat types 1120 ("Posidonia meadows") and 1170 ("reefs") respectivelly.

They also consist a “quality element” for the classification of marine coastal areas as described in the Water Framework Directive (WFD, 2000/60/EC).

Policy context (relevance of the indicator with reference to specific policy processes)

Posidonia oceanica, Cymodocea nodosa, Zostera noltii and *Cystoseira* species are mentioned as endangered ones in the Barcelona Conservation.

A specific Action Plan entitled "Mediterranean Marine Vegetation" is included to the UNEP MAP.

The marine Angiosperms (seagrasses) are already used in some EU states monitoring projects (e.g. France

At the EU level, for the implementation of the WFD the monitoring of phytobenthos is foreseen. The "good ecological status" for all waters bodies by 2015 is the goal of the WFD. Water management based on river basins "combined approach" of emission limit values and quality standards getting the citizen involved more closely streamlining legislation, is the basic concept.

Environmental context: (scientific soundness and choice and definition of the indicator)

The marine Angiosperms meadows (seagrasses) form the structural base for some of the most productive ecosystems of the world, including rocky and soft bottom intertidal and subtidal zones, reefs lagoons and salt marches

Seagrasses are perennial sessile organisms, and the different populations respond to abiotic and biotic aquatic environment, thus representing sensitive indicators of its changes (Table 1). Some species are considered to be indicators of high ecological quality (Pergent, 1991) especially when historic data – reference conditions exist. Typical examples is the species *Posidonia oceanica* in the Mediterranean

An index based on changes in coverage of seagrasses could be based on estimate of their abundance on hard and soft substrata. The abundance is usually expressed as surface (in hectares) covered by the macrophytes. A linear approach could also used (km of coastline), especially for seaweeds, which are developed on hard substrata as a narrow belt. On the other hand the simple presence of seagrass meadows in a given area does not mean that its ecological status is high. The density of shoots/m^2, the lower limits of the meadow, as well as the structure of the benthic community related to the meadow have to be considered in order to estimate the ecological status of the area, in the terms of the Water Frame Directive (2000/60/EC).

In the case of *Posidonia* meadows there is already a good basis for the typology (Giraud, 1977, pollution impact (Panayotidis 1980, Pergent 1987) and cartography (Paillard et al., 1993)

Assessment

Classification of the Ecological Status (in terms of the WFD) based on the depth limits of eelgrass *Zostera marina* is carried out in the Mediterranean Corsica, France The specific aims are to assess “reference conditions” in “water body types” and to evaluate the use of “type-specific” reference conditions and classification

However, reference conditions may vary markedly within a given water body type; Type-specific reference conditions therefore imply a risk of misinterpretation of ecological status; and Site-specific reference conditions seem to be a robust alternative

Mediterranean first results indicate that improvement of sewage treatment along the French Mediterranean coastline (reduction of pollution-induced regression) and direct protection regulation of *P. oceanica* (since 1988), has lead to improvement of the meadows (until 1990:

50% sites were in regression; after 90-93: 27 % are in regression, 46% are stable and 27 % are in progression)
Other parameters have been considered in the French monitoring for the classification of the Ecological Status (in terms of the WFD) namely:
1. upper limit (0-15m) of *P. oceanica* meadow: aeroplane survey, marking
2. vitality of *P. oceanica*: density, coverage, rhizome state, biometry
3. contamination of leaves of *P. oceanica*: heavy metal concentrations
4. GIS Multicriteria analysis

Data;
Case studies were used to demonstrate the usefulness of the indicator:
Mediterranean Sea: F. Bruchon (Seine Normady Water Agency) and G. Pergent (University of Corsica), France

Meta data

1. *Data source*: literature review
2. *Description of data:*

For Caulerpa racemosa In France there are large populations in the Gulf of Marseille and in the Bays of Toulon, Hyères and Villefranche-sur-Mer (Verlaque *et al*., 2000; Belsher *et al*., 2003; LEML-UNSA, 2003; Meinesz *et al*., 2003). Wide areas have been colonized in Libya (Nizamuddin, 1991), Greece (Panayotidis & Montesanto, 1994; 1998; 2001), Albania (Di Martino & Giaccone, 1995), Cyprus (Argyrou *et al*., 1999), Spain (Ballesteros *et al*., 1999; Aranda *et al*., 2003), Maltese Islands (Stevens, 1999; Mifsud *et al*., 2004), Tunisia (Djellouli, 2000; Langar *et al*., 2003), Turkey (Tolay *et al*., 2001; Cirik & Akçali, 2004) and Croatia (Žuljevic *et al*., 2003). In Italy, the spread of *C. racemosa* appears particularly threatening: the alga is now present along the coasts of Sicily (Giaccone & Di Martino, 1995; Serio & Pizzuto, 1999; Calvo, unpublished data), Liguria (Bussotti *et al*., 1996; Modena *et al*., 2000; Peirano, unpublished data), Tuscany (Piazzi *et al*., 1997a; 1997b; 2001; De Biasi *et al*., 1999), Sardinia (Cossu & Gazale, 1997; Cossu *et al*., 2003), Campania (Gambi & Terlizzi, 1998; Buia *et al*., 2001; Buia *et al*., 2003), Apulia (Buia *et al*., 1998; Bottalico *et al*., 2002; Costantino *et al*., 2002; Cecere & Petrocelli, 2004), Calabria (Cantasano, 2001; Di Martino, 2001) and Latium.

Geographical coverage:
Mediterranean coasts
Temporal coverage:
1984-2003,
Methodology and frequency of data collection:
per case study
Methodology of data manipulation, including making 'early estimates':
see comment on data case study
Quality information

Strength and weakness (at data level):

see comment on data

3. Reliability, accuracy, robustness, uncertainty (at data level):

see comment on data

4. Overall scoring 2

Relevancy: 1
Accuracy: 2
Comparability over time: 1
Comparability over space: 2

Further work required

The more sensitive coastal habitat types in the Mediterranean are defined and partly mapped (Spain, France, Italy, Greece). This could be easily accomplished for ALL Mediterranean countries if a protocol for rapid assessment surveys is developed and agreed upon. Based on the changes in habitat distribution of a few “Key species”, a clear sign of environmental degradation will be easily discerned and quantified. Rapid assessment techniques (e.g. rapid ecological assessment or side-scan for landscape diversity) and in particular specific surveys of species considered as “key-species” for marine biodiversity are gaining increased attention.

Although for the majority of Mediterranean countries the development of such schemes at national level is at early stages, the implementation of WFD will enforce them to develop monitoring and classification schemes based on marine benthic macrophytes. When these will be implemented the development of a proper fact sheet will take proper form and place.

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BIOMARKERS

Data management of Biomarkers: Tools for environmental management

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

Monitoring programmes have generally focused on traditional methodologies for assessing contamination but biomarkers are used increasingly to assess the environment. Many studies have, however, focused on single test organisms or limited arrays of biomarker responses and, in particular, there is little published work documenting the systematic use of multiple biomarkers to assess the health condition of complex ecosystems (Adams and Ryon, 1994). Thus, the multi-biomarker approach may be considered to be similar to common procedures in human epidemiology where many responses are interpreted to diagnose disease. Different data management systems were developed in order to provide usefull tools to environmental managers and to integrate biomarker in the routine methodology for environmental monitoring.

Rough set analysis was first used to classify sites and identify important biomarkers for specific questions (exposure, effects, environmental signification). This type of analysis is particularly useful since it is a simple and efficient method for classifying multivariable biomarker data and, furthermore, it is free from distributional assumptions. In order to standardize this approach and to give decision makers enough information expressed in a simplified form, we have developped a scoring approach (***Multi Marker Pollution Index, (Index)***, Narbonne *et al.,* 1999) for biomarkers of exposure. An ANOVA analysis was used to determine statistical significance of the individual biochemical variables among sites. Tukey's test was used to determine significance for individual variables between sites to determine the integrated response of mussel to the environmental conditions at each sampling site. All the individual biomarkers were considered jointly within a multivariate context using a canonical discriminant analysis procedure (Statistica software 6.0 StatSoft, Inc. Ed., 2002). A variable selection procedure (Adams *et al*., 1999) was also used to identify and select those variables that contributed most to the discrimination among the integrated biomarker response for each site. Moreover, a scale based on biomarkers of response must be able to be sensitive to different types of pollution. Therefore five biomarkers of exposure must be selected for scoring approach, among 5 clusters.

Contamination by Heavy metals: AChE, AOE, MT
Contamination by PAHs: EROD, BPH, GST
Contamination by Organic chemicals: GST, AOE

Contamination by Pesticides: AChE, GST, AOE
***Non specific stress* : NRR, Lysosomal parameters**

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

In lieu of a common expression of biomarker results (increased or decreased activity) we develop a simple scoring approach to provide a relative comparison among sites that exhibited mutiple biomarker responses. Multimarker pollution index (Index) for each site is calculated as the sum of each Biomarker pollution index (BPI). Finally a pollution scale was established including five levels (from lightly to highly contaminated). The Index of each site was converted in pollution level and associated to a colour (red, orange, yellow, green and blue for classes from 5 to 1). These colours may be reported on the map of the collected sites in order to visualize easily hot spots and the temporal changes in effects of pollution. The Index classification scale from 1 to 5 (Narbonne *et al.*, 1999) was firstly applied in European BIOMAR Program in Mediterranean sea (Greece, France, Italy and Spain) and Baltic Sea (Germany Poland) (Narbonne et al. 2001), a and validated during BEEP EU program (Italian, French and Spanish working sites, Narbonne et al 2005). Index was also used for monitoring impact of ERIKA oil spill in Britanny coast during 3 years. Moreover this procedure was applied to fresch water systems both in mesocosm and field conditions, by using both molluscs (corbicula and dressenia) and in fish, in connection with Water Agencies in order to be applied in the context of Water Framework Directive (Basseres el al. 2004, Vidal et al. 2001).

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

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

A rapid assessment of marine pollution (RAMP) programme has been developed by scientists from Plymouth University, UK (Wells *et al.*, 2001). The assessment included biomarkers of cellular (cell viability, lysosomal integrity) and physiological (heart rate, condition index) status, measures of genotoxicity (micronucleus formation) and immunotoxicity (spontaneous cytotoxicity). The relationships between biological responses and environmental data were investigated in Europe and South America. The RAMP program and its procedures have been disseminated to many countries through training workshops in the Central American region. Another RAMP pilot project was initiated in Vietnam in June 2000 (Bui *et al*., 2000).

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

The function of the Expert System is to rank the level of the pollutant-induced stress syndrome by integrating the data obtained from:

a) Early warning biomarkers: i.e. sensitive biomarkers of stress, or of exposure, revealing the effects of pollutants at the molecular and/or cellular level.

b) Biomarkers of stress, suitable to reveal the development of the stress syndrome at the tissue/organ level: i.e. histological biomarkers, but also biochemical biomarkers such as the GST (Glutathione Trasnferase) test recently developed (i.e. evaluation of the GST released from the cells and present in molluscan haemolymph).

c) Biomarkers of stress at the organism level: i.e. biomarkers able to show that the stress syndrome has decresed the mussel's capacity of survival and/or growth and reproduction (such as stress on stress response, scope for growth, gonad and gamete alterations, survival index).

A good interpretation of the development of the stress syndrome by the expert system depends on the possibility to utilize biomarkers of stress able to integrate the toxic effects of pollutants over the caging period. Among these, are those biomarkers that show a trend characterized by a continuous increase o decrease in the value of the selected parameter (such as lysosomal membrane stability, lysosomal lipofuscin accumulation, lysosomal neutral lipid accumulation , micronuclei frequency) in relation to an increase in toxicity.
Moreover, the expert system takes into account possible interferences among the different biomarkers.

Practical applications

The biomarker sampling campaigns are communly based on mussels or fish collected in sea ecosystems. However, the utilisation of fish in biomonitoring programmes poses some problems related to sampling of wild organisms and to collection system (net stress). In the other hand, the caging of specimens obtained from farms, and to the transport of samples to the laboratory for the analyses also induce stress and technical difficulties: however, due the importance of fish in the trophic chain and as commercial resource, the study of the effects of pollutants on these organisms cannot be underestimated and still is of utmost importance. An innovative approach for the organization of biomonitoring programs that utilizes molluscs as sentinel species may be based on the use of caged organisms for a better standardisation of the results and reduction in sample variability. Caged mussels should be maintained for 4 weeks at about 4 m depth at selected sites. This period of time is sufficiently long so that the biological effects of pollutant can be revealed, at the same time minimizing possible differences in gonad development that may rise in animals maintained at different sites due to exposure to local changes in environmental parameters, such as temperature and food availability.

Moreover, it is worth pointing out that the utilisation of caged organisms allows to correctly relate the results of chemical data (i.e. the amount of pollutants accumulated by mussels during the caging period) to the observed biological effects. This relationship cannot be clearly established in wild animals, due on one hand to the fact that pollutants may have very different biological half life, ranging from days (i.e. copper) to years (i.e. POPs), on the other that chemicals may be accumulated into non toxic forms in different tissues (in granules and lysosomes, bound to proteins etc…). Moreover, wild organisms may show different stages of gonad development and therefore differences in metabolism at the sampling time in different coastal areas.

A final important point in the organisation of a large biological “mussel watch” is the need to reduce the costs of the programme. It is in fact not feasible to ask environmental agencies to utilize a large number (10-15) of different biomarkers on mussels sampled in each site.

Therefore, a 2 tiers approach is suggested:

Tier 1) Utilization, as a first screening approach, a set of 2 to 5 sensitive and low-cost stress biomarkers, such as lysosomal membrane stability and/or lysosomal lypofuscin accumulation, or simple enzyme activities (AChE, GST, CAT) in samples obtained from all the sites of the biomonitoring programme.

Tier 2) On those selected sites where mussels show significant changes in selected biomarkers, utilization of the full battery of 8/12 biomarkers, and quantification of the stress syndrome utilising the expert system. Moreover, in these samples, the biological effects of pollutant should be related to the accumulation of toxic chemicals in the organisms.

Conclusions and recommandations

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

Adequate data management was now available leading to an integrated approach for biomarker selection and integration in site classification related to pollution level (Index) and pollution impact (Expert System). For Index approach based on a minimum (5) of biomarker of exposure, some limitations were identified (false negative results) in some limits conditions (hot spots). Thus Index procedure appeared to be particularly adequate for monitoring trends in low or moderately polluted sites. The integrated approach by Expert System, is able to integrate batteries of biomarkers of exposure and toxic effects measured at different levels (from genes to tissues) may constitute a general methodologie for multimarker data management in specific areas hot spots and pollution gradients.

Historical data in field application of biomarkers were produced from the last twenty years and disign and validation if practical approaches were carried out during national or international programs in fresh water (VALIMAR in Germany, FISH BIO in France) and in marine waters (BIOMAR, BEEP, Black See biomarker-based biomonitoring programme, IOC-IMO-UNEP funded programme of Global Investigation of Pollution of the Marine Environment).

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

In the actual state, Biomarkers provide, in a monitoring programme, what other methodologies, such as, the traditional measurement of chemicals in the environment or organisms, cannot. By their very nature, many biomarkers give information on response to pollution and this allows the environmental manager tools for site classification related to biological responses (i.e. classification from blue to red with Index or from A to E with Expert System). Moreover biomarker measurements provide data tofocus chemical analysis only on those contaminants which are main impacts. New tools in data management may now

possible biomarker-based monitoring programmes and risk assessments, such that financial resources are used wisely and cost effectively.

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Marine Pollution Indicator fact sheet

MED POL

Acetylcholinesterase activity in mollusc cells

Key message

In molluscs the acetylcholinesterase activity can be considered a biomarker of stress. A biomarker of stress is a biological parameter which value may change in relation to the toxic effects of pollutants.

A biomarker of stress is able to integrate the biological response to the total charge of pollutants bioavailable for the sentinel organisms.

Acetylcholinesterase activity is a simple, low cost, reproducible stress biomarker; it shows a low level of sensitivity in molluscs.

In fish this biomarker can be considered a biomarker of response showing an high sensitivity in particular to carbammate and organophosphoric compounds.

Policy relevance

Acetylcholinesterase activity was adopted as stress biomarker in different international programs such as the UNEP MAP Mediterranean Biomonitoring Program. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was considered a “core” biomarker.

Policy context

UNEP MAP Mediterranean Biomonitoring Program, RAMOGE activity and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate stress response in fish and molluscs.

Environmental context

Cholinesterases (ChEs) represent a well-known class of serine hydrolases. They are considered ubiquitous enzymes whose function is to eliminate acetylcholine from the synaptic cleft. The increase in the use of organophosphate (OP) and carbamate pesticides, two classes of compounds that are well known inhibitors of ChE activity at very low concentrations, posed the problem of the possible effects of these neurotoxic compounds on wildlife.

Pesticides enter waterways from both agricultural and urban discharges and can therefore also reach estuaries and marine coastal waters. Although these toxic compounds are known to hydrolyze quite rapidly in the environment, their brief life lasting only hours or days, their continuous increase addressed to the importance of evaluating their potential toxic effects on marine coastal organisms.

In vertebrates, two isoforms of ChEs were identified: acetylcholinesterases (AchE), which preferentially hydrolyse acetyl esters such as acetylcholine, and butyrylcholinesterases (BChE) which preferentially act on butyrylcholine (Chang,1991, Massouliè,1993).

AchE is considered to be mainly involved in the hydrolysis of neurotransmitters. On the contrary, for BChE, that is however able to hydrolyze acethylcholine, no specific substrate has been identified, and this enzyme is believed to be involved in the detoxification of natural compounds (Massouliè, 1993). ChEs were often found as polymorphic enzymes in invertebrates (Talesa, 1993). In Ostrea edulis and Mytilus spp. two forms of ChEs have been identified (Bocquené, 1997).

AchE activity in vertebrates is extremely sensitive to the toxic action of neurotoxic pesticides. However, it must be noted that in Mytilus spp. (and in molluscs in general) this biomarker seems to be less sensitive than in vertebrates. Mora (1999), reported data clearly showing that in molluscs AchE activity may be considered, due to its lack of sensitivity to organophosphate compounds, as a biomarker of acute exposure to pesticides. More recently (Rickwood, 2004; Galloway, 2002) it was suggested that the effects of pesticides may be evaluated primarily by typical biomarkers of stress, such as lysosomal membrane stability, and only in animals exposed to higher concentrations of chemicals it is possible to demonstrate significant changes in AChEs activity.

The response of AChE activity to pollutants shows a continuous decreasing trend. This biomarker should be considered a low sensitivity biomarker of stress, whose variation may be related to neurotoxic effects at the organism level (changes in valve closure cycle, gill cyclic beat, muscle movements, etc. in mussels; alteration of neural functions, etc. in fish). Due to the fact that different AChEs with different substrate preferences and sensitivity to pesticides are present in molluscs, it has been suggested that the enzyme activity may be evaluated by calculating the difference between total AChE activity and the same activity following incubation in 1 mM paraoxon, a well known AChE inhibitor (Ellman et al, 1961).

The evaluation of AChE activity is a simple and low-in-cost biomarker that is usually evaluated in the gills or in the whole body of mussel spp. (Mora, 1999; Rickwood, 2004; Galloway, 2002), Ostrea edulis (Valbonesi, 2003) and Crassostrea gigas (Bocquené, 1997). However, certain bivalve species may have only minimal AChE activity. This is the case again of Tapes philippinarum, where AChE activity was undetectable utilizing the usual methodologies (Valbonesi, 2003). The biomarker was largely used also in different vertebrates from fish to mammals (Viarengo, 1989)

Assessment of the indicator

From: “Beep Final Report”. Realized by dr. Gilles Bouquene and co-workers (IFREMER, France)

Introduction

The enzyme AChE is vital for the function of nerve endings using acetylcholine (ACh) as chemical transmitter of nerve signals. The AChE enzyrme hydrolyse ACh, forming acetate and choline, thereby the nerve signal is switched off, and the postsynaptic membrane repolarise again. The process is extremely rapid, cholinergic synapses may transfer 1000 impulses per second. In all higher organisms, neuromuscular junctions (motor end plates) utilise ACh. Inhibition of the AChE activity therefore leads to less efficient control of the muscles. This lowers the biological fitness and may ultimately lead to paralysis and death of the organism.

AChE can be used as an indicator of neuromuscular functions and serve as a biomarker of the effects of xenobiotic compounds known to inhibit the system, such as organophosphate (OP) and carbamate insecticides. More than 100 OP compounds are known to be relevant (parathion, malathion, fenitrothion, phosalone, etc.), likewise for carbamates more than 50 compounds are relevant (carbaryl, carbosulfan, aldicarb, carbofuran, propoxur, etc).

Reagents and solutions

1. Extraction and assay buffer: 0.02M phosphate buffer (PB).
Dissolve 2.76 g NaH2PO4 x H2O (M=137.99 g/mol) in 1 l distilled water. Add 0.1% Triton X 100. Adjust to pH 7.

2. 10 mM DTNB (dithiobisnitrobenzoate, M= 396 g/mol).
Dissolve 3.96 mg DTNB in 1 ml assay buffer. Can be stored several days at 4°C.

3. 100 mM ACTC (Acetylthiocholine iodide, M= 289 g/mol).
Dissolve 28.9 mg ACTC in 1 ml distilled water just prior to lysis.

Sample preparation
1. Use 0.1 - 1 g of sample tissue: Muscle, brain, or gills (bivalves).
2. Add 1:1 to 1:4 volume (depends on the organ) of PB buffer pH 7.0.
3. Ultra Turrax sample for 30 sec.
4. Obtain supernatant after a 10,000 x g 20 min centrifugation.
5. Analyse sample supernatant or freeze it in -20°C or in -80°C until analysis.

Analytical procedure (adapted far microplate reader)
(Method of Ellman et al. (1961) modified according to Bocquenè and Galgani)
1. Bring all reagents of to room temperature before analysis.
2. Add successively: 340 µl assay buffer (pH 7)
20µl 10 mM DTNB (0.5 mM final conc.)
10 µl supernatant sample

Modified to 1 ml
900 µl buffer
50 µl DTNB
30 µl sample
20 µl ACTC

3. Incubate for 5 min to allow reaction between DTNB and SH groups in extract.
4. Add 10 µl 0.1 M ACTC (2.6 mM final conc.) to start assay reaction. Be aware of substrate inhibition at > 10 mM ACTC.
5. Monitor enzyme reaction on microplate reader at 412 nm (possible range 405 nm to 420 nm) from 1 min to several min depending on the reaction velocity.
6. Blank wells without enzymatic extracts (buffer + DTNB + ACTC) are used to estimate non-enzyrmatic hydrolysis of ACTC and values are subtracted from the absorbance increase (per minute) of enzyrmatic samples.

Calculation of AChE activity
1 Unit = the amount of enzyme that hydrolyses 1µmol ACTC/min/mg protein

1 unit = (?A412 X VolT x 1000)/ (1.36 x 104 x lightpath x Vols x prot.conc).
?A 412 = change in OD412nm per min (after subtracted non-enzymatic values)
VolT = Total assay volume (0.380 ml)
1.36 X 104 = Extinction coefficient of TNB (thiobisnitrobenzoate)
lightpath = microplate well depth (1 cm)

Vols = Sample volume (in ml)
prot.conc. = mg protein / ml sample extract
Interpretation of results
Generally, a 50% (or more) inhibition of the AChE activity may be considered as a strong response. The concentration leading to a 50% inhibition is denoted IC 50. Exposure to OPs often leads to an irreversible inhibition, whereas carbamates may lead to reversible effects. A number of species may be used in studies of AChE, including: fish (muscle and brain), bivalves (Adductor muscle and gills, low activity), crustaceans (abdominal muscle), and birds.

Sex, age (size), GSI, water temperature should be recorded if possible. Fish maturation is a relevant factor, certain early stages may have high AChE activity. Temperature both in ambient seawater and in assay mix may influence AChE activity levels.

Cholinesterase inhibition by organophosphorus and carbamate compounds. Internal lab protocol at IFREMER DEL/EX, BP1105, 44311 Nantes Cedex, France

Data source

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Marine Pollution Indicator fact sheet

MED POL

Biomarker of stress: Acetylcholinesterase activity in mollusc cells

Indicator
Acetylcholinesterase Activity

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AchE activity in vertebrates is extremely sensitive to the toxic action of neurotoxic pesticides. However, it must be noted that in Mytilus spp. (and in molluscs in general) this biomarker seems to be less sensitive than in vertebrates. Mora (1999), reported data clearly showing that in molluscs AchE activity may be considered, due to its lack of sensitivity to organophosphate compounds, as a biomarker of acute exposure to pesticides. More recently (Rickwood, 2004; Galloway, 2002) it was suggested that the effects of pesticides may be evaluated primarily by typical biomarkers of stress, such as lysosomal membrane stability, and only in animals exposed to higher concentrations of chemicals it is possible to demonstrate significant changes in AChEs activity.

The response of AChE activity to pollutants shows a continuous decreasing trend. This biomarker should be considered a low sensitivity biomarker of stress, whose variation may be related to neurotoxic effects at the organism level (changes in valve closure cycle, gill cyclic beat, muscle movements, etc. in mussels; alteration of neural functions, etc. in fish). Due to the fact that different AChEs with different substrate preferences and sensitivity to pesticides are present in molluscs, it has been suggested that the enzyme activity may be evaluated by calculating the difference between total AChE activity and the same activity following incubation in 1 mM paraoxon, a well known AChE inhibitor (Ellman et al, 1961).

The evaluation of AChE activity is a simple and low-in-cost biomarker that is usually evaluated in the gills or in the whole body of mussel spp. (Mora, 1999; Rickwood, 2004; Galloway, 2002), Ostrea edulis (Valbonesi, 2003) and Crassostrea gigas (Bocquené,1997). However, certain bivalve species may have only minimal AChE activity. This is the case again of Tapes philippinarum, where AChE activity was undetectable utilizing the usual methodologies (Valbonesi, 2003). The biomarker was largely used also in different vertebrates from fish to mammals (Viarengo, 1989)
Assessment of the indicator

From: “Beep Final Report”. Realized by dr. Gilles Bouquene and co-workers (IFREMER, France)

Introduction

The enzyme AChE is vital for the function of nerve endings using acetylcholine (ACh) as chemical transmitter of nerve signals. The AChE enzyrme hydrolyse ACh, forming acetate and choline, thereby the nerve signal is switched off, and the postsynaptic membrane repolarise again. The process is extremely rapid, cholinergic synapses may transfer 1000 impulses per second. In all higher organisms, neuromuscular junctions (motor end plates) utilise ACh. Inhibition of the AChE activity therefore leads to less efficient control of the muscles. This lowers the biological fitness and may ultimately lead to paralysis and death of the organism.
AChE can be used as an indicator of neuromuscular functions and serve as a biomarker of the effects of xenobiotic compounds known to inhibit the system, such as organophosphate (OP) and carbamate insecticides. More than 100 OP compounds are known to be relevant

(parathion, malathion, fenitrothion, phosalone, etc.), likewise for carbamates more than 50 compounds are relevant (carbaryl, carbosulfan, aldicarb, carbofuran, propoxur, etc).

Reagents and solutions

1. Extraction and assay buffer: 0.02M phosphate buffer (PB).
Dissolve 2.76 g $NaH_2PO_4 \times H_2O$ (M=137.99 g/mol) in 1 l distilled water. Add 0.1% Triton X 100. Adjust to pH 7.

2. 10 mM DTNB (dithiobisnitrobenzoate, M= 396 g/mol).
Dissolve 3.96 mg DTNB in 1 ml assay buffer. Can be stored several days at 4°C.
3. 100 mM ACTC (Acetylthiocholine iodide, M= 289 g/mol).
Dissolve 28.9 mg ACTC in 1 ml distilled water just prior to lysis.

Sample preparation

1. Use 0.1 - 1 g of sample tissue: Muscle, brain, or gills (bivalves).
2. Add 1:1 to 1:4 volume (depends on the organ) of PB buffer pH 7.0.
3. Ultra Turrax sample for 30 sec.
4. Obtain supernatant after a 10,000 x g 20 min centrifugation.
5. Analyse sample supernatant or freeze it in -20°C or in -80°C until analysis.

Analytical procedure (adapted far microplate reader)
(Method of Ellman et al. (1961) modified according to Bocquenè and Galgani)
1. Bring all reagents of to room temperature before analysis.
2. Add successively: 340 µl assay buffer (pH 7)
20µl 10 mM DTNB (0.5 mM final conc.)
10 µl supernatant sample
Modified to 1 ml
900 µl buffer
50 µl DTNB
30 µl sample
20 µl ACTC

3. Incubate for 5 min to allow reaction between DTNB and SH groups in extract.
4. Add 10 µl 0.1 M ACTC (2.6 mM final conc.) to start assay reaction. Be aware of substrate inhibition at > 10 mM ACTC.
5. Monitor enzyme reaction on microplate reader at 412 nm (possible range 405 nm to 420 nm) from 1 min to several min depending on the reaction velocity.
6. Blank wells without enzymatic extracts (buffer + DTNB + ACTC) are used to estimate non-enzyrmatic hydrolysis of ACTC and values are subtracted from the absorbance increase (per minute) of enzyrmatic samples.

Calculation of AChE activity
1 Unit = the amount of enzyme that hydrolyses 1µmol ACTC/min/mg protein

1 unit = (?A412 X VolT x 1000)/ (1.36 x 104 x lightpath x Vols x prot.conc).
?A 412 = change in OD412nm per min (after subtracted non-enzymatic values)
VolT = Total assay volume (0.380 ml)
1.36 X 104 = Extinction coefficient of TNB (thiobisnitrobenzoate)
lightpath = microplate well depth (1 cm)
Vols = Sample volume (in ml)
prot.conc. = mg protein / ml sample extract
Interpretation of results

Generally, a 50% (or more) inhibition of the AChE activity may be considered as a strong response. The concentration leading to a 50% inhibition is denoted IC 50. Exposure to OPs often leads to an irreversible inhibition, whereas carbamates may lead to reversible effects. A number of species may be used in studies of AChE, including: fish (muscle and brain), bivalves (Adductor muscle and gills, low activity), crustaceans (abdominal muscle), and birds.

Sex, age (size), GSI, water temperature should be recorded if possible. Fish maturation is a relevant factor, certain early stages may have high AChE activity. Temperature both in ambient seawater and in assay mix may influence AChE activity levels.

Cholinesterase inhibition by organophosphorus and carbamate compounds. Internal lab protocol at IFREMER DEL/EX, BP1105, 44311 Nantes Cedex, France

Data source

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Marine Pollution Indicator fact sheet

MED POL

EROD activity

Key message

It is categorized as biomarker of exposure i.e. a biomarker able to evidentiate the biological response to a particular class of pollutants.

EROD activity is one of the first biomarker discovered and utilised at international level and it is able to evidentiate effects of organic aromatic xenobiotics such as polynuclear aromatic hydrocarbons, PCBs, etc. in different organisms (fish and in general vertebrates, but not in mussels).

Policy relevance

It was utilised in different regional/national biomonitoring programs in different States such as Canada, USA, France and Italy. It was adopted by UE in the Interreg Biomonitoring Programs. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was considered a “core” biomarker. It is a biomarker suggested by ICES and it is in the list of the biomarker intercalibration program realised by Belquam.

Policy context

UNEP MAP Mediterranean Biomonitoring Program,. RAMOGE activity and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate stress response in fish and molluscs.

Environmental context

In several animal cells an enzymatic system is present devoted to the oxidative metabolism of endogenous lipophilic compounds such as steroids (Goeptar, 1995; Stegeman, 1992; Goksøyr and Förlin, 1992). This enzymatic mono-oxygenase system is associated with the membranes of the smooth endoplasmic reticulum (Stegeman, 1992; Bucheli and Fent, 1995). It catalyzes the oxidation of lipophilic substrates utilizing O_2 and NADPH and involves the binding of CO to the cytochrome sP448-450 (whose name is derived from the maximum absorption wavelength of the CO-cytochrome complex). This enzymatic activity is present at a low level, since it is usually related to the degradation of endogenous lipophilic compounds; however, in organisms exposed to polycyclic aromatic hydrocarbons or PCBs (Polychlorinated Biphenyls) activity may be induced 10-100 times (Payne, 1984; Stegeman and Hahn, 1994; Bucheli and Fent, 1995; Goeptar, 1995).

Induction takes place due to binding of xenobiotics to a protein complex, i.e. the aromatic hydrocarbon receptor (Ah-receptor) and heat shock protein 90 (HSP90), this latter protein being subsequently released. The Ah-receptor complex binds to the Ah-receptor nuclear translocation factor (ARNT) and migrates into the nucleus (Bucheli and Fent, 1995; Safe, 2001). In the nuclear chromatin ARNT binds to a DNA region of the promoter upstream of the P450 genes, known as the xenobiotic regulatory element (XRE) or dioxin repressive element (DRE). Under these conditions, transcription factors may activate the promoter region of the

CYP1A gene and consequently mRNA synthesis is enhanced, leading to an increased protein level in the smooth endoplasmatic reticulum (Stegeman and Hahn, 1994).

Due to enzyme induction by xenobiotics, the evaluation of benzo[a]pyrene hydroxylase and EROD activity in fish liver has been often reported as a biomarker capable of detecting the biological effects of many aromatic xenobiotic co mpounds present in water and accumulated in fish tissues (Sijm and Opperhuizen, 1989; Bucheli and Fent, 1995; Stegeman and Lech, 1991; Stegeman and Hahn,1994; Viarengo, 1997; Stegeman, 1988).

Although evaluation of the EROD activity was found to be a powe rful biomarker, the determination of the amount of P450 evaluated by immunoblot analysis has also yielded excellent results. It must be noted, however, that EROD activity in fish may change in relation to biological factors such as organism size, although also environmental parameters such as seawater temperature may affect the enzymatic activity of the P448-450 cytochrome family. Moreover, heavy metals and oxidative stress, as well as an excess of substrate, are able to dramatically inhibit EROD activity (Viarengo, 1997; Omura and Sato, 1964). Finally, it has been demonstrated that the effects of different pollutants on the lysosomal vacuolar system may indirectly affect the level of EROD activity in the cells of pollutant-exposed organisms. In fact, pollutant induced destabilization of the lysosomal membranes triggers enhancement of protein catabolism. This in turn causes an increased autophagic rate, also involving the degradation of a portion of the smooth endoplasmatic reticulum and leading to a drastic reduction in EROD activity.

Overall, EROD activity remains one of the most powerful biomarkers suitable for detection of the effects of aromatic xenobiotic compounds in fish. However, the determination of EROD activity should be always associated with the utilization of a battery a biomarker of stress able to evidentiate the possible changes in hepatocyte physiology that could mask the specific effects of the aromatic pollutants.

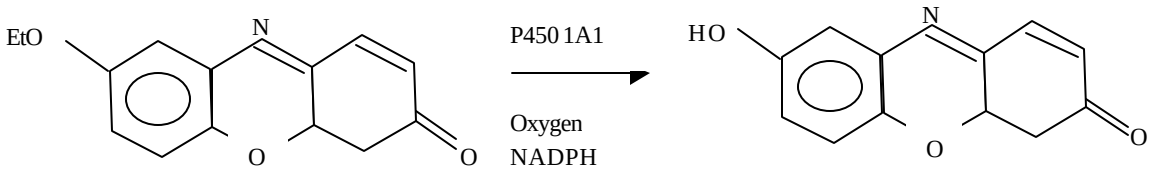
In mussels, the situation is more complicated: in fact, despite intensive research during the last two decades, a CYP1A gene in mussel DNA could not be identified; on the other hand, a P450 cytochrome was never purified from mussel tissues, and the attempts to utilize antibodies obtained against endoplasmic reticulum cytochromes purified from mussel digestive gland did not give the expected results: the protein identified by these antibodies seems to be insensitive to PCB exposure (Jonsson, 2003).

It must be also mentioned that EROD activity in mussel preparations is not detectable, the benzo[a]pyrene hydroxylase activity is at the limit of detection and it was found to be weakly induced by organic pollutants such as PCBs and PAHs only by some authors (Suteau,1988). For this reasons, the utilization of MFO activity in mussels as a biomarker of exposure is not recommended: more recent research seems to indicate that in molluscs both peroxisome proliferation and MXR may represent more powerful biomarkers of exposure to aromatic xenobiotic compounds, as mentioned in the final report of BEEP, the European Program related to the utilization of biomarkers in the evaluation of the biological effects of pollutants in marine coastal areas.
Assessment of the indicator

Background and principle

This section comprises the procedure to determine the activity of the cytochrome P450 multi-enzyme complex in fish as a biomarker of biological response to organic xenobiotics in the marine environment. It describes methods far preparing MFO containing subcellular fractions, determination of the catalytic activity of ethoxyresorufin-O-deethylase (EROD) and estimation of the protein content.

The catalytic estimation is based on the incubation of a substrate (ethoxyresorufin together with an enzyme preparation and cofactor (NADPH) in appropriate butter, the fluorescence increase due to resorufin production is then evaluated, by a spectrophotofluorimeter.



Ethoxyresorufin

Resorufin

This procedure has been tested both in the field and the laboratory as biomarker of exposure, mainly by PAH, PCB and chlorodibenzodioxins. It can also be applied to pentoxy- or benzyloxy-resorufin O de-alkylase (PROD and BROD) to indicate induction of other P450 isozymes (Burke and Mayer, 1983).

Sampling

A problem of great importance in field sampling of fish is the collection and storage of samples until they can be processed in the laboratory. The hemoprotein degrades rapidly in intact tissue or subcellular fractions; even the use of liquid nitrogen far storage may affect enzyme activity (Forlin and Andersson, 1985). Samples that have been thawed or stored at -20°C are of no use in catalytic measurements.

General guidelines for collection of fish:

– fish should be sampled outside the species-dependent spawning season and the gonadosomatic index should be recorded;

– fish should be sampled within a species-dependent defined length-range;

– either male (usually higher EROD levels than females) or female (higher induction ratio in comparison of high and low polluted sites) individuals should be used. Data from males and females should not be mixed;

– the numbers of individuals sampled must be representative for each site to enable appropriate statistical treatments (5-10 per site);

– only fish without external and internal visible diseases should be used for further processing, and

– bottom water temperature should be measured at the time and at the place of capture.

Equipment
Top-pan balance weighing to 0.1 9

- Conventional dissection instruments
- Ice bucket
- Range of small beakers
- Electric drill capable of 2700 rpm
- Potter-Elvehjem teflon-glass homogenizer (5 or 15 ml)
- Measuring cylinder (10 or 25 ml capacity)
- Refrigerated ultracentrifuge
- Graduated tubes, 5-15 ml;
- Pasteur pipettes
- Nalgene cryotubes
- Micropipettes with disposable tips to deliver 10, 25, 50, 100, 200,1000)µl
- Glass pipette to deliver 2 ml
- Fluorimeter

Solutions and chemicals

- Solution A
  150 mM KCl
- Solution B
  10 mM HEPES containing 250 mM sucrose; 1 mM Na2EDTA,
  adjust to pH 7.4 with KOH (34-36%, Prolabo)

- Solution C
  80% solution B + 20% glycerol (V/V)

- Solution D
  18.2% KH2PO4 (Prolabo) 50mM + 22.2% Na2HPO4 (Prolabo)
  200 mM bring to 100% with water, pH 7.44

Storage of solutions and chemicals

+ 4°C Solutions A, B, C, D, G-6PDH

-20°C NADP, G-6-P, Resorufin
-80°C Ethoxyresorufin (preferably)

Preparation of samples for analysis

This section describes the steps far preparing S9 fraction and microsomal samples prior to the measurement of MFOs. It is always convenient to prepare in advance as many reagents and solutions. Most, like those required for protein determinations, are stable and will withstand freezing and thawing if kept in plastic bottles. It is usually not possible to prepare nucleotide co-enzyme solutions in advance, however, and since (usually) small amounts of these are needed and as they are relatively expensive, it is desirable to preweigh appropriate amounts of these, and keep them (cooled and desiccated) in small vials.

S9 fractions are prepared by centrifugation (e.g. 9.000 x g for 15 minutes). Microsomal fractions are prepared by ultra-centrifugation (e.g. 100,000xg for 90 mins) of homogenates from fresh or frozen tissues (such as liver or hepatopancreas). Cytochrome P-450 activity measurements can be made both in the S9 supernatant and in the 100.000Xg resuspended microsomal fraction.

Tissue dissection and preparation for analysis

Kill fish by severing spinal cord at the level of the pectoral fins and by insertion of a scissor blade in the brain. Weigh fish with an accuracy of ± 1%.
Dissect out the liver and avoid rupturing the gall bladder, since bile may contain MFO inhibitors. Weigh the liver (±1 % accuracy) and place in a beaker on ice.

All subsequent operations should be performed at 4°C.

Homogenization of tissue

Mince weighed liver (ideally =1 g, weighed to ±0.1 g) with scissors, rinse it in solution A and blot dry on tissue paper.

Adjust solution B by 0.1 M PMSF (dissolved in ethanol).

Place the liver in a Potter glass homogeniser tube on ice and add solution B in ratio 5:1, v:w. Homogenize with 10 vertical strokes at high speed, keeping the tube led in ice. This produces the "crude homogenate".

Preparation of S-9 fraction and 100.000 x g Pellet (microsomes)

Place the homogenate in centrifuge tubes and spin for 15 minutes at 9000xg in a centrifuge. Collect the resultant supernatant (S-9), without the lipid phase, and subdivide it in small aliquots (100-200 µl) and store at -80°C. Utilize a Jot of S9 for protein determination using the Bradford method (Bradford, 1976).

Alternatively, prepare 100.000 x g fraction (microsomes). Transfer S-9 fraction to an ultracentrifuge tube and re-centrifuge again at 100.000 x g for 50 min at 4oC. Discard the supernatant (cytosol) and resuspend the microsomal pellet in 1 ml of sol C.

Transfer quantitatively this suspension into the potter, re-homogenize with 5 d strokes using a teflon tip, keeping the homogenizer cooled in ice. Transfer the homogenized suspension in a graduated tube and record its volume. Hold on ice.

This is the microsomal preparation that is now ready for quantification of protein concentration and enzymatic activities.

Freeze aliquots (100 and 200 µl) in Nalgene 1.5 mi cryotubes and stored at – 80°C or in dry ice if required far future reference.

Protein determination

This is in accordance with Bradford (1976).

1.5 ml polystirene spectrophotometer cuvettes are prepared containing various concentrations of Bovin Serum Albumine diluited with MilliQ water to a final volume of 20 microliter using 1, 2, 5, 10, 20 µg of BSA tram a 1 µg/µ1 stock solution.

10 µl of S9 are dispensed into the sample cuvettes. 10 µl of MilliQ water are added to obtain a final volume of 20 µl. 480 µl of MilliQ water are added to the reference and the samples. 500 µl of Pierce Protein Assay Reagent are dispensed in all the cuvettes.

The absorbance at 595 nm is read against a blank containing only the reagents without S9 supernatant. BSA calibration curve is plotted and the protein concentration is estimated according to the regression curve.

Ethoxyresorufin O-deethylase (EROD) determination (Suteau et al., 1988)
Preparation
Add sequentially in a tube:
1/100 volume of ethoxyresorufin (from a stock of 123 µM in DMSO)
1/10 volume of glucose-6-phosphate (25 mM in H2O)
1/10 volume NADPH (25 mM in H2O)

Bring the mixture to the desired final volume with solution D. Add glucose-6- phosphate dehydrogenase (G-6-PDH) to obtain a final concentration of 1 unit ml-1. Warm the medium for 5 minutes at 30°C in a water bath.

Enzymatic reaction

While the above medium is warming, dispense individual S9 samples (10 to 100 µg in solution C) into Falcon 2018 polypropylene tubes.
For each sample, set a time "zero" in duplicate and a time "five" in duplicate.
To set a time 'zero' reaction, add 2 ml cold acetone onto the S9.
Now transfer all the tubes to the water bath and every 20 seconds add 1 ml of the warm medium to the samples and the time 'zero' reaction using an Eppendorf Multipette fitted with a 50 ml syringe. Vortex the tubes immediately.

Stop the reaction after 5 minutes by adding 2 ml cold acetone except the time 'zero' reaction tube. Vortex again the tubes.

Centrifuge the samples far 5 minutes at 6000 x g to eliminate flocculated protein.

Quantification of the resorufin produced

Having checked the extinction coefficient of the resorufin standard, add 100 pmoles of resorufin to a new tube (using a 2 mM standard resorufin solution in di-methyl-sulphxide in 5 µl of a 1/100 dilution of solution C) and add 1 ml of reaction medium and 2 ml of cold acetone and vortex.

Measure the fluorescence using a spectrofluorimeter with an excitation wavelength of 537 nm and an emission wavelength of 583 nm.

Transfer the samples carefully into cuvettes leaving behind any precipitated protein.

Autoblank on the time 'zero' reaction tube.

Calculation of the activity

For the calculation of EROD activity expressed in pmoles of resorufin/min/mg protein you can use the following formula:

EROD activity= (IFc x c x VF)/(If x Vc x t x P)

In which,

IFc is the fluorescence of the sample
IF is the fluorescence of the standard (nmol/ml)

C is the concentration of the standard (nmol/ml)
VF is the final volume of the mix (ml)
V c is the volume of the sample (ml)
t is the reaction time
p is protein concentration (mg/ml)

Interpretation of results

The EROD measurement is a convenient way of assessing P-450 1A1 catalytic activity and has gained widespread use in biomonitoring studies with fish. The catalytic assay can be viewed as a very useful primary test to identify biological responses due to PAH contamination. The occurrence of hepatic lesions should be recorded; it is a good idea to preserve representative sub-samples of hepatic tissue for future histological examination. Confirmation of increased EROD response can be obtained by determining PAH adducts in fish as evidence that the EROD response is being mediated by organic aromatic xenobiotic compounds.

It is important to follow closely the proposed protocol as it is well known that standardization problems could arise due to: a) intercomparability of EROD activities S-9 and microsomal fractions. This often tends to produce somewhat ambiguous results. b) the use of different protein estimation methods by different laboratories. c) a more pressing issue, is the use by different authors of different extinction coefficients for the reaction product - resorufin (phenoxazone) - which was found to vary between 20 to 73 mM-1 cm-1.

Therefore, if the task is to intercompare and assess the EROD values among various regional laboratories, then it is important to fully standardise the catalytic assay before any actual biomonitoring takes place.

Future developments

Ongoing research is taking place to detect immunochemically the inductive response of P450 using antibody probes for both protein levels and mRNA levels (Goksoyr et al., 1991a). In this way, the amount of a specific antibody probe cross-reacting with the P450 protein is measured chemically.

Immunochemical detection of mRNA can prove highly advantageous since catalytic activity of induced P-450 (Gooch et al., 1989) may be inhibited by certain inducers (such as organochlorines). Consequently, analysis of catalytic activity alone might show no response, but strong induction can still be seen by immunochemical analysis of the P-450 protein or its mRNA. Apparently different types of inducers can also modulate the catalytic activity, as can endogenous compounds. In other cases the catalytic activity may be lost due to bad storage (e.g. In field sampling situations).or the sample or tissue may be too small to give measurable catalytic activity (as with fish eggs and larvae) (Goksoyr et al., 1991b). In all of these cases, immunodetection of P450 has been able to detect inductive responses that would not have been possible with catalytic measurements alone (Goksoyr et al., 1991 a).

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Frequency of micronuclei in molluscs and fish cells

Key message

The frequency of micronuclei is a well-established biomarker of genotoxicity.

This test represents a sensitive biomarker and is able to integrate the effects of the total amount of toxic chemical accumulated in the sentinel organisms. It is usually utilized to analyse both fish and molluscs samples.

The parameter increases with the increase of the exposure time of the organisms to the pollutants and with the pollutant concentration.

Policy relevance

The frequency of micronuclei was adopted as stress biomarker in different international programs such as the UNEP MAP Mediterranean Biomonitoring Program. This stress biomarker was also employed in the framework of the RAMOGE activity. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was included in the final report in the list of “suggested” biomarkers.

Policy context

UNEP MAP Mediterranean Biomonitoring Program, RAMOGE activity and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate genotoxicity response in fish and molluscs.

Environmental context

The micronuclei assay is a well-known cytogenetic method commonly utilized in the evaluation of genotoxic effects of enviro nmental stressors. In the cells, micronuclei appear when complete chromosomes or chromosome fragments fail to incorporate in the daughter nuclei in the anaphase during cell division and are incorporated in the cytoplasm where they remain during cell life. The presence of micronuclei evidentiates chromatin breakage caused by clastogens or spindle dysfunctions due to toxicants (Carrano and Natarajan, 1988; Heddle, 1983)

The micronuclei assay consists in the scoring of the cells containing in the cytoplasm one or more micronuclei associated to the main cell nucleus (Al-Sabti, 1986a; Al-Sabti and Metcalfe, 1995; Hofftman and Vink, 1981; Hofftman and de Raat, 1982; Scarpato, 1990; Majone, 1990; Siu, 2004).

The frequence of the observed micronuclei may be considered as a suitable index of accumulated genetic damage during the cell life span. Depending on the life span of each cell type and on their mitotic rate in a particular tissue, the micronuclei frequency may provide an index able to integrate the genotoxic effects of environmental pollutants; this is the case of mussel gills, where the exposure of caged mussels in the field to aromatic hydrocarbon polluted sea water (Petroleum Harbour of Genoa) determines a continuous

increase of micronuclei in the gill cells reaching a plateau after a month of caging (Bolognesi, 1996).

It must be mentioned that the micronuclei evaluation during the interphase is much easier than the analyses of the chromosomal alterations during metaphase, and for this additional reason this biomarker has been often adopted in biomonitoring programmes.

The micronuclei assay does not require sophisticated equipment (only a microscope) but it is time consuming, due to the fact that for every sample usually two thousand cells need to be scored (Bolognesi, 1996). Recently the utilization of 500 cells was proposed, although the authors emphasize that the sensitivity of micronuclei assay can always be improved analysing a sample of greater size (Siu, 2004).

Assessment of the indicator

From:"Manual on the Biomarkers recommended for the Med Pol Biomonitoring Programme". UNITED NATIONS ENVIRONMENTAL PROGRAMME MEDITARRANEAN ACTION PLAN. Athens 1999

Gill cells were fixed in methanol:acetic acid (3:1) for 20 min and centrifuged at 1.000 rpm. The resuspended cells were spread on slides, air dried and stained with Giemsa (3%). Two thousands cells with preserved cytoplasm per mussel were scored under a light microscope to determine the frequency of micronuclei.

Micronucleus test in fish.

Blood was collected from the caudal vein of fish and immediately smeared on a clean glass slide, dried overnight, fixed in methanol for 20 min and stained with Acridine Orange. Four thousand erythocytes per animal with 5-8 individuals per experimental point were analyzed by a fluorescence microscope (1000X magnification) to determine the frequency of micronucleated cells.

Determination of Micronuclei Frequency

Background

Micronuclei are small DNA-containing bodies, which can be present near the cell nucleus during interphase resulting from both chromosome breakage and spindle dysfunction.

The type of mutations that could contribute to micronuclei production include:

a) mutations to kinetochore proteins, centromeres and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase;
b) unrepaired DNA strand-breaks induced by environmental and endogenous genotoxic agents which may result in acentric chromosome fragments.

Studies indicate that the relative occurrence of micronuclei can provide an indication of accumulated genetic damage throughout the life span of the cells even during short phases of contamination. These considerations suggest the suitability of this test to monitor the extent of genotoxic damage in marine organisms in a time - integrated manner. The following protocol has been devised to assess the frequency of micronuclei in cells.

Equipment
- centrifuge,
- optical microscope.

Chemicals and solutions

HANKS' balanced salts solution 2X (HBSS 2X) 273.8 mM NaCl
- 10.73 mM KCl
- 0.81 mM MgSO4. 7H2O
- 2.52 mM CaCl2. 2H2O
- 0.674 mM Na2HPO4. 2H2O
- 0.88 mM KH2PO4
- 8.33 mM NaHCO3
- 10.09 mM D-Glucose. H2O

Dispase solution

Dispase I (neutral protease; grade I, Boehringer Mannheim, Germany) 0.1 mg/ml in HBSS 2X
- methanol: acetic acid (3: 1)
- 3% Giemsa

Method

Preparation of cell suspension Mussel haemolymph
Samples of haemolymph are drawn from the posterior adductor muscle sinus by a hypodermic syringe. The samples are diluted with an equal volume of Hanks' Balanced Salt solution and spinned at 1,000 rpm.

Gills of mussels

Gills are taken off and cells are isolated by enzymatic digestion with a solution of Dispase (Boehringer Mannheim, Germany), 0.1 mg/ml in modified (20%0) Hank's Balanced Salt solution, for 10 min at 37°C. The cellular suspension obtained by filtration is centrifuged at 1,000 rpm for 10 min.

Slide preparation

Aliquots of cellular pellet of mussel gills and hemolymph are fixed in methanol: acetic acid (3:1) for 20 min, then spread on slides, air dried and stained with 3% Giemsa. The slides are coded and scored blind.

Slide scoring

Two thousands cells with preserved cytoplasm per mussel are scored under oil immersion at 1,000 x magnification. Due to the high interindividual variability of the MN frequency, 8-10 animals must be analysed far each experimental point.

The following criteria have to be met during scoring:
- only intact cells are scored;
- chromatin structure and colour intensity similar to that of the main nucleus;
- on the same optical plane as the main nucleus;
- round or oval;
- not fragmented (to exclude small stain particles and apoptotic cells);
- located within 4-fold the shortest axis of the nearest nucleus.

Data source

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UNEP

Marine Pollution Indicator fact sheet

MAP

MED POL

Biomarker of stress: Lipofuscin lysosomal accumulation in molluscs and fish cells

Key message

A biomarker of stress is a biological parameter which value may change in relation to the toxic effects of pollutants.

A biomarker of stress is able to integrate the biological response to the total charge of pollutants bioavailable for the sentinel organisms.

Lipofuscin lysosomal accumulation is one of the more sensitive biomarker of stress related to the oxidative stress due to enhanced ROS production in the cells. The biomarker change shows a trend characterized by a continuous increase in molluscs and fish cells.

Policy relevance

Lipofuschin lysosomal accumulation was adopted in different international biomonitoring programs such as by the RAMOGE Biomonitoring Program and utilised experimentally by different both in the framework of the UNEP MAP Mediterranean Biomonitoring Program. This biomarker was included in the parameters selected in the final report of BEEP UE Program (Biological Effects of Pollutants in Marine Coastal Ecosystems).

Policy context

No policy has included this biomarker in the National or International Protocols and Conventions.

Environmental context

It is well known that Reactive Oxygen Species (ROS) produced in physiological conditions can induce a process of cellular membranes named lipid peroxidation. However, a complex antioxidant system is always present in the cells able to eliminate ROS produced in different subcellular compartments. Antioxidants consist of both soluble enzymes, such as SOD, catalase, etc… and ROS scavengers, both hydrophilic, such as GSH, ascorbate, metallothionein, and lipophilic, such as vitamin E and carotenoids. When ROS production exceeds the antioxidant defenses, oxidative stress occurs in the cells and membrane lipid per oxidation is one of the main effects.

Lipid peroxidation is a complex process consisting of an “initiation” step, in which free radicals react directly with the polyunsaturated fatty acid chains, abstracting a hydrogen atom and in this way originating free lipid-peroxyl radicals and semi-stable lipid hydroperoxides (Bus, 1979; Dianzani,1978; Viarengo, 1989). The latter decompose easily, producing new lipid radicals; therefore, once the process is initiated, it tends to continue in a chain reaction (“propagation” step). Finally, during the “termination” reactions, free lipid radicals are neutralized by reacting with each other, or with antioxidant compounds.

The whole process results in the production of lipid radicals and in the formation of a complex mixture of lipid degradation products (malondialdehyde and other aldheydes such as alkanals, alkenals, hydroxyalkenals, ketones, etc.), which are known to be extremely toxic for the cells (Esterbauer, 1985; Younes, 1984) because of their high reactivity towards other cellular components (such as soluble and membrane proteins, DNA, etc.). Both lipid radicals and their aldehydic products may react with SH-containing compounds, such as GSH, which are rapidly oxidized. This leads to alteration of the redox balance of the cell and, consequently, to a partial inhibition of SH-containing enzymes. (Bellomo, 1985; Ziegler, 1985). Moreover, during the propagation step, the lipid peroxyl radicals may abstract H atoms from proteins, resulting in lipid-protein and protein-protein cross-linking. In addition, as mentioned earlier, during the termination reactions, adjacent fatty acids may be joined by abnormal bonds, thus impairing structure and functions of cell membranes (Bus,1979).

The peroxidation products tend to accumulate in the lysosomal vacuolar system in the form of insoluble granules, containing undegradable fluorescent pigments, usually referred to as lipofuscin.

Those membranes damaged by ROS attack are rapidly removed by the lysosome vacuolar system, and new membrane synthesis is enhanced to compensate for cell damage due to the action of toxic chemicals able to induce oxidative stress.

The membranes are degraded in the lysosomes and their different components, such as amino acids and lipids, are released into the cytoplasm to be re-utilized in the cell; however, certain oxidized components of the membranes are not recognized as substrates by lysosomal acidic hydrolases, and therefore peroxidation products accumulate within the lysosomes as an insoluble growing body known as lipofuscin (Brunk and Collins,1981). As mentioned before in this paper, “growing” lipofuscin granules are able to sequester metals that are weakly bound to proteins and other cytosolic compounds. Metals that are strongly complexed in the tetrathiolate clusters of metallothioneins are not affected. During the evolution of secondary lysosomes into residual bodies (tertiary lysosomes), the lipofuscin granules “grow” as they accumulate the peroxidation end products. It seems possible that the metals which are loosely bound to the acid residues of the surface will become “trapped” by the additional lipofuscin and sterically prevented from moving in or out of the granule. The metals are therefore detoxified and they are finally eliminated by exocytosis of residual bodies.

This process can be interpreted as a general mechanism of heavy metal homeostasis, which is potentially present in the cells of all living organisms. It is particularly active, for example, in marine invertebrate kidney cells, which often have a lysosomal system rich in lipofuscin (Viarengo and Nott,1993).
From our knowledge about the lipofuscin lysosomal content in the digestive gland cells of molluscs or in fish hepatocytes, the accumulation of this pigment in the organelle represents a spy of the level of oxidative stress in the cells and in particular of the level of membrane lipid peroxidation. The accumulation of lipofuscin in the lysosomes will reach an equilibrium when the rate of exocytosis of residual bodies will balance the lipofuscin formation due to oxyradical action.

The trend of this biomarker during a pollutant-induced stress syndrome is represented by a continuous increase in the parameter till a maximum level related to the elimination rate of lipofuscin-rich residual bodies, a parameter which is characteristic of each different tissue in different organisms. Overall, the evaluation of lipofuscin as a cellular biomarker of oxidative stress seems to be more suitable than the evaluation of malondialdehyde (MDA) or thiobarbituric acid (TBA) reactive compounds: in fact these are intermediate products of lipid peroxidation and, as reactive toxic metabolites, they are rapidly eliminated. On the contrary, lipofuscin is an end-point of lipid peroxidation, it is accumulated in the lysosomes and it is

therefore easily detectable in the cells of stressed organisms, in comparison with the minimum level present in the cells of the organisms living in unpolluted waters.
Assessment of the indicator

Lipofuscin content of tertiary lysosomes was detected using the Schmorl reaction (Pearse 1972).

Duplicate cryostat sections (10 µm) were fixed for 15 min in calcium-formal at 4°C. Sections were then rinsed in distilled water and immersed in the reaction medium. This latter contained 1% ferric chloride and 1% potassium ferricyanide in a ratio of 3:1. Sections were stained for 5 min in this solution then rinsed in 1% acetic acid for 1 min, followed by rinsing in distilled water and mounting in aqueous mounting medium. The blue reaction product is quantified in terms of section staining by image analysis.

Data source

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Marine Pollution Indicator fact sheet

MED POL

Biomarker of stress: Lysosomal membrane stability in molluscs and fish cells

Key message

A biomarker of stress is a biological parameter which value may change in relation to the toxic effects of pollutants.

A biomarker of stress is able to integrate the biological response to the total charge of pollutants bioavailable for the sentinel organisms.

Lysosomal membrane stability is the best-acknowledged stress biomarker: it is extremely sensitive and the change of its value is extremely rapid in pollutant-exposed organisms.

Policy relevance

Lysosomal membrane stability was adopted as stress biomarker in different international programs such as the UNEP MAP Mediterranean Biomonitoring Program. This stress biomarker was also employed in the framework of the RAMOGE activity and in the UNIDO activity of the Black Sea. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was considered a “core” biomarker. It is a biomarker suggested by ICES and it is in the list of the biomarker intercalibration program realised by Belquam.

Policy context

UNEP MAP Mediterranean Biomonitoring Program,. RAMOGE activity and UNIDO and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate stress response in fish and molluscs.

Environmental context

Lysosomes are cytoplasmic, single membrane organelles, that show peculiar characteristics: they contain more than 40 different classes of hydrolytic enzymes (such as proteases, nucleases, lipases, etc.) with optimal activities at acidic pHs. These enzymes are able to hydrolyze essentially all biological molecules, from proteins and nucleic acids and nucleotides to complex sugars and lipids. The lysosomal matrix has a pH of 4,5-5; this low pH is maintained by active proton pumping due to the activity of a H+-ATPase present in the membrane of the organelle and by the acidic proteins within the lysosomal matrix.

In the cells, lysosomes show heterogeneous shapes; this is mainly due to the fact that, after their assembling in the Golgi apparatus, they have an initial size of about 0,5 µm: these vesicles, having a pH of about 6 and an increasing amount of hydrolytic enzymes, are not functionally active, and are usually named ‘primary lysosomes’. When mature lysosomes directly take up components from the cytoplasm or fuse with autophagosomes or hetero phagosomes, their size increases up to several µm and the organelles, whose pH is about 5, are actively involved in the digestion of biological macromolecules (‘secondary lysosomes):

at the final phase of their activity a residual body containing un-degradable material (mainly lipofuscin) and with minimal hydrolytic activity is formed ('tertiary lysosomes', i.e. residual bodies). In those cells able to active exocytosis the content of the residual bodies is released into the extracellular fluids.

The functions of lysosomes in different cell types and tissues of different organisms (from protozoa to mammals) may be specific and very different, but in all organisms the lysosomal vacuolar system is involved in the degradation of the material assumed in the cell by endocytosis, as well as in the regulation of the catabolic rate of cellular macromolecules, proteins in particular (Moore et al., 1985; Viarengo, 1989).

It has long been known that lysosomes, despite their acidic internal pH, are also able to accumulate metal cations: this seems to be mainly due to the presence in the lysosomal matrix of lipofuscins, end-products of peroxidation processes, that are able to trap inside their growing granules different metal cations (Viarengo, 1989). Lysosomes are also able to accumulate lipophilic organic compounds, such as aromatic hydrocarbons and PCBs (Viarengo,1989; Viarengo and Nicotera,1991). This fact is of particular importance in organisms, such as mussels, whose mixed function oxygenase (MFO) acticity is extremely low, and a typical cytocrome p450 is absent: in this case, most aromatic compounds are not metabolized but accumulated in these organelles, that thus represent one of the most important sites of accumulation of organic pollutants, and therefore of their action at the cellular level.

It is now widely accepted that the accumulation of contaminants within these organelles may represent the main mean by which toxic chemicals are able to alter lysosomal physiology, and consequently increase the protein catabolic rate stimulating the autophagic activity in the target cells. Moreover, these organelles show a high capacity of pollutant accumulation and are at the same time extremely sensitive to minimal concentrations of toxic chemicals that penetrate into the cells (in mussels, nanomolar concentration of both inorganic and organic pollutants are able to destabilize the lysosomal membranes and to activate protein catabolism).

Moreover, recent data obtained in mussels showed that inorganic pollutants, such as Cu, Hg, Cd, can alter lysosomal activity by affecting Ca-dependent cell signalling. It was demonstrated that the metal-induced increase in cytosolic [Ca2+] concentrations is able to activate a Ca-dependent phospholipase A2 (PLA2). PLA2 binds to lysosomal membranes and activates the process of vacuole fusion with the associated increase in protein catabolism (Burlando et al., 2002). Moreover, organic contaminants known as endocrine disrupters have been shown to alter lysosomal membrane stability of mussel cells through activation of components of kinase-mediated cell signalling, in particular of the stress-activated Mitogen Activated Protein Kinase p38 and protein kinase C (PKC) (Canesi et al., 2004).

An increase in lysosomal size indicates an enhanced rate of fusion of primary lysosomes with auto/hetero phagosomes, but in the case of pollutant-exposed cells, lysosomal enlargement usually indicates increased autophagy, which may greatly contribute to induction of cell disfunctions and, as an ultimate consequence, to cell death.
The evaluation of lysosomal membrane stability is a suitable parameter to quantify the changes in the lysosomal activity induced by pollutants. This parameter was found to be sensitive to the presence of minimal, nM concentrations of toxic chemicals in the marine environment and accumulated in the target cells of different organisms. In mussels, lysosomal membrane stability represents the simplest, most sensitive and low-cost biomarker to evaluate the physiological status of the organisms.

Two methodologies have been developed to evaluate lysosomal membrane stability: the former is applied to living cells (such as haemolymph cells of mussels), the latter to cryostatic tissue sections.

The in vivo lysosomal membrane stability assay utilizes the lipid-soluble dye Neutral Red (NR) that is rapidly taken up by lysosomes; once protonated, is it sequestered in the lysosomal matrix. The assay evaluates the lysosomal membrane stability on the basis of the NR retention time by the organelles: control cells, with intact lysosomal membranes, usually show retention times over 60 min. In the cells of organisms exposed to pollutants, the NR retention is reduced proportionally to lysosomal membrane damage induced by toxic chemicals present in the water and accumulated in the cells. The method is very simple, low-in-cost, extremely sensitive, and it only needs a basic equipment for the analysis (a microscope in the simplest setting).

The evaluation of lysosomal membrane stability in tissue cryostatic sections is also highly sensitive, but it needs more training for the histological analyses of the samples, and a more complex instrumentation (at least a cryostat and a microscope).
It must be said, however, that this method allows not only to collect data of lysosomal membrane stability, but also a good evaluation of the lysosomal size, a biomarker often utilized in biomonitoring programs; moreover, the analysis of the cells of mussel digestive tubules also permits to estimate histological changes due to the increased lysosomal activity (i.e. reduction in the volume of cell cytoplasm) and also to evaluate possible changes in the proportion of digestive/secreting cells in the tubules (Cajaraville, 1995).

Both these parameters are of great importance in the integration of the biomarker data by the expert system to rank the stress syndrome of the animals.

Assessment of the indicator

Background

It is very difficult to evaluate the molecular changes affecting the permeability of the lysosomal membrane. These analyses require extensive purified lysosomal membrane preparations and their examination at molecular level. An easier way to assess this parameter is to examine whether its normal physiological function has been altered or disrupted following exposure to pollutants.
An approach that links the results of morphological and biochemichal analysis to describe pathological alterations is cytochemistry. In addition to the requirement of very small amounts of tissue samples, this technique is also ideal to detect changes in particular target cells and tissues.

Cytochemistry has been successfully applied to assess lysosomal integrity by visualising the hydrolytic enzymes within the lysosomes, and has proved to be a rapid and sensitive tool for evaluating the effects of organic xenobiotics and other injurious agents at very low intracellular concentrations. This generalised response occurs in all cell types ranging from fungi to vertebrates, so that such a cytochemical test can be applied on a fairly widespread basis.

Assessment of Lysosomal membrane stability: cytochemical assay on cryostat sections

Principle

The following protocol is a cytochemical procedure far the determination of lysosomal membrane stability, based on the evaluation of the activity of N-acetyl- ß-hexosaminidase, a

lysosomal enzyme. Lysosomal destabilisation is measured as the increased permeability of the substrate (naphthol AS-BI N-acetyl-ß-glucosaminide) visualized by the reaction with the enzyme into the lysosomes in presence of diazonium salt. The preparation of tissues for the examination of cell structures requires the use of specialised methodolo gy to produce high-quality stained sections. In this section all observations are related to frozen material, and this preparative technique will be described.

Solutions and chemicals
Lysosomal membrane labilising buffer (Solution A)

0.1 M Na-citrate Buffer - 2.5% NaCl w:v, pH 4.5
Substrate incubation medium (to be prepared just 5 minutes before use) (Solution B)

20 mg of naphtol AS-BI N-acetyl-p-D-glucosaminide (Sigma, N4006) are dissolved in 2.5 ml of 2-methoxyethanol (Merck, 859) and made up to 50 ml with solution A, containing also 3.5 g POLYPEP (Sigma, P5115; low viscosity polypeptide to act as a section stabiliser).

Diazonium dye (Solution C)

0.1M Na-phosphate buffer, pH 7.4, containing 1 mg/ml of diazonium dye Fast Violet B salts (Sigma, F1631) (Note: saturated solution)

Other dyes can be utilised such as:
Fast Garnet GBC (Sigma)
Fast Red Violet LB (Difco)
Fast Blue BB (Sigma)
Fast Blue RR (Sigma)

Fixative (Solution D)

- calcium formol: 2% Ca-acetate w:v + 10% Formaldehyde v:v

Mounting Medium: aqueous Mounting Medium (Vector Laboratories H1000) or Kaiser glycerin gelatin

- Liquid Nitrogen

Preparation of tissue
Rapidly excise 5 small pieces (3-4mm) of the organ/tissue (usually digestive gland of mollusc or fish liver) obtained from five different animals and rapidly place them on an aluminium cryostat chuck (i.e. aligned in a straight row across the centre).

While dissecting, leave the chuck on ice and then place it for 40 seconds in a small plastic box containing pre-cooled N-hexane3 at -70°C using liquid nitrogen. Seal the chuck with 4-5 pieces of Parafilm and immediately store at -80°C. (At this temperature the tissue preparations maintain their integrity for months).

Using a Bright's Cryostat or other equivalent equipment (cabinet temperature below -25°C), cut 10 µm thick sections using a 15° knife angle. Transfer the sections to "warm" slides (at room temperature) to flash-dry them. The slides can be stored in the cryostat (for at least 4 hours).

Enzymatic determination of membrane stability
Place the sections in a Hellendal jar containing solution A for different times (O, 3, 5, 10, 15, 20, 30, 40 minutes) at 37°C in order to find out the range of pre-treatment time needed to

completely labilise the lysosomal membrane (i.e. labilisation period). In the last five minutes use shaking water-bath.

Transfer the set of slides to solution B and incubate the slides far 20 minutes at 37°C in a Hellendal jar preferably in a shaking water-bath.

Wash the slides in filtered sea-water at room temperature or with a saline solution (3% NaCl) at 37°C far 2 to 3 minutes. Transfer the slides to solution C containing the diazonium coupler far 10 min at room temperature. Rapidly rinse the slides in running tap water far 5 minutes. Fix the sections far 10 minutes in solution D at 4°C (or mount directly with glycerol gelatin), rinse in distilled water and mount in aqueous mounting medium.
Interpretation of results

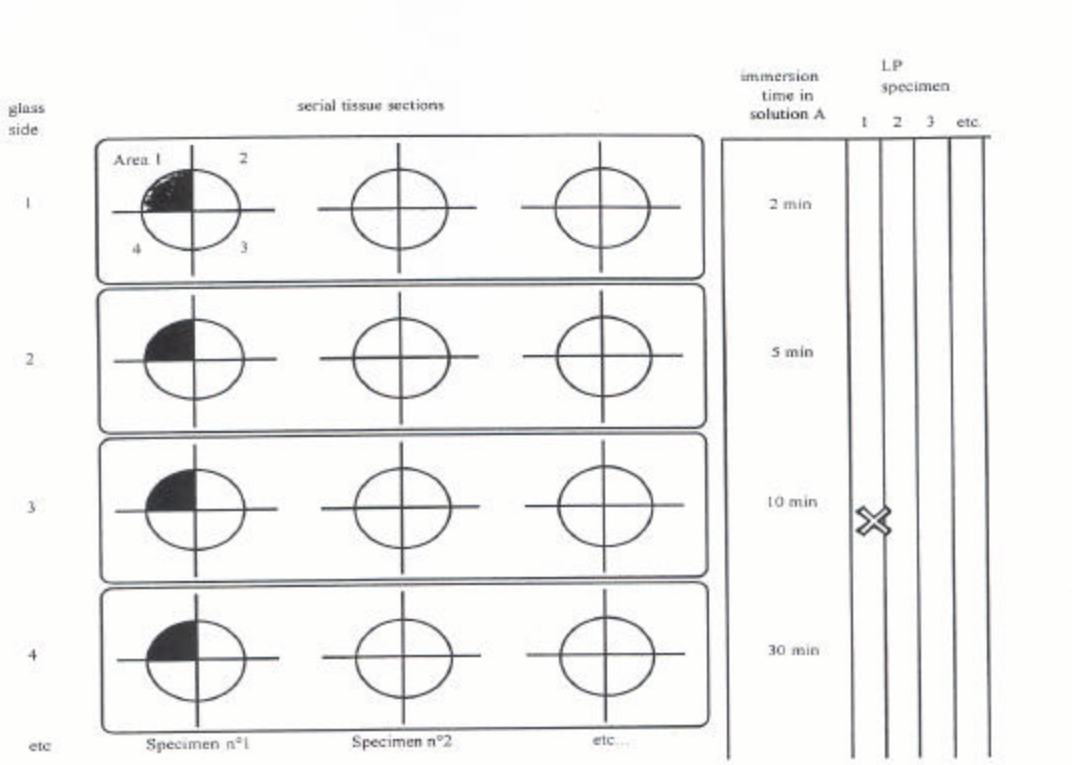

Fig. 2 Staining Intensity:

Area 1 (10 min) > Area 1 (30 min) > Area 1 (15 min) ...etc.
Area 2 (10 min) > Area 2 (30 min) > Area 2 (20 min) ...etc.
Area 3 (15 min) > Area 3 (20 min) > Area 3 (30 min) ...etc.
Area 4 (15 min) > Area 4 (30 min) > Area 4 (20 min) ...etc.

View the slides under a microscope and divide each section into four areas (quarters) for statistical interpretation (see Fig. 2).

Lysosomes will stain reddish-purple due to the reactivity of the substrate with N-acetyl-β-hexosaminidase. The average labilisation period (LP) far each section corresponds to the average incubation time in the acid buffer that produces maximal staining reactivity. LPs for the other samples (in this case n=5) are similarly obtained.

LP value for specimen 1 = mean of 4 areas = 12.5 minutes

Now analyze one quarter of each set of sections pertaining to the same animal and find out the section quarter showing maximal staining (from which the LP value is derived). Repeat

this analysis for the remaining three quarters of the first specimens analyzed. The mean of the results obtained represents the LP value of specimen 1. The same procedure is adopted for the other sections present on the slides derived from the other four remaining animals. Finally, a mean value of lysosomal membrane stability of the sample will be calculated utilizing the 5 data obtained from the 5 animals analyzed.

Compare test samples with those taken from reference area and determine gradient of cytotoxicity. Reduction in the LP along the expected pollution gradient would indicate cellular stress due to pollution.

Any decrease in staining intensity in successive sections following that with maximal staining may be due to loss of enzyme by diffusion from fully labilised lysosomes. If there are two peaks of staining intensity, then consider only the main staining peak as the LP. This may be due to differential latent properties of the lysosomal hydrolase concerned.

"0" time will be utilised only to verify the correct Lysosomal enzyme activity and it will not be considered in the evaluation of the maximal staining intensity peak.

Intervals of 3 or 5 min are generally satisfactory far most test situations. The data can then be statistically tested using the Mann-Whitney U-test (Speigel, 1961) and compared with reference data. For mussel digestive gland, timing intervals of 3, 5, 10, 15, 20, 30 and 40 minutes are normally utilized.
Determination of Lysosomal membrane stability in living cells: neutral red retention assay
Neutral red is a lipophilic dye and as such will freely permeate the cell membrane. Within cells the compound becomestrapped by protonisation in the lysosomes and accumulated in these organelles, where it can be visualised microscopically. The degree of trapping of this lysosomotropic marker depends on the pH of the lysosome as well as the efficiency of its membrane associated proton pump (Segien, 1983).

The acidic environment of lysosomes is maintained by a membrane Mg2+- ATPase dependent H+ ion proton pump (Ohkuma et al., 1982); the neutral red retention assay reflects the efflux of the lysosomal contents into the cytosol following damage to the membrane and, possibly, impairment of the H+ ion pump (Lowe et al., 1992). So any impairment of this latter system will result in a reduction of the dye retention assay. Studies indicate that, similarly to the cytochemical method described above, the neutral red retention assay is sensitive to the main classes of chemical pollutants (Lohse, 1990). The following protocol has been specifically adapted to be used on mussels.

Chemicals and solutions

- Physiological saline

20 mM (4.779) Hepes
436 mM (25.489) NaCl
53 mM (13.069) MgSO4
10 mM (0.759) KCl
10 mM (1.479) CaCl2

Dissolve these in 1 liter of distilled water. Gas far 10 minutes (95%02:5% CO2) and adjust to pH 7.3 with 1 M NaOH. Store solution in refrigerator, but use it at room temperature.

- Neutral Red dye

Prepare stock solution by dissolving 20 mg of neutral red powder in 1 ml di-methyl-sulfoxide (DMSO). Transfer 5 1.11 of stock solution into 995 1.11 of physiological saline (working

solution). Keep neutral red in the dark and in fridge when not utilized. The working solution must be prepared freshly before analysis.

Practical evaluation

The following procedures is according to Lowe et al. (1992) and Lowe et al. (1995)

Fill the eppendorf tubes with sigmacote (SIGMA) far 10-30 minutes, then return sigmacote to container (it is reusable). Put 2 µl of Poly-L-Lysine (SIGMA), diluted 1 to 10 with distilled water, on a microscope slide and spread out with a cover slip. Leave to dry in a humidity chamber.

Insert scissors half way along the ventral surface of the mussel and partially disclose the valves to allow the insertion of the hypodermic syringe. Drain the water from the shell. Fill an hypodermic syringe with 0.5 ml of physiological saline and then aspirate 0.5 ml of haemolymph from the posterior abductor muscle of the mussel. After obtaining the haemolymph sample, discard the needle and expel the content in a siliconised eppendorf tube.

Dispense 40 µl of haemolymph-saline mixture on the slide, in the same position where the poly-l-lysine was added and incubate in humidity chamber for 30 minutes to allow the cells to attach. Carefully drain the excess solution from the slide by placing the slide on its side and letting the liquid run off. Add 40 µl of the neutral red working solution and leave in a humidity chamber for 15 min (maintained 15- 16°C during the analysis). Apply a coverslip and inspect the preparation under a microscope.

Look at the slides every 15 minutes for the first hour then every 30 minutes for the next two hours thereafter. Determine the time at which 50% of the lysosomes in the cells leaches out neutral red in the cytosol. Derive a mean value far each specimen and then a global mean far all specimens pertaining to the same pool. Compare samples from monitored field sites with those taken from reference field sites and determine gradient of cytotoxicity. An increase in leaching rates would indicate cellular stress due to pollution.

Results

Samples	0	15	30	45	60	90
control	+	+	+	+	+	+
treated	+	+	±	-	-	-

Key:
+ more than 50% of the lysosomes in the cells retaining neutral red
- less than 50% of the lysosomes in the cells retaining neutral red

Data source

B. Burlando, B. Marchi, I. Panfoli, A. Viarengo. Essential role of Ca2+-dependent phospholipase A2 in estradiol-induced lysosome activation. Am J Physiol Cell Physiol. 283(5):C1461-8 2002.

M.P. Cajaraville, Y. Robledo, M. Etxeberria, I. Marigomez. Cellular biomarkers as useful tools in the biological monitoring of environmental pollution: molluscan digestive lysosomes. Cell Biology in Environmental Toxicology. M.P. Cajaraville editor pp.. 29-55 1995.

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Marine Pollution Indicator fact sheet

MED POL

Biomarker of stress: Lysosomal membrane stability in molluscs and fish cells

Key message

A biomarker of stress is a biological parameter which value may change in relation to the toxic effects of pollutants.

A biomarker of stress is able to integrate the biological response to the total charge of pollutants bioavailable for the sentinel organisms.

Lysosomal membrane stability is the best-acknowledged stress biomarker: it is extremely sensitive and the change of its value is extremely rapid in pollutant-exposed organisms.

Policy relevance

Lysosomal membrane stability was adopted as stress biomarker in different international programs such as the UNEP MAP Mediterranean Biomonitoring Program. This stress biomarker was also employed in the framework of the RAMOGE activity and in the UNIDO activity of the Black Sea. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was considered a “core” biomarker. It is a biomarker suggested by ICES and it is in the list of the biomarker intercalibration program realised by Belquam.

Policy context

UNEP MAP Mediterranean Biomonitoring Program,. RAMOGE activity and UNIDO and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate stress response in fish and molluscs.

Environmental context

Lysosomes are cytoplasmic, single membrane organelles, that show peculiar characteristics: they contain more than 40 different classes of hydrolytic enzymes (such as proteases, nucleases, lipases, etc.) with optimal activities at acidic pHs. These enzymes are able to hydrolyze essentially all biological molecules, from proteins and nucleic acids and nucleotides to complex sugars and lipids. The lysosomal matrix has a pH of 4,5-5; this low pH is maintained by active proton pumping due to the activity of a H+-ATPase present in the membrane of the organelle and by the acidic proteins within the lysosomal matrix.

In the cells, lysosomes show heterogeneous shapes; this is mainly due to the fact that, after their assembling in the Golgi apparatus, they have an initial size of about 0,5 µm: these vesicles, having a pH of about 6 and an increasing amount of hydrolytic enzymes, are not functionally active, and are usually named ‘primary lysosomes’. When mature lysosomes directly take up components from the cytoplasm or fuse with autophagosomes or hetero phagosomes, their size increases up to several µm and the organelles, whose pH is about 5, are actively involved in the digestion of biological macromolecules (‘secondary lysosomes): at the final phase of their activity a residual body containing un-degradable material (mainly lipofuscin) and with minimal hydrolytic activity is formed (‘tertiary lysosomes’, i.e. residual

bodies). In those cells able to active exocytosis the content of the residual bodies is released into the extracellular fluids.

The functions of lysosomes in different cell types and tissues of different organisms (from protozoa to mammals) may be specific and very different, but in all organisms the lysosomal vacuolar system is involved in the degradation of the material assumed in the cell by endocytosis, as well as in the regulation of the catabolic rate of cellular macromolecules, proteins in particular (Moore et al., 1985; Viarengo, 1989).

It has long been known that lysosomes, despite their acidic internal pH, are also able to accumulate metal cations: this seems to be mainly due to the presence in the lysosomal matrix of lipofuscins, end-products of peroxidation processes, that are able to trap inside their growing granules different metal cations (Viarengo, 1989). Lysosomes are also able to accumulate lipophilic organic compounds, such as aromatic hydrocarbons and PCBs (Viarengo,1989; Viarengo and Nicotera,1991). This fact is of particular importance in organisms, such as mussels, whose mixed function oxygenase (MFO) acticity is extremely low, and a typical cytocrome p450 is absent: in this case, most aromatic compounds are not metabolized but accumulated in these organelles, that thus represent one of the most important sites of accumulation of organic pollutants, and therefore of their action at the cellular level.

It is now widely accepted that the accumulation of contaminants within these organelles may represent the main mean by which toxic chemicals are able to alter lysosomal physiology, and consequently increase the protein catabolic rate stimulating the autophagic activity in the target cells. Moreover, these organelles show a high capacity of pollutant accumulation and are at the same time extremely sensitive to minimal concentrations of toxic chemicals that penetrate into the cells (in mussels, nanomolar concentration of both inorganic and organic pollutants are able to destabilize the lysosomal membranes and to activate protein catabolism).

Moreover, recent data obtained in mussels showed that inorganic pollutants, such as Cu, Hg, Cd, can alter lysosomal activity by affecting Ca-dependent cell signalling. It was demonstrated that the metal-induced increase in cytosolic [Ca2+] concentrations is able to activate a Ca-dependent phospholipase A2 (PLA2). PLA2 binds to lysosomal membranes and activates the process of vacuole fusion with the associated increase in protein catabolism (Burlando et al., 2002). Moreover, organic contaminants known as endocrine disrupters have been shown to alter lysosomal membrane stability of mussel cells through activation of components of kinase-mediated cell signalling, in particular of the stress-activated Mitogen Activated Protein Kinase p38 and protein kinase C (PKC) (Canesi et al., 2004).

An increase in lysosomal size indicates an enhanced rate of fusion of primary lysosomes with auto/hetero phagosomes, but in the case of pollutant-exposed cells, lysosomal enlargement usually indicates increased autophagy, which may greatly contribute to induction of cell disfunctions and, as an ultimate consequence, to cell death.
The evaluation of lysosomal membrane stability is a suitable parameter to quantify the changes in the lysosomal activity induced by pollutants. This parameter was found to be sensitive to the presence of minimal, nM concentrations of toxic chemicals in the marine environment and accumulated in the target cells of different organisms. In mussels, lysosomal membrane stability represents the simplest, most sensitive and low-cost biomarker to evaluate the physiological status of the organisms.
Two methodologies have been developed to evaluate lysosomal membrane stability: the former is applied to living cells (such as haemolymph cells of mussels), the latter to cryostatic tissue sections.

The in vivo lysosomal membrane stability assay utilizes the lipid-soluble dye Neutral Red (NR) that is rapidly taken up by lysosomes; once protonated, is it sequestered in the lysosomal matrix. The assay evaluates the lysosomal membrane stability on the basis of the NR retention time by the organelles: control cells, with intact lysosomal membranes, usually show retention times over 60 min. In the cells of organisms exposed to pollutants, the NR retention is reduced proportionally to lysosomal membrane damage induced by toxic chemicals present in the water and accumulated in the cells. The method is very simple, low-in-cost, extremely sensitive, and it only needs a basic equipment for the analysis (a microscope in the simplest setting).

The evaluation of lysosomal membrane stability in tissue cryostatic sections is also highly sensitive, but it needs more training for the histological analyses of the samples, and a more complex instrumentation (at least a cryostat and a microscope).
It must be said, however, that this method allows not only to collect data of lysosomal membrane stability, but also a good evaluation of the lysosomal size, a biomarker often utilized in biomonitoring programs; moreover, the analysis of the cells of mussel digestive tubules also permits to estimate histological changes due to the increased lysosomal activity (i.e. reduction in the volume of cell cytoplasm) and also to evaluate possible changes in the proportion of digestive/secreting cells in the tubules (Cajaraville, 1995).

Both these parameters are of great importance in the integration of the biomarker data by the expert system to rank the stress syndrome of the animals.

Assessment of the indicator

Background

It is very difficult to evaluate the molecular changes affecting the permeability of the lysosomal membrane. These analyses require extensive purified lysosomal membrane preparations and their examination at molecular level. An easier way to assess this parameter is to examine whether its normal physiological function has been altered or disrupted following exposure to pollutants.
An approach that links the results of morphological and biochemichal analysis to describe pathological alterations is cytochemistry. In addition to the requirement of very small amounts of tissue samples, this technique is also ideal to detect changes in particular target cells and tissues.

Cytochemistry has been successfully applied to assess lysosomal integrity by visualising the hydrolytic enzymes within the lysosomes, and has proved to be a rapid and sensitive tool for evaluating the effects of organic xenobiotics and other injurious agents at very low intracellular concentrations. This generalised response occurs in all cell types ranging from fungi to vertebrates, so that such a cytochemical test can be applied on a fairly widespread basis.

Assessment of Lysosomal membrane stability: cytochemical assay on cryostat sections

Principle

The following protocol is a cytochemical procedure far the determination of lysosomal membrane stability, based on the evaluation of the activity of N-acetyl- ß-hexosaminidase, a lysosomal enzyme. Lysosomal destabilisation is measured as the increased permeability of the substrate (naphthol AS-BI N-acetyl-ß-glucosaminide) visualized by the reaction with the enzyme into the lysosomes in presence of diazonium salt. The preparation of tissues for the examination of cell structures requires the use of specialised methodology to produce high-

quality stained sections. In this section all observations are related to frozen material, and this preparative technique will be described.

Solutions and chemicals

Lysosomal membrane labilising buffer (Solution A)

0.1 M Na-citrate Buffer - 2.5% NaCl w:v, pH 4.5
Substrate incubation medium (to be prepared just 5 minutes before use) (Solution B)

20 mg of naphtol AS-BI N-acetyl-p-D-glucosaminide (Sigma, N4006) are dissolved in 2.5 ml of 2-methoxyethanol (Merck, 859) and made up to 50 ml with solution A, containing also 3.5 g POLYPEP (Sigma, P5115; low viscosity polypeptide to act as a section stabiliser).

Diazonium dye (Solution C)

0.1M Na-phosphate buffer, pH 7.4, containing 1 mg/ml of diazonium dye Fast Violet B salts (Sigma, F1631) (Note: saturated solution)

Other dyes can be utilised such as:
Fast Garnet GBC (Sigma)
Fast Red Violet LB (Difco)
Fast Blue BB (Sigma)
Fast Blue RR (Sigma)

Fixative (Solution D)

- calcium formol: 2% Ca-acetate w:v + 10% Formaldehyde v:v

Mounting Medium: aqueous Mounting Medium (Vector Laboratories H1000) or Kaiser glycerin gelatin

- Liquid Nitrogen

Preparation of tissue
Rapidly excise 5 small pieces (3-4mm) of the organ/tissue (usually digestive gland of mollusc or fish liver) obtained from five different animals and rapidly place them on an aluminium cryostat chuck (i.e. aligned in a straight row across the centre).

While dissecting, leave the chuck on ice and then place it for 40 seconds in a small plastic box containing pre-cooled N-hexane3 at -70°C using liquid nitrogen. Seal the chuck with 4-5 pieces of Parafilm and immediately store at -80°C. (At this temperature the tissue preparations maintain their integrity for months).

Using a Bright's Cryostat or other equivalent equipment (cabinet temperature below -25°C), cut 10 µm thick sections using a 15° knife angle. Transfer the sections to "warm" slides (at room temperature) to flash-dry them. The slides can be stored in the cryostat (for at least 4 hours).

Enzymatic determination of membrane stability
Place the sections in a Hellendal jar containing solution A for different times (O, 3, 5, 10, 15, 20, 30, 40 minutes) at 37°C in order to find out the range of pre-treatment time needed to completely labilise the lysosomal membrane (i.e. labilisation period). In the last five minutes use shaking water-bath.

Transfer the set of slides to solution B and incubate the slides far 20 minutes at 37°C in a Hellendal jar preferably in a shaking water-bath.

Wash the slides in filtered sea-water at room temperature or with a saline solution (3% NaCl) at 37°C far 2 to 3 minutes. Transfer the slides to solution C containing the diazonium coupler far 10 min at room temperature. Rapidly rinse the slides in running tap water far 5 minutes. Fix the sections far 10 minutes in solution D at 4°C (or mount directly with glycerol gelatin), rinse in distilled water and mount in aqueous mounting medium.
Interpretation of results
View the slides under a microscope and divide each section into four areas (quarters) for statistical interpretation (see Fig. 2).

Lysosomes will stain reddish-purple due to the reactivity of the substrate with N-acetyl-β-hexosaminidase. The average labilisation period (LP) far each section corresponds to the average incubation time in the acid buffer that produces maximal staining reactivity. LPs for the other samples (in this case n=5) are similarly obtained.
LP value for specimen 1 = mean of 4 areas = 12.5 minutes

Now analyze one quarter of each set of sections pertaining to the same animal and find out

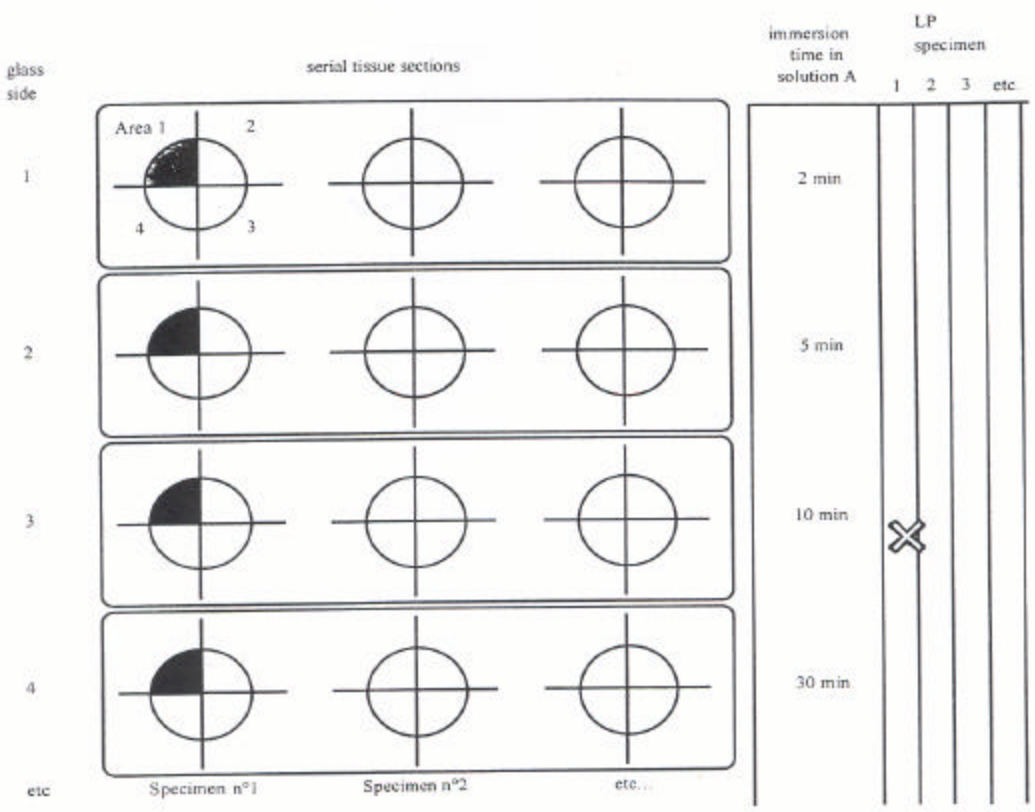

Fig. 2 Staining Intensity:

Area 1 (10 min) > Area 1 (30 min) > Area 1 (15 min) ...etc.
Area 2 (10 min) > Area 2 (30 min) > Area 2 (20 min) ...etc.
Area 3 (15 min) > Area 3 (20 min) > Area 3 (30 min) ...etc.
Area 4 (15 min) > Area 4 (30 min) > Area 4 (20 min) ...etc.

the section quarter showing maximal staining (from which the LP value is derived). Repeat this analysis for the remaining three quarters of the first specimens analyzed. The mean of the results obtained represents the LP value of specimen 1. The same procedure is adopted for the other sections present on the slides derived from the other four remaining animals. Finally, a mean value of lysosomal membrane stability of the sample will be calculated utilizing the 5 data obtained from the 5 animals analyzed.

Compare test samples with those taken from reference area and determine gradient of cytotoxicity. Reduction in the LP along the expected pollution gradient would indicate cellular stress due to pollution.

Any decrease in staining intensity in successive sections following that with maximal staining may be due to loss of enzyme by diffusion from fully labilised lysosomes. If there are two peaks of staining intensity, then consider only the main staining peak as the LP. This may be due to differential latent properties of the lysosomal hydrolase concerned.

"0" time will be utilised only to verify the correct Lysosomal enzyme activity and it will not be considered in the evaluation of the maximal staining intensity peak.

Intervals of 3 or 5 min are generally satisfactory far most test situations. The data can then be statistically tested using the Mann-Whitney U-test (Speigel, 1961) and compared with reference data. For mussel digestive gland, timing intervals of 3, 5, 10, 15, 20, 30 and 40 minutes are normally utilized.
Determination of Lysosomal membrane stability in living cells: neutral red retention assay
Neutral red is a lipophilic dye and as such will freely permeate the cell membrane. Within cells the compound becomes trapped by protonisation in the lysosomes and accumulated in these organelles, where it can be visualised microscopically. The degree of trapping of this lysosomotropic marker depends on the pH of the lysosome as well as the efficiency of its membrane associated proton pump (Segien, 1983).

The acidic environment of lysosomes is maintained by a membrane Mg2+- ATPase dependent H+ ion proton pump (Ohkuma et al., 1982); the neutral red retention assay reflects the efflux of the lysosomal contents into the cytosol following damage to the membrane and, possibly, impairment of the H+ ion pump (Lowe et al., 1992). So any impairment of this latter system will result in a reduction of the dye retention assay. Studies indicate that, similarly to the cytochemical method described above, the neutral red retention assay is sensitive to the main classes of chemical pollutants (Lohse, 1990). The following protocol has been specifically adapted to be used on mussels.

Chemicals and solutions

- Physiological saline

20 mM (4.779) Hepes
436 mM (25.489) NaCl
53 mM (13.069) MgSO4
10 mM (0.759) KCl
10 mM (1.479) CaCl2

Dissolve these in 1 liter of distilled water. Gas far 10 minutes (95%02:5% CO2) and adjust to pH 7.3 with 1 M NaOH. Store solution in refrigerator, but use it at room temperature.

- Neutral Red dye

Prepare stock solution by dissolving 20 mg of neutral red powder in 1 ml di-methyl-sulfoxide (DMSO). Transfer 5 1.11 of stock solution into 995 1.11 of physiological saline (working solution). Keep neutral red in the dark and in fridge when not utilized. The working solution must be prepared freshly before analysis.

Practical evaluation

The following procedures is according to Lowe et al. (1992) and Lowe et al. (1995)

Fill the eppendorf tubes with sigmacote (SIGMA) far 10-30 minutes, then return sigmacote to container (it is reusable). Put 2 µl of Poly-L-Lysine (SIGMA), diluted 1 to 10 with distilled water, on a microscope slide and spread out with a cover slip. Leave to dry in a humidity chamber.

Insert scissors half way along the ventral surface of the mussel and partially disclose the valves to allow the insertion of the hypodermic syringe. Drain the water from the shell. Fill an hypodermic syringe with 0.5 ml of physiological saline and then aspirate 0.5 ml of haemolymph from the posterior abductor muscle of the mussel. After obtaining the haemolymph sample, discard the needle and expel the content in a siliconised eppendorf tube.

Dispense 40 µl of haemolymph-saline mixture on the slide, in the same position where the poly-l-lysine was added and incubate in humidity chamber for 30 minutes to allow the cells to attach. Carefully drain the excess solution from the slide by placing the slide on its side and letting the liquid run off. Add 40 µl of the neutral red working solution and leave in a humidity chamber for 15 min (maintained 15- 16°C during the analysis). Apply a coverslip and inspect the preparation under a microscope.

Look at the slides every 15 minutes for the first hour then every 30 minutes for the next two hours thereafter. Determine the time at which 50% of the lysosomes in the cells leaches out neutral red in the cytosol. Derive a mean value far each specimen and then a global mean far all specimens pertaining to the same pool. Compare samples from monitored field sites with those taken from reference field sites and determine gradient of cytotoxicity. An increase in leaching rates would indicate cellular stress due to pollution.

Results

Samples	0	15	30	45	60	90
control	+	+	+	+	+	+
treated	+	+	±	-	-	-

Key:
+ more than 50% of the lysosomes in the cells retaining neutral red
- less than 50% of the lysosomes in the cells retaining neutral red

Data source

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M.N. Moore. Cellular responses to pollutants. Mar. Pollut. Bull. 16: 134-139 1985.

Marine Pollution Indicator fact sheet

MED POL

Biomarker of exposure: Metallothionein in molluscs cells

Key message

This biomarker can be categorized as biomarker of exposure i.e. a biomarker able to evidentiate the biological response to a particular class of pollutants, but only in mussels. In fish it must be considered a biomarker of stress.

The concentration of metallothionein in tissues is considered a biological response to the accumulation in the cells of heavy metal cations such as Cu, Cd, Hg, Zn, Ag, etc.
In fish cells the metallothionein concentration increases in the organisms exposed to heavy metals but also in organisms in which pollutants accumulations (organic aromatic xenobiotics) are able to cause an oxidative stress. The effect of hormones is also evident.

Policy relevance

It is a biomarker suggested by ICES and it is in the list of the biomarker intercalibration program realised by Belquam. It has been utilised only at local level and it was included in the biomarker list of the UE Program BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) final report.

Policy context

UNEP MAP Mediterranean Biomonitoring Program,. RAMOGE activity and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate stress response in fish and molluscs.

Environmental context

Metallothioneins are soluble, low molecular weight proteins with a high metal content. These metalloproteins have a characteristic aminoacid composition showing the absence of aromatic amino acid and of histidine (and methionine in mussels) and an extremely high cysteine content (up to 30% of the aminoacid content).

The peculiar aminoacid composition and the typical position of the cysteine residues confer to metallothioneins characteristic biochemical properties: the metalloprotein lacks of absorbance at 280 nm in relation to the absence of aromatic aminoacids, and due to the cystein content the protein shows high affinity for heavy metal cations (both essential metals such as Zn and Cu and non essential and high toxic metals such as Hg, Cd and Ag - metals of class I and II).(Hamer,1986; Kägi and Kojima,1987; Shaw,1992; Viarengo,2000). Metals bind to metallothionein by high affinity tetrathiolate clusters, this conferring to the metalloproteins a highly stable tertiary structure.
Concerning the physiological role of metallothionein, their function in the detoxification of cellular metal cation excess (both essential metals such as Zn and Cu and metal without established biological role such as Cd and Hg) is well known. Metallothioneins also act as cellular reserve of essential metals (Brenner, 1987). Moreover, it has been demonstrated that

metallothioneins play an important role as oxyradical scavangers as part of the cellular antioxidant defence system (Sato and Bremner, 1993). Finally, recent evidence showed that metallothioneins might play a role as regulators of the activity of Zn finger proteins in modulation of gene expression (Zeng, 1991; Roesijadi, 1998).

The possible utilization of metallothioneins as biomarkers of exposure is based on the fact that the synthesis of these metalloproteins is highly stimulated by heavy metal cations that penetrate into the cells in metal-exposed organisms (Viarengo and Nott 1993). This has been widely demonstrated both for metal exposed fish and mussels (Webb, 1987; Bremner, 1987). However, more recent data showed that, in fish, metallothioneins are induced not only by heavy metals but also by aromatic compounds able to determine oxidative stress in the hepatocytes by activation of ROS production (Pedrajas, 1995; Kling, 1996).

It seems therefore that in fish these metalloproteins may be considered as a general stress response to environmental pollutants. This hypothesis is also confirmed by the studies concerning the structure of the metalloprotein genes A and B. It was in fact found that the MT promoters contain AP1sequences typical of oxidative stress regulation, as well as sequences typical of hormone and heavy metal regulation (Bonham, 1987; Olsson, 1995).

Therefore, in fish, metallothioneins may be considered as a biomarker of stress, with a bell-shaped response to increasing concentration of the pollutants.

With regards to mussels, the most recent results concerning the structure of the promoters of metallothionein genes indicate that also in this case it is possible to identify nucleotide sequences with high homology with the sequences AP1, GRE and MRE identified in fish (Dondero, 2003).

Utilizing RT quantitative PCR probes specific for the metallothionein genes MT10 and MT20 it has been demonstrated that MT10 is a constitutive gene activated mainly by Zn and Cu, but the MT20 gene is typically activated by Cd, Hg and, to a lower extent, by Cu and minimally by Zn: the gene is also activated by oxyradical production (Dondero et al., in press). On the basis of these results, metallothioneins, also in mussels, may be considered as a biomarker of general stress response; however, in these organisms, aromatic pollutants are weak MFO inducers (Viarengo, 2000) and therefore these toxic compounds are able to produce ROS in the cytosol of the cells only at a low level, usually not sufficient to activate MT20 synthesis.

This hypothesis was confirmed by the fact that no increase in metallothionein concentration was observed in the digestive glands of mussels caged for 30 days at a site highly polluted by PAHs (Petroleum Harbour of Genoa); in addition, in mussels exposed in laboratory experiments to high (mM) concentrations of Paraquat, no significant evidence of metallothionein increase was detected (Viarengo, unpublished data).

It is important to note that these results concerning the metallothionein concentration in the tissues of molluscs exposed to organic xenobiotics may be also interpreted as due to an activation of the lysosomal vacuolar system, and to the consequent increase of the metallothionein catabolic rate.

Overall, these data seem to indicate that in molluscan digestive gland cells the metabolism of organic xenobiotics is different from what observed in fish (and vertebrate in general) hepatocytes; therefore, although the activation of the metallothionein gene promoter seems to show at least important characteristics common to the two groups of organisms, metallothioneins may be considered, from our present knowledge, a biomarker of exposure in mussels. Also in this case, the biomarker shows a bell-shaped response to increasing heavy metal concentrations.

Assessment of the indicator

Background

Routine quantification of MT levels often proves to be problematical due to the lack of a measurable biological activity of this metalloprotein. This has forced investigators to explore unique structural features to be exploited for the evaluation of the MT concentration. Research efforts have relied so far on estimates of (1) metal content bound to this protein (e.g. by competitive metal displacement or direct quantification techniques) and (2) physical (e.g. absorption measurements), and chemical (e.g. measurement of sulphyhydryl groups and immunochemical affinities) characteristics.

Each of these approaches has its own strength and weakness. One major disadvantage common to most of these methods is the indirect estimation of metallothioneins, which may lead to inconsistent results concerning the absolute value of the metallothionein concentration in the tissues. In addition, most of these procedures require expensive laboratory equipment (e.g. ultracentrifuges, MS, chromatographic systems, etc.) and sample preparation and assay optimization require large commitments of time.

Biomolecular assay, such as the measurement of MT m-RNA, are providing some hope in developing a very sensitive technique (Swapan et al., 1991). Specific immunoassays far MT are available, but the limited inter-species compatibility provides a challenge far future development (Kay et al., 1991).

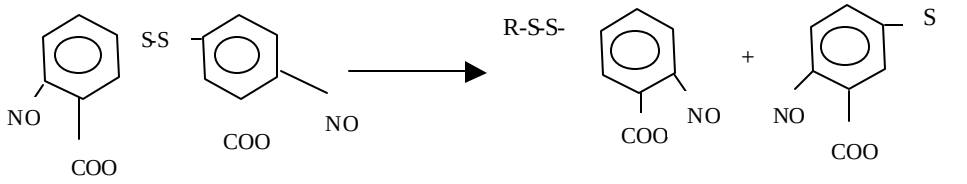
Recently, investigators are adapting simpler but still accurate and sensitive techniques to quantify the levels of MT in biological tissues.

The following methodology is based on the estimation of the sulfhydryl content of MT proteins. This method has been reported to be a sensitive, time saving, and low-cost technique able to detect metallothionein content in the tissues marine organisms (Viarengo et al.. 1997) and is currently being intercalibrated and standardized by a number of laboratories within the Mediterranean.
Spectrophotometric determination of the -SH groups using Ellman's reagent

Principle

The method here described consists of the ethanol/chloroform fractionation of the 30.000xg cytosolic containing fraction, to obtain a partially purified metallothionein fraction. Metallothionein concentration in the samples is then quantified by evaluating the SH residue content by a spectrophotometric method,using Ellman's reagent (DTNB: 5,5 dithiobis 2 nitrobenzoic acid) (Ellman, 1959). As known, metallothioneins are characterized by an extremely high cysteine content (about 20-30%) when compared to other proteins eventually present in the ethanolic extracts and therefore the metallothionein determination based on the SH detection allows a more selective evaluation of these metalloproteins.
Illustrated below is the reaction between DTNB and protein SH groups. The reaction produces stechiometric amounts of TNB (thionitrobenzoate), a yellowish compound with maximum absorbance at 412 nm.

RS +



Thionitrophenyla ed protein

Coloured thionitrobenzoa te anion

The analytical procedure has been adapted to be used on mussels, although other organisms can be used.

Practical evaluation

To detect metallothionein content in biological tissues by the DTNB reaction the samples have to be prepared under rigorous reducing conditions (0.01 % ß-mercaptoethanol) as described in the protocol. The ethanol/chloroform fractionation allows both the elimination of low molecular weight soluble thiols, which reacting with DNTB, could interfere with metallothionein quantification, and the partial concentration of metallothioneins whose level in the tissues of uncontaminated animals is often low. During ethanolic fractionation the addition of RNA as co-precipitant and acid is essential to allow a quantitative metallothionein recovery. A final "washing" of the metallothionein extracts eliminates thiol contaminants, such as reducing agents present in the cells (glutathione, cysteine, etc.) or thiols added during sample preparation (ß-mercaptoethanol). The concentrated MT pellet is resuspended in 0.25 M NaCl with addition of HCl and EDTA (to remove heavy metal cations still bound to metallothioneins), followed by the addition of a known amount of DTNB reagent in a high ionic strength medium (to completely denature metallothioneins). A calibration curve of reduced glutathione (GSH) or purified Cd, Zn thionein (commercially available) can be utilized to quantify the metallothionein content in mollusc tissues. Absorbance is evaluated at 412 nm.

Equipment

- ✓ Cooling high speed centrifuge (having swing- and fixed-type angle rotors)
- ✓ Spectrophotometer
- ✓ Motor driven teflon/glass Potter homogenizer with teflon tip
- ✓ Freezer
- ✓ Nitrogen gas cylinder

Solutions and Chemicals

Homogenization buffer

0.5M Sucrose-20mM TRIS pH 8.6.

Leupeptin stock solution (1 mg/ml) (SIGMA L2884).

Ethanolic stock solution of Phenylmethylsulfonyl fluoride (PMSF) (58 mg/ml) (SIGMA P7626).

To the desired volume of Sucrose- TRIS buffer add 3.0 µl/ml leupeptin, 1.5 µl/ml PMSF and 0.1 µl/ml ß-mercaptoethanol (equivalent to 0.01 %) (MERCK 805740).

- GSH (SIGMA G 4521) stock solution: freshly prepared before analysis
- 0.25M NaCl
- 0.2M phosphate buffer pH 8 containing 2M NaCl (store at room temperature)
- DTNB (SIGMA D 8130)
- RNA (SIGMA R 7250) (100 mg/ml) store at -20°C.
- cold (-20°C) absolute ethanol
- chloroform
- 37% HCl
- 1 N HCl/EDTA 4 mM (store at room temperature)

Preparation of sample and enriched MT fraction

Homogenization

Rapidly dissect out and blot the digestive gland on 3 µm filter paper. Weigh a pool of tissues from at least 10 animals and homogenize about 1 g tissue in 3 volumes of homogenizing buffer containing ß-mercaptoethanol, PMSF and leupeptine, with 8 strokes in a motor driven teflon/glass Potter homogenizer.

Centrifugation

Centrifuge the homogenate at 30,000 x g for 20 minutes to obtain a nuclei and mitocondria free soluble fraction containing MTs. The centrifuge rotor must be at 0-4°C.

Note on safety: equilibrate the tubes before centrifuging. Employ (16ml) PYREX or COREX glass tubes or organic solvent higly resistant plastic tubes.

Ethanolic precipitation

Precipitate the high molecular weight proteins present in the supernatant using cold (-20°C) absolute ethanol. To 1 ml of the 30,000xg supernatant add 1.05 ml cold (-20°C) absolute ethanol and 80 µl of chloroform. Vortex for few seconds. Centrifuge in a fixed angle or swinging rotor at 6,000xg far 10 minutes at 0-4°C. Collect the supernatant and measure the volume using a pipette.

To the 6,000xg supernatant add 40 µl of 37% HCl and 10 µl of a solution of RNA (1 mg/10 µl) followed by 3 volumes of cold ethanol. Store at -20°C for 1 hour.

Re-centrifuge at 6,000xg for 10 minutes using the swinging rotor. Discard the supernatant and wash the pellet with an ethanol/chloroform/homogenizing buffer (cold -20°C) solution (87: 1: 12 v/v) without the additio n of ß-mercaptoethanol, PMSF and leupeptin.

Centrifuge for 10 minutes at 6,000xg using a swinging rotor. Remove supernatant and dry pellet under nitrogen gas stream far about 10 min.
Note: tubes must be kept on ice during all steps.
Note on safety: Use (16ml) PYREX or COREX glass tubes or organic- acid solvent resistant plastic tubes.

Resuspension of the MT enriched fraction

Add to the pellet 150 µl of 0.25 M NaCl solution and 150 µl of a solution made of 1 N HCl containing 4 mM EDTA (destabilising solution). Put a glass stirrer the tube and vortex a few seconds for a complete resuspension of the sample.

DTNB assay (Ellman's reaction)
Glutathione reference standard curve
Prepare a glutathione stock solution at 1 mg/ml concentration in 0.25 M NaCl. Store in ice. Prepare at least 3 GSH reference standard concentrations and a blank in accordance to Table
MT spectrophotometric evaluation
Just before analysis dissolve 0.43 mM (7.14 mg/42 ml) DTNB in 0.2 M phosphate buffer pH 8 containing 2M NaCl. Store the solution in darkness at room temperature.
Add to blank, standards and samples 4.2 ml DTNB solution. Centrifuge metallothionein samples at 3.000xg for 5 min. at room temperature.
Measure the absorbance, ABS412, using a spectrophotometer set at 412 nm utilizing reduced glutathione (GSH) as a reference standard.

	GSH stock solution	0,25 M NaCl	1N HCl 4 mM EDTA	DNTB solution	Final volume
Test samples	-	150µl	150µl	4,2 ml	4,5 ml
Blank	-	150µl	150µl	4,2 ml	4,5 ml
Standard					
Stnd 20 (14.4 nmol/ml)	20 µl	130µl	150 µl	4,2 ml	4,5 ml
Stnd 40 (28,8 nmol/ml)	40 µl	110 µl	150µl	4,2 ml	4,5 ml
Stnd 80 (57,8 nmol/ml)	80 µl	70 µl	150 µl	4,2 ml	4,5 ml

Calculation and interpretation of result

Plotting of a standard curve

Express glutathione GSH reference standard as nmol/ml.

Plot a standard curve in which ABSGSH412 is a linear function of GSH concentration (nmol/ml).

ABSGSH412 = e [-SH]

Where (unit of measure between brackets):

ABSGSH412, (OD412), is the Optical Density of GSH samples at 412 nm ,

e,(OD412/nmol/ml), is a constant representing the extinction coefficient for GSH,

[-SH], (nmol/ml), represents the concentration of sulphydrylic groups in GSH samples.

Quantitative determination of MT content

Interpolate ABSMT412 values obtained for metallothionein samples on the reference curve. The corresponding values found on the X-axis represents the concentrations of sulphydrylic

groups belonging to metallothionein present in the samples. Taking into account the molecular features of mussel metallothionein (Mackay et a/., 1993), for which cystein residues are 21 and molecular weight is 8600 DA, the final volume of DTNB reaction (4.5ml) and the dilution factor of the homogenate (4), the concentration of mussel metallothionein (ng/g tissue w.w.) present in the samples can be obtained using the following formula:
[x/21]*8,600 * 4.5 * 4 ,

where :

x (nmol/ml) represent the X-axis value coming from interpolation of ABSMT 412 value on the reference curve.

Alternatively, determine graphically or by the use of a specific software (such as Microsoft Excell) the equation ABSGSH412 = e [-SH] and use the following formula to determine metallothionein (ng/g tissue w.w.) content in the samples:

[[(ABSMT412/eGSH)/21]*8,600] * 4.5 * 4 , (1)

where :

ABSMT412 is the OD value read for metallothionein sample
eGSH is a constant representing the extinction coefficient for GSH

21 is the number of cysteine residues of mussel metallothionein (Mackay et a/., 1993)

8.600 is the molecular weight of mussel metallothionein (Mackay et al., 1993)

4,5ml is the final volume of DTNB reaction

4 is the dilution factor of the homogenate.

(1) it can be thus simplified:

(ABSMT 412/eGSH) * 7.37 *103 , (2)

A higher level of sulphyhydryl content (=MT) relative to the basal pre-existing level in reference clean samples would generally indicate the presence of a metal pollution stress in the animals at the sampling location. However, for the reasons already discussed in the introductory part of this manual, high MT levels can also be related to other factors which can influence its synthesis. For a correct approach to the use of Metallothionein as biomarker of exposure to heavy metal pollution (Viarengo et al., in press).

Coastal areas where the organisms show higher MT level than those belonging to reference unpolluted areas should be further investigated. In this case, chemical analysis of biota and sediments is necessary to identify the type of insulting metal in that particular area. Therefore it would be right to set aside a set of samples far metal analysis.

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Marine Pollution Indicator fact sheet

MED POL

Biomarker for the evaluation of DNA damage in mollusc and fish cells

Key message

DNA damage is a biomarker able to evidentiate breaks in the nuclear DNA of the cells.

This biomarker is able to integrate the DNA alterations induced by the total amount of the pollutants accumulated in the cells of the sentinel organisms.

This important biomarker is not particularly sensitive and this is due to the high value of standard deviation often observed in the reported data.

Policy relevance

DNA damage was adopted as genotoxicity biomarker in different international programs such as the UNEP MAP Mediterranean Biomonitoring Program. This genotoxicity biomarker was also employed in the framework of the RAMOGE activity. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was included in the final report in the list of the "suggested" biomarker.

Policy context

UNEP MAP Mediterranean Biomonitoring Programme, RAMOGE activity and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate genotoxicity response in fish and molluscs.

Environmental context

Thousands of toxic compounds capable of inducing alterations in the physiological status of organisms may be present in the marine environment. Among these, carcinogenic compounds are of particular interest; tumours have been frequently described in marine fish and shellfish (Bolognesi, 1990; Gopal, 1993; Malins, 1988; Mix, 1986; Mix, 1988).

Recently, the importance of POPs (Persistent Organic Pollutants) has been underlined (Siu, 2004). POPs are usually present in the marine environment at very low concentrations but, due to their persistence, they are accumulated in the tissues of marine organisms at high concentrations, several orders of magnitude higher than those present in seawater.

POPs do not only exert general toxic effects but they are also known to be genotoxic, i.e. able to modify DNA structure altering its integrity, either directly or through their metabolites (Shugart, 1995). In general, genotoxic compounds such as POPs may cause mutagenesis (Siu, 2004).

It should be mentioned that the metabolism of aromatic xenobiotics might greatly differ in vertebrate (mammalian and fish) and invertebrate species. In particular, it was demonstrated that the mixed function oxigenase (MFO), the most important pathway in the metabolism of

organic xenobiotic compounds in fish, is present in mussels at very low level (Ade, 1982; Livingstone, 1984; Stegeman, 1985; Roggeband, 1993; Livingstone, 1996). In addition, more recent molecular studies could not demonstrate the presence of a CYP1A-like gene in the mussel genome. On the other hand, it was demonstrated that mussels are able to metabolise B[a]P into Benzo[a]pyrene quinones: these compounds interact with DNA or undergo redox cycling and generate superoxide anion radicals (O2-) and other reactive oxygen species (ROS) (Flowers,1996; Devanesan,1996, Shou,1993). The formation of DNA adducts, an important event in genotoxicity in mammalian and fish tissues (Cavalieri, 1995; Chen, 1996), has been also demonstrated in the gill cells of M. galloprovincialis exposed to B[a]P (Kurelec,1988; Venier,1996; Bolognesi,1996).

Although the detection of DNA adducts using P52-postlabelling represents a direct evidence of the genotoxic effects of organic pollutants, it should be stressed that, due to the complexity of the methodology, this test is not usually considered a suitable biomarker for routine use in large biomonitoring programmes.

Chemical-induced DNA alterations include single and double strand breaks, modified bases and DNA-DNA protein cross links. The breaks in the DNA may be due to indirect action of ROS or by action of the excision repair enzymes, as well as to consequences of apoptotic or necrotic processes.

Among the methods usually adopted to detect DNA damage, 3 tests seem to be the most suitable for use in biomonitoring programmes. The first is the Alkaline Elution method, that is based on the fact that the rate at which DNA single strand fragments pass through a membrane filter under alkaline conditions is related to the length of the DNA strand (Kohn, 1976). This method, opportunely modified, was successfully utilized to evaluate the genotoxic effects of pollutants in fish and molluscs exposed to chemicals both in the field and in laboratory experiments (Bolognesi,1996).

A second method, the COMET assay, is now largely used to assess the genotoxic effects of pollutants: individual cells are directly embedded in agarose (to minimize mechanical shearing artefacts) and cellular membranes are disrupted using a lysing solution containing high salt concentrations and detergents. The nuclear DNA is then electrophoresed under neutral conditions: the cleaved DNA fragments migrate away from the chromatinic nucleosomal residual core structure.

DNA staining with a specific dye and examination under a fluorescence microscope shows a “comet” in which the distance of DNA migration from the core (i.e. the comet tail length) reflects the level of double strand DNA breakage (Siu, 2004).

It is important to note that the incorporation in the methodology of an alkaline unwinding step allows the sensitive detection of both double and single strand DNA breaks and permits the generation of breaks at the alkaline labile sites due to the DNA adduct formation. A large body of studies confirms the successful application of this methodology both to fish and molluscan cells (Bolognesi, 1996).

Another approach to evaluate DNA strand breaks is based on the rate of unwinding under alkaline conditions measured by the incorporation of a fluorescent dye in double strand DNA (Bolognesi, 1996). Recently PicoGreen, a new fluorescent dye, has been utilized to evaluate DNA unwinding. A novel micromethod has been developed (Batel, 1999) and utilized to estimate DNA damage in fish and invertebrate cells and tissues. PicoGreen is able to bind to intact double strand DNA with high affinity and fluorescence response, with negligible fluorescence background of the unbound dye. These characteristics of PicoGreen allow the estimation of minimal levels of DNA fragmentation. Specific conditions of analysis, such as

temperature, pH, lysis buffer have been found to optimize the evaluation of the denaturation kinetic curves and the calculated strand scission factors.

However, standardisation of the protocol as well as intercalibration procedures are needed before routine application in the near future of this new method and of the “comet single cell assay” in the evaluation of DNA damage in biomonitoring programmes.
Assessment of the indicator

Alkaline Filter Elution Method
From:” Manual on the Biomarkers recommended for the Med Pol Biomonitoring Programme”. UNITED NATIONS ENVIRONMENTAL PROGRAMME MEDITARRANEAN ACTION PLAN. Athens 1999.

The following protocol, commonly known as the alkaline filter elution method (AFE), is a widely used method to determine the extent of genetic damage in a wide range of marine organisms (Erickson et al., 1980). DNA single strand breaks or weak points in the alkali are identified by measuring the rate at which single-stranded DNA passes through a membrane filter of known porosity under alkaline denaturing conditions.

The sensitivity of the method depends on the complexity of the DNA, which differs considerably among the different taxa. Thus, DNA from a lower taxon will elute faster than one of a higher one, even if completely undamaged. One good advantage in using this method is that it allows the determination of genotoxic damage in live animals, and in many instances, small tissue biopsies may be sufficient. Additionally, microfluorometric DNA determination (Cesarone et al., 1979) increases the sensitivity and reproducibility of the alkaline elution method.

Equipment

- peristaltic pumps with multiple channels (flow rate 1-10 ml/h);
- spectrofluorometer: excitation: 360 nm / emission: 450 nm.;
- fraction collector;
- pH meter able to measure pH>12;
- filters (Millipore), Type GV 0.22 µm, GVWP 02500;
- inverted microscope;
- centrifuge;
- micro-syringe filter holder, luer inlet (XX30 02500)4 ;
- micro-syringe stainless support screen4 ;
- O-ring teflon filter sealing4
- flat gasket, teflon

Chemicals and Solutions

- Homogenisation buffer 0.14 M NaCl
  1.47 mM KH2PO4
  2.7 mM KCl
  8.1 Na2HPO4
  0.1 M EDTA
  Bring the solution to pH 7.4 using NaOH.

- HANKS' balanced salts solution 2X
  273.8 mM NaCl
  10.73 mM KCl
  0.81 mM MgSO4. 7H2O
  2.52 mM CaCl2. 2H2O

0.674 mM Na2HPO4. 2 H2O
0.88 mM KH2PO4
8.33 mM NaHCO3
10.09 mM D-Glucose. H2O

- Lysing solution
  2 M NaCl
  0.02 M EDTA
  0.2% N-laurylsarcosinate, sodium salt (Sigma L5125)
  bring solution to pH 10.2 using NaOH 1 N.

- Washing solution

  0.02 M EDTA
  bring solution to pH 10.2 using NaOH 1 N.

- Eluting solution

  0.04 M EDTA. Bring to pH 12.3 using tetraetylammonium hydroxide (Merck 822149.0250).

- Working BIS solution

  Prepare 1.5 x 10-4 M of BIS solution by dissolving 8 mg of bisbenzimide (33258 Hoechst: 2-[2-(4-hydroxy-phenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)-benzimidazole trihydrochloride (Farbwerke Hoechst, Frankfurt, Germany), MW: 533.9) in 100 ml distilled water. Make 1 ml aliquots in Eppendorf tubes and stare at -20°C. This solution remains stable for at least 1 week when stored at 4°C in dark glass bottles and wrapped in tinfoil. Prepare the working solution by add 100 ml of water containing 0.154 M NaCl and 0.015 M Na3citrate (SSC buffer, pH 7.0) to 2 ml of BIS stock solution. The final solution is the working BIS solution.

- DNA standard

  Calf thymus DNA was purchased from Sigma Chemical Company (St. Louis, Mo.), dissolved in sterile SSC, pH 7.0, sonicated far 10 s to increase the solubility, and diluted to a concentration of 1 mg/ml. This stock solution was diluted with SSC, pH 7.0.

Sample preparation from tissues of aquatic organisms
Avoid damaging DNA during handling procedures by using high EDTA concentrations (0.03-0.1 M). Always keep materials on ice and try to work fast.

For fish liver
Excise liver and wash in homogenization buffer to remove blood residues. Proceed immediately to the next step or stare at -80°C. Homogenize the liver in the buffer using 1:5 w/v.

For mussel gill cells
Open mussels and remove gill cells. Isolate gill cells by enzymatic digestion with a solution of dispase (Boehringer Mannheim), 0.1 mg/ml in modified (2X) Hanks' Balanced Salt solution, far 10 min at 37°C. The cellular suspension obtained by filtration is centrifuged at 1,000 rpm far 10 minutes.

For mussel haemolymph

Introduce a hypodermic syringe in the large adductor muscle and draw out some haemolymph. Dilute the sample with equal volume of Hanks' solution 2X.

Sample application

Prepare the elution apparatus. Place a filter (0.22 µm pore size) on a stainless support screen set on a filter holder, then put an O-ring Teflon filter sealing and screw a stainless extension barrel.
Load sample onto filter (10-20 mg fish liver per filter; 1 to 2 X 106 haemocytes or gill cells per filter). Count cell concentration using a counting chamber.

Cell lysis

Wash the filters with 4.5 ml of lysing solution. Repeat washing using 2.5 ml of washing solution at the same flow rate.

Elution of single stranded DNA

Perform the elution under reduced light conditions.
Elute DNA through Durapore filters (25 mm diameter, 0.2 I.µm pore size)
placed on filter-holders (Millipore Corp. USA) with 10 ml of eluting solution at a flow rate of 0.05 ml/min (i.e. 2 ml. per fraction). Collect this volume in 5 tubes each containing 2 ml.
Recover the remaining DNA by removing the filter and immersing it in 4 ml of eluting solution. Cut it into small pieces. Shake vigorously.
Rinse the filter holder and tubes with 4 ml of eluting solution. This is denoted as 'dead' volume.

Microfluorimetric determination of DNA

Place 1 ml aliquots of each elution fraction, the DNA retained on the filter, and a wash of the filter holder in disposable glass test tubes. Neutralise each tube with 0.4 ml of 0.2 M KH2PO4, and add 0.6 ml of water to bring the volume up to 2 ml. Finally, add 1.0 ml of working BIS solution and vortex. Determine the increased fluorescence, due to binding of the fluorochrome to DNA, using a spectrofluorometer with the excitation wavelength set at 360 nm and the emission at 450 nm.

Calculation

Calculation of the slope of the elution curves (elution rate)
Elution slopes are calculated from semi-logarithmic plots of the fraction of DNA retained on the filter versus eluted volume or elution time and are expressed as the average rate of elution. The slope of the elution curve is a measure of the number of breaks in arbitrary units. Since the elution rate is faster for broken than for intact DNA, the amount of DNA retained on the filter is a measure of DNA single strand breaks. The elution rate decreases exponentially with elution time or volume (fraction number).
When the elution profile approaches a straight line we could use the first order kinetics equation.

For first order kinetics of alkaline elution:
$y = ae^{-kv}$
where:
y = the fraction of DNA retained on the filter after the elution of the volume v
a = the quantity of DNA present on the filter at the O solution volume
v = vol

ln y = -kv + ln a
and
K = -ln y/v

The elution rate (K) could also be expressed versus t (the time of elution).

Calculation of Strand Scission Factor (SSF)

A value characterising the relative number of DNA-strand breaks, referred to as a "strand scission factor", was calculated by taking the absolute value of the log10 of the percentage of DNA retained in the treated sample eluted divided after a known elution volume by the percentage of DNA retained in the control sample eluted into the same amount of volume. Therefore, a strand scission factor of 0 indicates no DNA strand breaks. Values greater than 0 indicate a relative value far DNA breaks in the exposed cells (Meyn and Jenkins, 1983).
SSL=log [(% DNA eluted in 6 ml from test sample)/(% DNA eluted in 6 mi from control sample)]
Table 1
Grid table showing presentation of arbitrary data

Control sample

DNA Standard: 1 µg	Fluorescence 160					
Fraction number	Volume (ml)	X value total volume (ml)		Total fluorescence	Y (%retained)	ln Y
1	1.8	1.8	45	81	98.4	4.58
2	1.9	3.7	45	85	96.7	4.57
3	1.8	5.5	42	76	95.2	4.55
4	1.8	7.3	39	70.2	93.8	4.54
5	2.1	9.4	40	82	92.3	4.52
Dead	4		38	152		
Filter	4		1138	4552		
		Total fluorescence		5098		
		Total DNA (µg)		31.8		

For control sample	For treated or polluted sample
K=0.0088	K=0.0575
Y=70.8	Y=95.2

Treated or exposed sample

DNA Standard: 1 µg	Fluorescence 160				
Fraction number	Volume (ml)	X value total volume	Total fluorescence	Y (%retained)	ln Y

		(ml)				
1	2.0	2.0	248	496	87.8	4.48
2	2.0	4.0	205	410	77.8	4.35
3	2.0	6.0	142	284	70.8	4.26
4	2.0	8.0	98	196	66.0	4.19
5	2.0	10.0	92	184	61.5	4.12
Dead	4		65	206		
Filter	4		562	2248		
		Total fluorescence		4078		
		Total DNA (µg)		25.4		

SSF = log (70,8/95,2) = -0,129 at 6 ml of elution

Dna Unwinding Using Fluorometry (Picogreen ds DNA Quantitation)
From: "Beep Final Report". Realized by Dr. Claudia Bolognesi and co-workers (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy)

Pico Green is a fluorophore that selectively binds dsDNA and it appears to exhibit high affinity for DNA and a large fluorescence enhancement upon DNA binding. Pico Green is very stable and little background occurs since the unbound dye has virtually no fluorescence. The fluorescence enhancement of PicoGreen dye on binding to DNA is nearly 2000 fold for dsDNA but is very low upon binding to ssDNA or RNA.

All these characteristics allow to evaluate low levels of DNA fragmentation induced by genotoxic insult.

The method could be applied to detect and quantitate small amounts of DNA and allows to develop a microplate-based assay to evaluate DNA single strand breaks in small amount of tissues.

The method has been applied in fish liver and in digestive glands of mussels in laboratory experiments as well as in the field, revealing a sufficient efficiency and discrimination power in the classification of the coastal sites along a pollution gradient. The most critical point in the application of the method is the DNA denaturation which is obtained in different condition in mussels and fish.

PicoGreen Method
Liver fragments or Digestive glands were homogenized in TE buffer (10 mM Tris HCl buffer, Na2 EDTA 1 mM - pH 7.4) with the pestle cooled in liquid nitrogen. Equalized amounts of the homogenates were then applied to a microplate.
Lysis of homogenates was performed by addition of lysing solution (9 M urea pH10.0 containing dye PicoGreen). The samples were incubated at 0°C for 1 hour in the dark.
DNA unwinding was obtained by addition of working NaOH solution in order to reach a 12.30 and 12.00 pH value in fish liver and mussel digestive gland respectively.
To achieve proper DNA denaturation condition the working NaOH solution is prepared daily by diluting the 0,1 M stock NaOH with bidistilled water in subsequent 1:1 steps and checking the final pH of the denaturation mixture.

The DNA denaturation was checked by measuring the fluorescence of ds-DNA Pico Green complex at room temperature every 5 min starting from 0 min and for at least 30 min. The extent of ssDNA was calculated as % dsDNA after 30 min vs % dsDNA at 0 min. The results could be expressed as Strand Scission factor (SSF) using an internal standard (such as V79 cells, human lymphocytes or homogenate of mussel from an unpolluted site).
SSF = log10 (% ds DNA treated sample/% ds DNA control sample)
SSF = 0 indicate no additive DNA damage.

Data source

International references

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Marine Pollution Indicator fact sheet

MED POL

Neutral lipid lysosomal accumulation in molluscs and fish cells

Key message

A biomarker of stress is a biological parameter which value changes in relation to the toxic effects of pollutants.

A biomarker of stress is able to integrate the biological response to the total charge of pollutants bioavailable for the sentinel organisms.

Neutral lipid accumulation is a se nsitive biomarker of stress. It is related to chemical induced oxidative stress syndrome.

Policy relevance

Neutral lipid lysosomal accumulation was adopted as stress biomarker in different international programs such as the UNEP MAP Mediterranean Biomonitoring Program. This stress biomarker was also employed in the framework of the RAMOGE activity. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was included in the final report in the list of “suggested” biomarker.

Policy context

UNEP MAP Mediterranean Biomonitoring Programme,. RAMOGE activity, UNIDO and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate stress response in fish and molluscs.

Environmental context

The effects of pollutants are often associated with degeneration of fatty acid metabolism and with the accumulation in the lysosomal vacuolar system of high levels of unsaturated neutral lipids (Dianzani, 1978; LÜllman-Rauch, 1979). This lysosomal accumulation of neutral lipids was found to be a useful indicator of alterations of cell physiology in the digestive gland of mussels, as well as in fish liver hepatocytes (Moore, 1988).

The relationship between lysosomal accumulation of neutral lipids and the pathological changes in cells is confirmed by the fact that neutral lipid accumulation is usually associated with lysosomal membrane destabilization, and therefore to increased autophagy.

As reported by Moore (Moore, 1988) neutral lipid lysosomal accumulation in mussel digestive gland cells may be described as a form of lipidosis induced by toxic chemicals. In fact, cytochemical data clearly indicate an accumulation of neutral lipids in the cytoplasm of the cells of pollutant-exposed organisms. The lipids (probably in form of droplets) are then internalised into the lysosomes by autophagic uptake. It is important to note that the observed increase in neutral lipid lysosomal accumulation may be related either to an

increase in the cytoplasmatic lipid content or to a decrease in the utilization of neutral lipids, this leading to an enhanced autophagy of the neutral lipids accumulated in the cells.

Moreover, it is important to underline that, depending on the gametogenic cycle, the response of this biomarker to pollutants is not always detectable in the digestive gland cells of mussels: this is probably due to the large lipid utilization related to gonadal changes and in particular to gamete formation.

In addition in some molluscs, such as Tapes philippinarum, neutral lipid lysosomal accumulation is scarcely affected by toxic chemicals, and therefore the test should be utilized only in molluscs such as mussels or fish species previously studied, to avoid to collect data of difficult interpretation. (Fabbri, personal comunication and Viarengo, unpublished data).

Assessment of the indicator

From: “Beep Final Report”. Realized by Prof. Aldo Viarengo and co-workers (DiSAV University of astern Piedmont, Alessandria, Italy)

Duplicate cryostat sections (10 µm) were post-fixed in calcium-formol for 15 min at 4°C, then rinsed in distilled water and placed in 60% triethylphosphate in distilled water for 3 min.

Sections were stained in a 1% solution of oil red 0 in 60% triethylphosphate at 20°C for 15 min (Bancroft 1967), then washed in 60% triethylphosphate for 30 s, rinsed in distilled water and mounted in UV-free aqueous mounting medium.

The lipid content is quantified in terms of section staining by image analysis.

Data source

International references

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Peroxisome proliferation

Key message

It is categorized as biomarker of exposure i.e. a biomarker able to evidentiate the biological response to a particular class of pollutants.

Peroxisome proliferation is a novel biomarker able to evidentiate the biological response to organic aromatic xenobiotic compounds such as PA,H PCBs, etc. It is of particular relevance because it is able to evidentiate the stress response to organic aromatic compounds also in mussels, usually utilised as sentinel organisms in biomonitoring programs (in mussels EROD activity is not detectable).

Policy relevance

It has been utilised only at local level and it was included in the biomarker list of the UE Program BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) final report.

Policy context

No policy has included this biomarker in the National or International Protocols and Conventions.

Environmental context

Peroxisomes are single membrane organelles involved in different cellular functions such as lipid metabolism and the biochemistry of oxygen reactive species (ROS)(Mannaerts and Van Veldhoven, 1993; Singh, 1996). These organelles in mammalian and also in fish and molluscan cells contain a number of antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Dhaunsi, 1992; Singh, 1994; Orbea, 2000).

Initially peroxisomes were studied in relation to their important role in lipid and ROS metabolism; however, it was successively demonstrated that both in vertebrates and invertebrates peroxisomes also play an important role in the metabolism of aromatic xenobiotics. It has in fact been demonstrated that in vertebrates different organic toxic chemicals are able to stimulate peroxisome proliferation (Beier, 1991; Gibson, 1993; Reddy, 1994). Peroxisome proliferation has been confirmed also utilizing invertebrate species as experimental organisms (Cajaraville, 1997; Fahimi and Cajaraville, 1995; Krishnakumar, 1997; Peng, 1997; Cancio, 1998).

It is now well established that peroxisomal proliferation is characterized by an increase in volume and number of peroxisomes: these changes are often associated to an increase of the enzymes involved in fatty acid oxidation, as well as of enzymes such as catalase involved in ROS metabolism. The fact that the two aspects do not always show similar trends

of change during peroxisome proliferation renders the biochemical/cyto chemical approach an essential aspect in the utilization of this new biomarker.

From what reported above, it appears clear that peroxisome proliferation seems to be an important biomarker of exposure, able to evidentiate both in fish and mussels the exposure of the organisms to aromatic xenobiotics.

Although the kinetic of the response of this biomarker to increasing xenobiotic concentrations should have a continuously increasing trend, different studies evidentiated that in pollutant exposed organisms the change observed in peroxisome proliferation may represent a transient effect and the biomarker can often show a bell shaped curve of stress response (Cajaraville, 1997).

The evaluation of peroxisome proliferation has been long carried out utilizing the evaluation of catalase activity/cytochemical determination; however, more recent data collected in the framework of BEEP European Program demonstrated that AOX (acyl-CoA oxydase activity) determination represents the best approach to evaluate peroxisome proliferation. AOX activity is evaluated in whole homogenates of digestive glands and quantification of peroxisomal volume density (Vvp) is carried out using cryostat sections stained with the alkaline DAB technique for demonstration of the peroxisomal marker enzyme catalese. Therefore the methodology presented, developed by the group of M. Cajaraville, was obtained from the BEEP final report. Only in the next years the procedure for the evaluation for this biomarker will be available in the international literature and it will be then possible to critically evaluate the results obtained in different laboratories.

As a final point it should be also stressed that low levels of aromatic xenobiotics do not seem to be able to stimulate peroxisomal proliferation, as evaluated by the catalase biochemical/cytochemical test (Orbea, 2002); therefore it should be further demonstrated that the new methodology is able to render this biomarker more sensitive and suitable for biomonitoring studies.

Assessment of the indicator
Removal of paraffin and dehydration
1.- Xylol 2x10 min
2.- Ethanol 100º 2 min
3.- Ethanol 96º 2 min
4.- Ethanol 80º 2 min
5.- Ethanol 70º 2 min
6.- H2Od 2x5 min

Antigen retrieval

1.- Choose between different pretreatments

a) Trypsin: place slides in a wet chamber at 37ºC with a drop in each slice of the corresponding trypsin dilution
a.1) Trypsin 0.1% 5 min 37ºC
a.2)Trypsin 0.01% 5 min 37ºC

b) Microwave: place slides in a (plastic) kopling (do not use glass, it gets broken), in 0.01 M citrate buffer for 15 min in a household microwave (740W).
Wait 20 min at room temperature, without removing the slides from the microwaved buffer.

c) Microwave plus trypsin: proceed with trypsin digestion as in a.2) and then follow the protocol as in b).

2. - Wash with TBS 2x5 min

Immunolabeling
1. - Peroxidase blocking: place slides in 3% H2O2 diluted in methanol 10 min

2- Wash with TBS 3x10 min
11.- Blocking solution 1 h at room temperature
TNB (Belongs to a Perkin Elmer TSA-amplification kit)
12.- Wash with TBS 2x5min
13.- Specific primary antibody, diluted in TNB (1:8)+0.05% Tween, overnight at 4ºC
14.- Wash with TBS 2x5 min
15.- Biotinylated goat anti-rabbit secondary antibody (1:20) diluted in TNB+0.05% Tween
16.- Wash with TBS 2x5 min
17.- Extravidin coupled to HRP (1:20) diluted in TBS
18.-Wash with TBS

Visualization of the immunoprecipitation

Novared (follow manufacturer´s instructions), 4-8 min
DAB 10 min
Prepare DAB at a concentration of 60 mg/ml and keep frozen in 750 µl aliquots.
Use 1 aliquot and dilute in 75 ml TBS. Add 25 µl H2O2.
20.-Wash with TBS
21.- Contrast with hematoxylin 20-30 sec (optional)
22.- Wash with H2Od
23.- Mount with glycerine-gelatine.

Data source

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Marine Pollution Indicator fact sheet

MED POL

Stress on stress (survival in air) in molluscs

Key message

A biomarker of stress is a biological parameter which value may change in relation to the toxic effects of pollutants.

A biomarker of stress is able to integrate the biological response to the total charge of pollutants bioavailable for the sentinel organisms.

Stress on stress represent a simple and low cost biomarker at organism level i.e. a biomarker able to show the pollutant induced alterations in the organism physiology that render the animal more sensitive to further environmental changes. Bivalve molluscs such as mussels are usually utilised for this test.

Policy relevance

Lysosomal membrane stability was adopted as stress biomarker in different international programs such as the UNEP MAP Mediterranean Biomonitoring Program. This stress biomarker was also employed in the framework of the RAMOGE activity. In the BEEP (Biological Effects of Pollutants in Marine Coastal Ecosystems) UE Program it was included in the final report and in the list of the suggested biomarkers.

Policy context

UNEP MAP Mediterranean Biomonitoring Program,. RAMOGE activity and different regional biomonitoring programs have proposed the utilisation of this biomarker to evaluate stress response in molluscs.

Environmental context

Among the biomarkers utilized to evidentiate the stress syndrome in marine mussels there is the need to select simple, low-in-cost and reliable tests.

The time of survival in air of mussels has been found to be a biomarker matching these characteristics (Viarengo et al., 1995; Thomas et al., 1999; Eertman et al., 1995; Smaal et al., 1991; Veldhuizen-Tsoerkan et al., 1991). It was demonstrated in laboratory experiments that mussels survival in air decreases proportionally to the pollutant concentration in the sea water; in fact exposure to Aroclor 1254 at the concentration of 0.3 and 0.6 µM have dose-dependent effects on mussels survival (LT50) (Viarengo et al., 1995).

Exposure to 9,10-dimetyl 1,2 benzanthracene (0.4-0.6 µM) resulted in a LT50 of 6 and 4 days, and exposure to nM concentration of Cu (360 nM) showed similar effects (Viarengo et al., 1995). These data were collected leaving the mussels (n 40) to die in an humidified chamber at the temperature of 15°C (Viarengo et al., 1995).

The biological meaning of this test assumes particular importance when the definition of "stress" is considered: in fact, stress is usually defined as a measurable alteration of biochemical and/or physiological parameters induced by an environmental change which results in a reduced capacity of the animals to adapt to further environmental changes (Bayne, 1986).

Therefore, the evaluation of the survival time in air of mussels should be more correctly named as "stress on stress" response, i.e. a biomarker able to interpret at the organism level the effects of environmental stressors, and that can clearly show if the change of the physiological status has affected the capacity of molluscs to survive to a further environmental change (i.e. air exposure).

For its nature this extremely simple biomarker is, together with scope for growth (Widdows et al., 1992; Widdows et al. 2002), the only able to evidentiate the effects of pollutants at the organism level, and also to indicate the possible potential effects at the population level.
The response of this biomarker shows a typical curve characterized by a continuous decrease of the parameter (LT50) with increasing pollutant concentrations, although in some experiments in the presence of minimal amount of pollutants a slight increase was also observed probably due to an hormesis effect (Eertman, 1995).

The stress on stress response was successfully utilized as biomarker of stress in biomonitoring programmes such as Ramoge. Several recent papers have emphasized the importance of utilizing this simple biomarker to evaluate the effects of aromatic polycyclic hydrocarbons as in the case of Exxon Valdez (Thomas, 1999) or in the Halifax Harbour biomonitoring (Hellou and Law, 2003), as well as in studying the effects of the pollutants present in untreated sewage (Moles and Hale, 2003).

In conclusion, the stress on stress response appears to be an excellent biomarker for evaluating the long term effects of pollutants, and in particular of crude oils and heavy metals. This does not mean that stress on stress may be considered an highly sensitive early warning biomarker of stress, such as lysosomal membrane stability or lipofuscin lysosomal accumulation, but it should be considered an important "end point", i.e. a biomarker able to show when the pollutant induced stress syndrome has reached the organism level and may affect the capacity of the organisms to survive in their environment due to their higher sensitivity to further environmental changes.

Assessment of the indicator

This test consist in the exposure of animals to air at 15 °C in a humidity chamber. Mortality is recorder daily, until 100% is reached. As known bivalves can survive for a long time in air, but individual stressed by pre-exposure to pollutants show higher mortality than controls.

Data source

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CHEMICAL INDICATORS

Introduction

The preparation of the "Chemical Indicators" was based on a list like the one presented as Annex I to the Document ***Concept Paper on Mediterranean Marine Pollution Indicators*** under the heading ***Chemical Indicators, Core Set*** at the **Expert Meeting on Marine Pollution Indicators (MPIs)**, Athens, Greece, 4-5 April 2005.

The work reported addresses the assessment of the state of the Chemical Indicators (as listed) in the Mediterranean Sea much in the way the EEA has been carrying out over the last years. As a matter of fact, the EEA's published Indicators were used, to some extent, as a model for the preparation of the present Set of Indicators.

One of the questions raised during the drafting was the strong connection between several of the Indicator's subjects. For example, ***Nitrate***, ***Ammonium***, ***Nitrite***, ***Total nitrogen*** were individual MPIs to be developed for ***Sea water*** but their strong and immediate interconnection, resulting in high redundancy, justified their merging into one single Indicator named ***Nitrate and other forms of Inorganic Nitrogen in transitional, coastal and marine waters of the Mediterranean Sea***.

Furthermore, during the Expert Meeting, it was proposed further merging of many interconnected and/or complementary indicators to conclude with a much smaller set of MPIs:

1. ***Loads of Trace Elements and other hazardous substances discharged to the coastal environment and levels in marine biota.***

2. ***Loads of Nutrients (organic and inorganic) discharged to the coastal environment and Eutrophication.***

3. ***Natural and man-made long-term basin-scale changes in sea water properties related to Climate Change.***

Therefore, the set of MPIs presented to the Expert Meeting under the heading ***Chemical Indicators, Core Set*** should be combined under one of the three above headings and/or redrafted in the following way:

1. List of items referred to hazardous substances

    - *Heavy metals in Effluents*
    - *HH (+PAH) in Effluents*
    - *Total Mercury in Biota*
    - *Total Cadmium in Biota*
    - *Bacterial levels in bathing waters*
    - *Bacterial levels in shellfish-growing areas*

2. List of items referred to Eutrophication (transitional and coastal environments)

    - *Nutrients in Effluents (loads)*
    - *BOD/COD in Effluents (loads)*
    - *Orthophosphate*
    - *Total Phosphorus*
    - *Orthosilicic acid*
    - *Dissolved Oxygen*
    - *Nitrate, Nitrite, Ammonium*
    - *Total Nitrogen*

- *Chlorophyll a*
- *Temperature*
- *Salinity*
- *Transparency*
- *pH*

3. List of items referred to the (open) marine environment related to Climate Change

   - *Temperature*
   - *Salinity*
   - *Dissolved Oxygen*
   - *Nitrate, Nitrite, Ammonium*
   - *Orthophosphate*
   - *Orthosilicic acid*
   - *pH, Alkalinity*
   - *Transparency*
   - *Chlorophyll a*
   - *Nutrient deposition (atmospheric)*

The above proposal not only would allow a more homogeneous treatment of the various MPIs involved but would eliminate redundancies and provide a higher coherence in the ensuing Monitoring Programmes. The difference in technical and human skills required by the three proposed Indicators would also allow different strategies to be followed in their monitoring:

1. Monitoring the MPI 1 ***Loads of Trace Elements and other hazardous substances discharged to the coastal environment and levels in marine biota*** is typically a health-related undertaking that falls perfectly within the responsibilities of a Ministry of Health.

2. Monitoring the MPI 2 ***Loads of Nutrients (organic and inorganic) discharged to the coastal environment and Eutrophication*** is a purely environmental task that falls under the responsibilities of a Ministry of Environment.

3. Monitoring the MPI 3 ***Natural and man-made long-term basin-scale changes in sea water properties related to Climate Change*** is a purely research objective responsibility of the oceanographic community.

Not only the human and instrumental resources differ between the three above areas but also the sampling/measurement strategies. Just to put some examples mentioned in the Expert Meeting, the MPI 1 would require seasonal to annual sampling (or whenever the Biomarkers activity would trigger the alarm). The MPI 2 might require continuous automatic measurements of some parameters (Dissolved Oxygen, Chlorophyll a, etc.) at least during critical periods (e.g. summer). The MPI 3 may well be carried out at supra-annual frequencies and not necessarily for the purpose of assessing this Indicator.

Finally, the author recommends that the presently available MPI documents be combined and better focussed to the above goals. This should allow a much better definition of the environmental problems being addressed and include an adequate conceptual model of the phenomenon as well as some of the indices (such as the TRIX in the case of Eutrophication) which could not be applied when dealing with individual-parameter MPIs as they deal across them.

Much of the work to be done is of an editorial nature. The rest can be done by corresponding between the author, the Secretariat and other participants to the Expert Meeting in close collaboration.

Marine Pollution Indicator fact sheet

MED POL

Biochemical Oxygen Demand in effluents discharging to the Mediterranean Sea

Key message

Discharges of high-BOD waters to the coastal environment should be restricted unless a sufficiently high degree of dispersion is guaranteed by the outfall to avoid accumulation of POM in its surroundings and assure that oxygen requirements for oxidation of DOM/POM in the water column will be lower than the oxygen saturation.

Policy relevance

The land-based sources Protocol aims at the reduction of BOD in effluents discharging to the marine and coastal environment through proper waste-water treatment plants.

Policy context

The Contracting Parties agreed to the achievement of the reduction of pollutant levels from urban discharges to the coastal areas of the Mediterranean Sea

Environmental context

Whether treated or not, effluent waters always contain dissolved and particulate organic matter that makes up the substratum for various types of micro-organisms. Particularly, bacteria decompose most organic materials using oxygen dissolved in the water. The rate of consumption of oxygen by the native bacterial population feeding on the existing organic material is the base for determination of the Biochemical Oxygen Demand (BOD) sometimes also called Biological Oxygen Demand.

High BOD values can be caused by:

- high levels of organic pollution, caused usually by poorly treated waste water, or
- high nitrate levels, which trigger high plant growth.

An excessive load of dissolved or particulate organic matter may lead to the generation of hypoxic or anoxic environments shifting the system from being oxygen-controlled to be sulphur-controlled with the appearance of nasty smelling hydrogen sulphide and toxic sulphides for the biota.

Ordinary sea water contains low or very low concentrations of organic matter either in soluble (DOM) or particulate (POM) forms and near-saturation dissolved oxygen (DO) concentrations. The amount of organic matter (autochthonous) that can possibly be synthesized on the basis of the nutrient concentrations present in any sea area is always less than would be required for consumption of the oxygen dissolved in such water. However, when additional nutrients or organic matter (allochtonous) are carried out by rivers

or effluents to a coastal area (or confined sea), the respiratory processes may well upset the above-mentioned equilibrium initiating the process of ***eutrophication***.

Only a few, well known, sea areas are naturally eutrophic. The Black Sea is one case in which oxygen depletion and sulphide production occur below about 150 m depth. This is due to the high nutrient loads received from the rivers (Danube, etc.) and the relatively slow exchange of open-sea water through the shallow sills of the Bosphorus. Slight eutrophication phenomena are also occurring in such areas as the eastern subtropical Pacific or the Sea of Arabia and in the Cariaco Trench off Venezuela.

In parts of the Adriatic Sea, under the direct influence of the Po river discharges, seasonal appearance of hypoxia and even anoxia at the sediment/water interface has been reported with the consequent mortality of benthic organisms unable to escape. Exceptionally, fish and other pelagic organisms may also be stranded on the beaches of the Emilia Romagna when anoxia is generalized to the entire water column.

Discharges of high-BOD waters to the coastal environment should therefore be restricted unless a sufficiently high degree of dispersion is guaranteed by the outfall to avoid accumulation of POM in its surroundings and assure that DOM/POM concentrations in the water column will be lower than the DO at saturation.

Assessment of the indicator

Most of the urban/agricultural effluents are collected by the hydrologic network and end up in the rivers. A compilation of average BOD values in different Mediterranean rivers is given in UNEP (2003, MTS # 141). The values for the period 1985– 1990 gave a median of 3.5 mg/l, higher than the median for European rivers (EEA, 1999). Organic pollution in Mediterranean rivers is therefore, a greater problem than in northern European rivers. Besides, due to the very irregular flow of the Mediterranean rivers, they are more vulnerable to organic pollution, particularly in the dry season, when even small amounts of urban and/or agricultural waste waters may be sufficient to cause environmental problems within the rivers and also in the coastal zone.

Effluents directly discharged to the coastline rely on the dispersion of the waste water within a large body of seawater. Provided the discharge is made following the proper technical guidelines, very little effect should be appreciated on the coastal ecosystems. However, when high BOD waters are discharged in small bays or harbours, the effect may be quite visible if not completely obnoxious since the end result is the production of nasty smell and turbidity in the near shore area.

The test for determination of BOD consists in monitoring the consumption of oxygen in a flask containing an aliquot of water during a defined incubation period. Although other lengths have been reported, the usual time spent in this incubation is five days, originally thought as the maximum flushing time for a river in the UK, and the parameter is called BOD_5.

There are other techniques possibly more adapted to the quantification of dissolved and particulate organic matter; however, the BOD_5 is considered a standard method to assess the degradable organic matter, mainly in fresh waters. If the oxygen required by the amount of organic matter present in the water is above the existing dissolved oxygen, the test cannot be carried out.

In general, BOD_5 data suffer from difficulties in the sampling and the long time required for its determination in the laboratory. A similar parameter, Chemical Oxygen Demand (COD), based on the chemical oxidation of organic matter, has the advantage that measurements

can be made within a few hours, or even minutes, while BOD_5 measurements take not less than five days.

Data source

1. River data from UNEP 2003

Quality information

All these measurements suffer from difficulties in the frequency of sampling and correlation with water flow.

Further work required

Work on this issue should concentrate on elaborating an inventory of all effluents and in monitoring BOD_5 in the effluent waters with methods and frequencies agreed upon. In view of this, the issue should be incorporated in the next phase of MED POL.

Marine Pollution Indicator fact sheet

MED POL

Chemical Oxygen Demand in effluents discharging to the Mediterranean Sea

Key message

Discharges of high-COD waters to the coastal environment should be restricted unless a sufficiently high degree of dispersion is guaranteed by the outfall to avoid accumulation of POM in its surroundings and assure that oxygen requirements for oxidation of DOM/POM in the water column will be lower than the oxygen saturation.

Policy relevance

The land-based sources Protocol aims at the reduction of COD in effluents discharging to the marine and coastal environment through proper wastewater treatment plants.

Policy context

The Contracting Parties agreed to the achievement of pollutant levels from urban discharges to the coastal areas of the Mediterranean Sea
Environmental context

Whether treated or not, effluent waters always contain dissolved and particulate organic matter. Particularly, most organic materials may be oxidised using a chemical oxidant. The consumption of oxidant by the existing organic material is the base for determination of the Chemical Oxygen Demand (COD).

High COD values can be caused by:

- high levels of organic pollution, caused usually by poorly treated wastewater, or
- high nitrate levels, which trigger high plant growth.

An excessive load of dissolved or particulate organic matter may lead to the generation of hypoxic or anoxic environments shifting the system from being oxygen-controlled to be sulphur-controlled with the appearance of nasty smelling hydrogen sulphide and toxic sulphides for the biota.

Ordinary sea water contains low or very low concentrations of organic matter either in soluble (DOM) or particulate (POM) forms and near-saturation dissolved oxygen (DO) concentrations. The amount of organic matter (autochthonous) that can possibly be synthesized on the basis of the nutrient concentrations present in any sea area is always less than would be required for consumption of the oxygen dissolved in such water. However, when additional nutrients or organic matter (allochtonous) are carried out by rivers or effluents to a coastal area (or confined sea), the respiratory processes may well upset the above-mentioned equilibrium initiating the process of ***eutrophication***.

Only a few, well known, sea areas are naturally eutrophic. The Black Sea is one case in which oxygen depletion and sulphide production occur below about 150 m depth. This is due to the high nutrient loads received from the rivers (Danube, etc.) and the relatively slow exchange of open-sea water through the shallow sills of the Bosphorus. Slight eutrophication phenomena are also occurring in such areas as the eastern subtropical Pacific or the Sea of Arabia and in the Cariaco Trench off Venezuela.

In parts of the Adriatic Sea, under the direct influence of the Po river waters, seasonal appearance of hypoxia and even anoxia at the sediment/water interface has been reported with the consequent mortality of benthic organisms unable to escape. Exceptionally, fish and other pelagic organisms may also be stranded on the beaches of the Emilia Romagna when anoxia is generalized to the entire water column.

Discharges of high-COD waters to the coastal environment should therefore be restricted unless a sufficiently high degree of dispersion is guaranteed by the outfall to avoid accumulation of POM in its surroundings and assure that DOM/POM concentrations in the water column will be lower than the DO at saturation.
Assessment of the indicator

Most of the urban/agricultural effluents are collected by the hydrologic network and end up in the rivers. A compilation of average COD values in different Mediterranean rivers is given in UNEP (2003, MTS # 141). The values for the period 1985– 1990 gave a median of 3.5 mg/l, higher than the median for European rivers (EEA, 1999). Organic pollution in Mediterranean rivers is therefore, a greater problem than in northern European rivers. Besides, due to the very irregular flow of the Mediterranean rivers, they are more vulnerable to organic pollution, particularly in the dry season, when even small amounts of urban and/or agricultural waste waters may be sufficient to cause environmental problems within the rivers and also in the coastal zone.

Effluents directly discharged to the coastline rely on the dispersion of the wastewater within a large body of seawater. Provided the discharge is made following the proper technical guidelines, very little effect should be appreciated on the coastal ecosystems. However, when high COD waters are discharged in small bays or harbours, the effect may be quite visible if not completely obnoxious since the end result is the production of nasty smell and turbidity in the near shore area.

The test for COD determination consists in the titration of the dissolved and particulate organic matter with a strong oxidant. There are other techniques possibly more adapted to the quantification of dissolved and particulate organic matter; however, the COD is considered a standard method to assess the degradable organic matter, mainly in fresh waters.

In general, COD data suffer from difficulties in the sampling but, unlike BOD, does not require a long time for its determination in the laboratory.

Data source

River data from UNEP 2003

Quality information

All these measurements suffer from difficulties in the frequency of sampling and correlation with water flow.

Further work required

Work on this issue should concentrate on elaborating an inventory of all effluents and in monitoring COD in the effluent waters with methods and frequencies agreed upon. In viewof this, the issue should be incorporated in the next phase of MED POL.

Chlorophyll a in transitional, coastal and marine waters of the Mediterranean Sea

Key message

In spite of Chlorophyll a measurements being one of the parameters more studied with the satellite mounted remote sensors, very few scientific works deal with its general distribution in the Mediterranean Sea.

An extra effort ought to be done to produce, standardized maps of Chlorophyll a at regular time intervals covering the entire basin.

Policy relevance

The objective of this indicator is to assess the effects of nutrient load reductions on the proliferation of phytoplankton organisms in transitional, coastal and marine waters of the Mediterranean Sea.

Policy context

The Land-based sources Protocol deals with the reduction of nutrient loads being discharged to the coastal waters around the Mediterranean Sea. Nutrient loads in river-born discharges or in sewage outfalls cause phytoplankton blooming in coastal areas with reduction of dissolved oxygen and sudden appearance of fish-killing hydrogen sulphide and other nuisances (red tides, mucilage, etc.). Measurement of Chlorophyll a, whether from *in situ* observations or with remote sensing equipment, is one of the most practical ways of assessing the degree of eutrophication of any given coastal area.

Environmental context

Chlorophyll a is one component of the plant system required for the photosynthetic growth of phytoplankton usually used in marine and terrestrial ecology as an indicator of plant biomass and sometimes as an indicator of primary productivity. In order for phytoplankton (and plants in general) to grow, there are strong requirements not only of appropriate light conditions but of available nutrients as well. Nitrogen, phosphorus and other substances (silicate, iron, etc.) are taken up by phytoplankton organisms in order to produce biomass.

Like in all mid-latitude seas, the Mediterranean Sea environment is mostly oligotrophic due to the stratification occurring over most of the year in all areas except in those affected by particularly intense hydrodynamic conditions (fronts, upwelling, etc.) or by important land runoff. In addition, the low or very low concentrations of nutrients in the intermediate and deep waters of the Mediterranean as compared to the oceans also contribute to the limited phytoplankton development.

However, there are a number of coastal and estuarine areas where nutrient discharges are in excess of the losses through dispersion and cause the development of chlorophyll containing

phytoplankton organisms to eutrophication levels. In such cases, the Chlorophyll a indicator allows to assess phytoplankton biomass and, indirectly, also other environmental processes such as rates of water replacement.
Often, blooming phytoplankton organisms or their products may be harmful for seafood, humans or they may impair human activities such as bathing, fishing or even navigation (toxic dinoflagellates, mucilage, etc.).

This indicator describes the general biological quality of seawater and its capacity to produce higher trophic level organisms (molluscs, crustaceans, fish) and the ecological conditions leading to eutrophication (or resulting from it) caused by excessive nutrient loads.

Trends in this indicator at particular points do not necessarily relate to the success of environmental measures being taken but does relate to them in a broader environmental context.

Assessment of the indicator

There are a number of coastal lagoons and estuarine areas where nutrient discharges are in excess of the losses through dispersion and cause the development of chlorophyll containing phytoplankton organisms to eutrophication levels. In such cases, the Chlorophyll a indicator allows to assess phytoplankton biomass and, indirectly, also other environmental processes such as rates of water replacement.

Often, blooming phytoplankton organisms or their products may be harmful for marine biota or to humans through seafood, or they may impair human activities such as bathing, fishing or even navigation (toxic dinoflagellates, mucilage, etc.).

The thermal balance of the Mediterranean Sea, with large seasonal fluctuations in surface temperature, causes stratification to occur along most of the year all over the region. Only winter conditions (cooling and evaporation of surface waters) in the northern parts of the region (e.g. Gulf of Lions) are such that vertical mixing completely destroys the thermocline and nutrients from intermediate and deep waters may reach the euphotic zone. Under these conditions, a shallow Chlorophyll a maximum may appear with maximum values around 1.5 to 2.5 :g L^{-1}.

However, the Chlorophyll a maximum is found, most of the time, as a subsurface maximum layer with values dropping above and below it. Summer surface concentrations of Chlorophyll a may be as low as 0.1 :g L^{-1} in large areas of the southern and eastern parts of the Mediterranean. The depth at which the maximum Chlorophyll a values are found varies depending on the hydrodynamic and climatic conditions ranging from 50 m in the NW to 120 m in the SE. On the other hand, the concentrations reached at this Deep Chlorophyll Maximum (DCM) which seems to be related to the depth at which nutrient supply is equivalent to light availability range between 1.5 and 0.3 µg L^{-1}.

No trend could be found from comparison of data obtained over the last decades in the open seawater of the Mediterranean Sea.

Sub-Indicator

Chlorophyll in open sea waters of the Mediterranean Sea

Key message

No trend is observed in Chlorophyll a concentrations from open sea areas of the Mediterranean Sea. No excess in nutrient loads is to be expected in these open sea areas.

Assessment of the sub-indicator

The thermal balance of the Mediterranean Sea, with large seasonal fluctuations in surface temperature, causes stratification to occur along most of the year all over the region. Only winter conditions (cooling and evaporation of surface waters) in the northern parts of the region (e.g. Gulf of Lions) are such that vertical mixing completely destroys the thermocline and nutrients from intermediate and deep waters may reach the euphotic zone. Under these conditions, a shallow Chlorophyll a maximum may appear with maximum values around 1.5 to 2.5 :g L^{-1}.

However, the Chlorophyll a maximum is found, most of the time, as a subsurface maximum layer with values dropping downwards and upwards. Summer surface concentrations of Chlorophyll a may be as low as 0.1 :g L^{-1} in large areas of the southern and eastern parts of the Mediterranean (Fig. 1). The depth at which the maximum Chlorophyll a values are found varies depending on the hydrodynamic and climatic conditions ranging from 50 m in the NW to 120 m in the SE. On the other hand, the concentrations reached at this Deep Chlorophyll Maximum (DCM) which seems to be related to the depth at which nutrient supply is equivalent to light availability range between 1.5 and 0.3 :g L^{-1}.

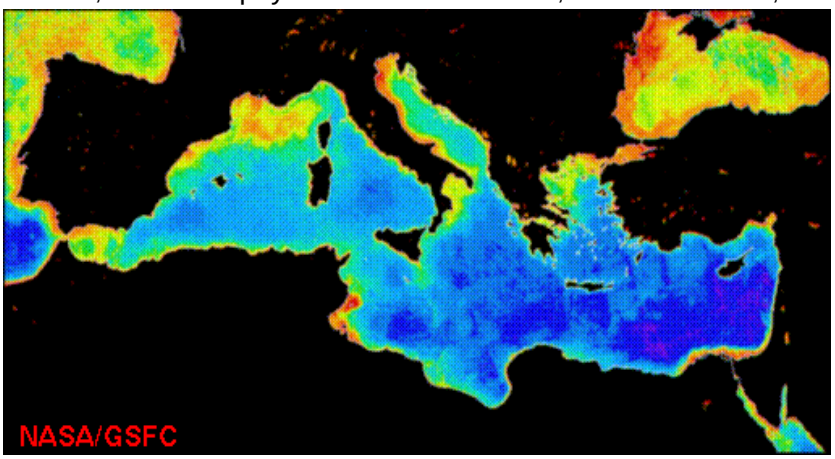

Fig. 1. Chlorophyll a distribution in the Mediterranean Sea as seen by the Nimbus 7 – CZCS sensor. Composition of images obtained during May 1980. (Courtesy NASA/GSFC Project).

No trend could be found from comparison of data obtained over the last decades in the open seawater of the Mediterranean Sea.

Sub-Indicator

Chlorophyll a in coastal waters of the Mediterranean region

Key message

No significant trend is observed in Chlorophyll a concentrations from coastal areas of the Mediterranean Sea. However, enhanced Chlorophyll a values should be expected in areas receiving large nutrient loads from rivers and/or effluents.

Assessment of the sub-indicator

There are a number of coastal and estuarine areas where nutrient discharges are in excess of the losses through dispersion and cause the development of chlorophyll containing phytoplankton organisms to eutrophication levels. In such cases, the Chlorophyll a indicator allows to assess phytoplankton biomass and, indirectly, also other environmental processes such as rates of water replacement.

Often, blooming phytoplankton organisms or their products may be harmful for seafood, humans or they may impair human activities such as bathing, fishing or even navigation (toxic dinoflagellates, mucilage, etc.).

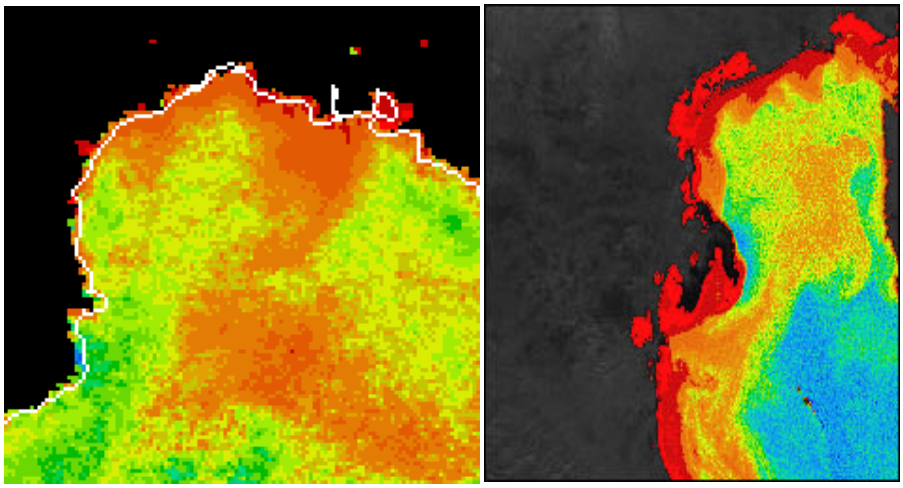

Fig. 2. Surface Chlorophyll distribution in the Gulf of Lions (left) and the North Adriatic Sea (right) as observed by the Nimbus 7 – CZCS remote sensor.

Sub-Indicator:

Chlorophyll a in coastal lagoons and estuaries of Mediterranean states

Key message

Chlorophyll a concentrations in coastal lagoons and estuaries of the Mediterranean Sea show enhanced Chlorophyll a values due to large nutrient loads received from rivers and/or effluents (Fig. 2).

Assessment of the sub-indicator

There are a number of coastal lagoons and estuarine areas where nutrient discharges are in excess of the losses through dispersion and cause the development of chlorophyll containing phytoplankton organisms to eutrophication levels. In such cases, the Chlorophyll a indicator allows to assess phytoplankton biomass and, indirectly, also other environmental processes such as rates of water replacement.

Often, blooming phytoplankton organisms or their products may be harmful for seafood, humans or they may impair human activities such as bathing, fishing or even navigation (toxic dinoflagellates, mucilage, etc.).

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Further work required

Though Chlorophyll a is an easily measurable magnitude with automatic sensors either *in situ* or remote, there are relatively few scientific references referring to the general distribution of Chlorophyll a in the Mediterranean basin.

Nonetheless, like in the case of Sea Surface Temperature (SST), there exists sufficient technology to produce near real time maps of Sea Surface Colour, with standard palettes in order to provide end-users with the information required in the monitoring of any open or coastal sea area.

The MAP ought to endeavour to obtain such products on a routine basis.

Marine Pollution Indicator fact sheet

MED POL

Dissolved oxygen in transitional, coastal and marine waters of the Mediterranean Sea

Key message

Oxygen is one of the gaseous components of the atmosphere necessary for the respiratory metabolism of most organisms, including many kinds of microorganisms. The respiratory system is based on the use of oxygen as the electron acceptor (oxidant) for oxidising organic matter constitutive of the body or used as substrate (aerobic respiration).

Oxygen is not the only electron acceptor. Nitrate, sulphate, iron (III), manganese or carbon dioxide may be used by some microorganisms in absence of oxygen (anaerobic respiration). When oxygen has been depleted in organic matter-rich environments (eutrophic), microorganisms shift to nitrate and sulphate respiration producing ammonium and hydrogen sulphide. Methane and other reduced substances may also be produced in such a phase.

Policy relevance

The GESAMP definition of marine pollution includes the discharge of substances, heat and other forms of energy into the marine environment detrimental to the legitimate uses of the sea.

Policy context

No policy or management principles have been included in the Barcelona Convention and Protocols regarding the discharge of oxygen into the coastal areas.

Environmental context

Oxygen is one of the gaseous components of the atmosphere necessary for the respiratory metabolism of most organisms, including many kinds of microorganisms. The respiratory system is based on the use of oxygen as the electron acceptor (oxidant) for oxidising organic matter constitutive of the cells and/or tissues or used as substrate (aerobic respiration).

Oxygen is not the only electron acceptor in the environment. Nitrite, nitrate, sulphate, iron (III), manganese or carbon dioxide may be used by some microorganisms in absence of oxygen (anaerobic respiration). When oxygen has been depleted in organic matter-rich environments (eutrophic), microorganisms shift to nitrate and sulphate respiration producing ammonium and hydrogen sulphide respectively. Methane and other reduced substances may also be produced in such a phase.

Whether or not atmospheric oxygen was generated in the marine environment, the fact is that oxygen in the atmosphere is in global equilibrium with oxygen dissolved in surface sea water. Concentration of oxygen in surface sea water is controlled by the difference between atmospheric partial pressure of oxygen, sea surface temperature and processes occurring in the upper layers of the ocean. These processes are photosynthetic oxygen production and respiratory oxygen consumption.

Below the surface, depending on the hydrodynamic and other conditions, there may be a net production or consumption of oxygen thus promoting the existence of vertical oxygen gradients that drive oxygen transport across the sea surface. Cold, recently up welled oxygen-deficient water will facilitate the dissolution of atmospheric oxygen. While warming, highly productive spring waters tend to exhale oxy gen to the atmosphere. These processes are facilitated by well-stirred, choppy surface waters and rendered difficult by stagnant, slick covered surface waters.

Dissolved oxygen concentration at depth is the resulting balance between the equilibrium values when the corresponding water parcel was last in contact with the atmosphere (saturation) and the consumption due to metabolic respiration, mostly of micro-organisms. The longer the time spent since the last contact with the atmosphere (age of water mass), the farther from equilibrium will be the dissolved oxygen concentration. In the deep sea, depending on the origin and history of the water masses, dissolved oxygen is plentiful, though a minimum oxygen layer is normally found in all oceans with lower concentrations in this layer in the Pacific and Indian than in the Atlantic Ocean.

Assessment of the indicator

Dissolved oxygen in the open Mediterranean Sea is close to saturation both because water parcels are relatively “young”, having been in contact with the atmosphere in a not too distant past, and because the metabolic respiration is weak due to the relatively low productivity of the upper layers. The minimum oxygen layer is often associated to the Levantine Intermediate Water at depths shallower than would be in the oceans. Therefore, the downwards flux of oxygen from the maximum oxygen layer under the thermocline is significant and keeping the values at the minimum layer not too far from saturation.

The same is true in coastal waters. Depending on the local rate of photosynthetic production, the waters will be more or less supersaturated. Shallow waters will, in addition, be subject to wind stirring and therefore equilibrium with atmosphere easily attained. However, in shallow areas, particularly in coastal lagoons, day and night conditions may greatly differ since photosynthetic production will dominate during the day and metabolic consumption during the night. In highly productive coastal waters this may lead to temporary hypoxia or anoxia.

This is especially true in coastal areas receiving high nutrient loads since the productivity in the surface layer is above the oxidative capacity of the dissolved oxygen in the lower layer (the upper layer looses part of the oxygen produced to the atmosphere). The disequilibrium between the upper and lower layers is even stronger when the nutrient load is connected to the presence of fresh water at the surface forming a halocline (parts of the Adriatic Sea).

Sub-Indicator:

Dissolved oxygen in open sea waters of the Mediterranean Sea

Key message

There is no concern regarding dissolved oxygen in open seawaters.

Assessment of the sub-indicator

Dissolved oxygen in the open Mediterranean Sea is close to saturation both because water parcels are relatively “young”, having been in contact with the atmosphere in a not too distant past, and because the metabolic respiration is weak due to the relatively low productivity of the upper layers. The minimum oxygen layer is often associated to the Levantine

Intermediate Water at depths shallower than would be in the oceans. Therefore, the downwards flux of oxygen from the maximum oxygen layer under the thermocline is significant and keeping the values at the minimum layer not too far from saturation.

Sub-Indicator:

Dissolved oxygen in coastal waters of the Mediterranean region

Key message

There is no concern regarding dissolved oxygen in coastal waters in general. In areas receiving large nutrient loads from rivers hypoxia or even anoxia events may take place.

Assessment of the sub-indicator

In coastal waters, depending on the local rate of photosynthetic production, the waters will be more or less supersaturated. Shallow waters will, in addition, be subject to wind stirring and therefore equilibrium with atmosphere easily attained. However, in shallow areas, particularly in highly productive coastal waters this may lead to temporary hypoxia or anoxia. This is especially true in coastal areas receiving high nutrient loads since the productivity in the surface layer is above the oxidative capacity of the dissolved oxygen in the lower layer (the upper layer looses part of the oxygen produced to the atmosphere). The disequilibrium between the upper and lower layers is even stronger when the nutrient load is connected to the presence of fresh water at the surface forming a halocline (parts of the Adriatic Sea).

Sub-Indicator:

Dissolved oxygen in coastal lagoons and estuaries of Mediterranean states

Key message

In shallow coastal areas, particularly in coastal lagoons, day and night conditions may greatly differ since photosynthetic production will dominate during the day and metabolic consumption during the night and may lead to temporary hypoxia or anoxia.

Assessment of the sub-indicator

Depending on the local rate of photosynthetic production, the waters will be more or less supersaturated. Shallow waters will, in addition, be subject to wind stirring and therefore equilibrium with atmosphere easily attained. However, in shallow areas, particularly in coastal lagoons, day and night conditions may greatly differ since photosynthetic production will dominate during the day and metabolic consumption during the night. In highly productive coastal waters this may lead to temporary hypoxia or anoxia.

This is especially true in coastal areas receiving high nutrient loads since the productivity in the surface layer is above the oxidative capacity of the dissolved oxygen in the lower layer (the upper layer looses part of the oxygen produced to the atmosphere).

UNEP

Marine Pollution Indicator fact sheet

MAP

MED POL

Nitrate and other forms of Inorganic Nitrogen in transitional, coastal and marine waters of the Mediterranean Sea

Key message

Nitrate, ***Nitrite*** and ***Ammonium*** constituting, with the organic fraction, the ***Total Nitrogen (TN)*** are chemical entities naturally existing in the environment, of great importance for the maintenance of the ecosystem since they are required, as sources of Nitrogen, by marine plants and other micro-organisms for the production of particulate organic matter (POM) and, eventually, dissolved organic matter (DOM).

The Mediterranean Sea has lower nutrient concentrations in the intermediate and deep waters than those in the world's oceans and a higher than normal N/P ratio which makes this sea possibly P-limited instead of N-limited as is considered normal for most oceanic waters.

Of the 545 cities with population above 10,000 inhabitants, with about 60 Million inhabitants "found", 70 % have plants treating 9 Million cubic meters of sewage per day. A similar amount of sewage (8 Million cubic meters per day) remains untreated.

An extra effort remains to be done to achieve the treatment of the remaining sewage unless it can be proved that submarine outfalls achieve a proper disposal of the sewage, particularly in small cities and other urban agglomerations.

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large nitrogen loads is detrimental to marine ecosystems. Concentration of nitrate and ammonium are excellent indicators for coastal water quality.

Policy context

In 1985, the Contracting Parties to the Barcelona Convention adopted ***The Genoa Declaration***. Amongst the targets approved, one of the priorities was the establishment of sewage treatment plants in all cities around the Mediterranean Sea with more than 100,000 inhabitants and appropriate outfalls and appropriate treatment plants for all cities with more than 10,000 inhabitants.

Environmental context

Nitrate is a chemical entity naturally existing in the environment. Other forms in which nitrogen may be made available in the environment are nitrite, ammonium, organic nitrogen, etc. Nitrate, however, is the most stable form in oxidised marine environments. Elemental nitrogen (gas), present everywhere in the atmosphere and dissolved in the seawater, may be converted to one of the other forms by micro-organisms in the nitrogen-fixation process and

the reverse is also true, nitrate and other forms of nitrogen may be converted into elemental nitrogen through denitrification.

Nitrate is one of the chemical forms possible for nitrogen in seawater and largely the most abundant one in the open sea. Together with other nutrients, particularly phosphorus and silicon, nitrate or other forms of nitrogen are indispensable for the production of mainly plant material through the photosynthetic process. Nitrogen may also be constitutive of organic matter either in dissolved (DON) or particulate (PON) form. However, nitrogen present in all the matrices, in oxygenated environments, end up in the final chemical state nitrate.

Seawater is a large reservoir of nutrients cycling with the global hydrological cycle (Conveyor Belt). Nutrients alternate between the dissolved inorganic and organic forms by virtue of the two major opposing biogeochemical processes: photosynthesis and metabolic oxidation. Nutrients flow to the illuminated upper layers of the sea (euphotic zone) mainly by advection and turbulent diffusion, i.e. by transport of water and of dissolved/dispersed materials from greater depths due to existing vertical gradients and kinetic/turbulent energy-driven motions.

Local productivity is thus the result of a combination of factors that include prevailing meteorological and hydrodynamic conditions, solar radiation and existence of nutrients or nutrient gradients in the water column, all parameters subject to strong seasonal and regional variability. Part of the organic material resulting from the photosynthetic activity will be metabolically oxidised regenerating inorganic nutrients that will be re-used within or near the place of “first” utilisation thus generating a small cycle, often known as the microbial loop. The remaining of the organic material produced will be metabolised at greater depths entering the global cycle of remineralisation of carbon, nitrogen, phosphorus, etc.

The Mediterranean Sea, like all other parts of the world’s ocean system, has a pool of nutrients albeit a reduced one, since the concentrations of nitrate in the deep and intermediate waters of the Mediterranean Sea are 2 to 5 times lower than those in the oceans. This is due to the particular hydrological conditions of the Mediterranean Sea at the outflow in Gibraltar where intermediate and deep water flowing out carry in solution a large amount of nutrients compensated by the combined inflows at Gibraltar and from land-based sources through the rivers, effluents and the atmosphere. The loss of nitrate to the ocean through the Straits of Gibraltar may be estimated at not less than 112 Kg of nitrogen per second (or 3.5 Million tonnes per year).

Another specific characteristic of the Mediterranean Sea is the high N/P ratio, the proportion of nitrate to orthophosphate (the so-called Redfield ratio), with values that range from 21 to 27 when expressed in molar form and even higher compared to a ratio of about 16 that is normal for all oceans. This ratio seems to be controlled, in the biogeochemical sense, by the average composition of the marine organisms and the high values point out to a limitation by orthophosphate of biological productivity. However, nitrogen-fixation has been mentioned as a natural way of increasing the biological productivity in highly oligotrophic surface waters such as those of the Mediterranean Sea. This issue is, however, to be demonstrated since so far only circumstantial evidence has been provided.

Sources of nutrients external to the marine system all have large nitrogen relative to phosphorus loads. Atmospheric nitrogen deposition to the Mediterranean Sea has been estimated at 0.3 – 4 $g.m^{-2}.yr^{-1}$ for nitrogen (MTS #133), values comparable to the nitrogen exported through Gibraltar with the Mediterranean out flowing waters. However, the diffuse atmospheric deposition does not generate any significant increase in fertility except when a heavy rainfall event may trigger the development of short-lived surface phytoplankton blooms.

Also rivers draining large basins and receiving effluents from urban and agricultural areas contribute with significant amounts of phosphorus to the seawater. A surplus of productivity may be given to transitional and coastal waters depending on nutrient availability. Concentrations of nitrate in river water may be order-of-magnitude higher than those in the surface receiving waters and, in the last years a steady increase in nitrogen concentrations have been obtained in most European rivers through the use of fertilizers in agriculture. This is a controversial issue since nitrogen may also be bound to particles in organic and inorganic forms with the possibility of returning to the dissolved state after transiting in the sediments. Therefore, the N availability cannot be determined only on the basis of the dissolved inorganic form and also the particulate and organic forms have to be taken into account.

On the other hand, direct discharge of nutrients to the marine environment, through effluents, has the effect of promoting the photosynthetic uptake and the production of organic matter in a limited area. Weather of urban, agricultural or industrial origin effluents always contain important nutrient loads that, unless properly disposed of, may lead to eutrophication with discolouration of waters, reduced transparency, unsightliness and impairing recreation. This is most evident when the effluents discharge into coastal lagoons or embayments with restricted water exchange with the open sea.

The most serious manifestations of eutrophication are appearance of algal blooms (red tides), algal scum, enhanced benthic algal growth and, at times, a massive growth of submersed and floating macrophytes that may choke shallow channels, lagoons and estuaries impairing fishery and navigation. When aging, the organic material produced decays through complex microbial activity, consuming and, in serious cases depleting, the limited oxygen reserve of the water, causing an array of secondary problems such as mortality, formation of undesirable substances such as CH_4, H_2S, NH_3, organic acids, toxins, etc, many of which produce intense noxious odour.

Discharge of nutrients to the marine environment may also upset the balanced proportions of naturally occurring nutrients thus creating additional ecological problems like the production of extra cellular materials such as polysaccharides or muco-polysaccharides (mucillagine). The N/P ratio in atmospheric deposition is clearly unbalanced towards a greater N load than required by the P deposition favouring the high N/P ratio observed in Mediterranean Sea waters, more in the Eastern than in the Western basin. The same is true of N/P in river and in effluent waters.

Assessment of the indicator

The test for determination of nitrate in seawater (and fresh water as well) consists in a standard photometric technique based on the reduction of nitrate to nitrite with copperised cadmium and then formation of a dye with sulphanilamide and nafthyl-ethylene-diamine. The second step also reacts with nitrite. Usually, nitrite is determined separately by the same technique without the reducing step although, often, the parameter Nitrate includes Nitrite as well. The precision of this technique is very high; however, concentrations in surface waters may be near detection level.

Other nitrogen ions and fractions may be analysed, depending on whether the aliquot of water has been previously filtered and/or digested:

- Dissolved inorganic Nitrogen (DIN) as the sum of nitrate, nitrite and ammonium
- Dissolved organic Nitrogen (DON)
- Total Dissolved Nitrogen (D N)
- Particulate organic Nitrogen (PON)

- Total Nitrogen (TN)

From an environmental point of view, the state in which the nutrient is present in the effluent is quite irrelevant, since the transit from one form to another is readily carried out by one or other kind of the omnipresent micro-organisms. Perhaps, it might be noted that the transformation of organic onto inorganic nutrients (also ammonium onto nitrate) are oxygen-consuming processes dealt with in the BOD test and the particulate form will also contribute to turbidity dealt with elsewhere.

From a purely technical point of view, it should be stressed that all analytical procedures and techniques should be subject to inter-calibration and quality control protocols.

Subindicator

Nitrite in transitional, coastal and marine waters

Key message

Nitrite is a chemical entity naturally existing in the environment contributing, as a source of Nitrogen, to the maintenance of the ecosystem. Although free nitrite is toxic to all kinds of higher organisms, marine plants can take it up and some micro-organisms can transform it onto nitrate, ammonium or even nitrogen gas. Nitrite will, eventually, end up contributing to the production of particulate organic matter (POM) and/or dissolved organic matter (DOM).

In the Mediterranean Sea, like in many other oligotrophic areas, there is always (or nearly) some nitrite at the low end of the euphotic zone. The discussion still goes on over whether the nitrite maximum is the result of nitrification carried out by bacteria on organic matter produced above or is produced and excreted by phytoplankton organisms when nitrate is taken up but not completely assimilated due to the dim light available. The fact is that the nitrite maximum is close to the deep chlorophyll maximum and not far (a few meters) from the dissolved oxygen maximum and, as such, does not constitute any symptom of pollution.

However, the existence of nitrite in other environments (surface, halocline, bottom waters, sediment, etc) may be an indication of reducing conditions caused by nitrifying and other heterotrophic bacteria and is an early symptom of eutrophication.

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large nitrite loads is detrimental to marine ecosystems. Concentration of nitrite is an excellent indicator for coastal water quality.

Policy context

In 1985, the Contracting Parties to the Barcelona Convention adopted **The Genoa Declaration**. Amongst the targets approved, one of the priorities was the establishment of sewage treatment plants in all cities around the Mediterranean Sea with more than 100,000 inhabitants and appropriate outfalls and appropriate treatment plants for all cities with more than 10,000 inhabitants.

Environmental context

Nitrite is a chemical entity naturally existing in the environment contributing, as a source of Nitrogen, to the maintenance of the ecosystem. Although free nitrite is toxic to all kinds of

higher organisms, marine plants can take it up and some micro-organisms can transform it onto nitrate, ammonium or even nitrogen gas. Nitrite will, eventually, end up contributing to the production of particulate organic matter (POM) and/or dissolved organic matter (DOM).

In the Mediterranean Sea, like in many other oligotrophic areas, there is always (or nearly) some nitrite at the low end of the euphotic zone. The discussion still goes on over whether the nitrite maximum is the result of nitrification carried out by bacteria on organic matter produced above or is produced and excreted by phytoplankton organisms when nitrate is taken up but not completely assimilated due to the dim light available. The fact is that the nitrite maximum is close to the deep chlorophyll maximum and not far (a few meters) from the dissolved oxygen maximum.

The existence of nitrite in other environments is an indication of reducing conditions caused by the nitrifying and other heterotrophic bacteria and may be an early symptom of eutrophication.

Nitrite is one of the chemical forms of nitrogen relatively stable in seawater under slightly oxidising and/or reducing conditions. Together with other forms of nitrogen (nitrate, ammonium) and other nutrients, particularly phosphorus and silicon, nitrite may contribute to the production of mainly plant material through the photosynthetic process.

If not taken up by plants, nitrite will be readily transformed by bacteria into either nitrate (nitrification) or ammonium (nitrite reduction). Eventually, nitrite may be transformed into molecular nitrogen (gas) by denitrifying bacteria. This process takes place in eutrophic environments when oxidising/reducing conditions alternate.

Nitrite is not very relevant within the global hydrological cycle (Conveyor Belt). Nitrite concentration in the bulk of oceanic waters is near undetectable levels. However, in oceanic deep and intermediate areas experiencing oxygen depletion (tropical Eastern Pacific, Sea of Arabia, etc), a sizeable part of the nitrate may be reduced to nitrite and eventually converted to N_2 through denitrification.

Local productivity is not significantly affected by nitrite in the open sea. However, nitrite is part of the inorganic nitrogen pool carried out by rivers and effluents onto the coastal areas. It may also be a significant part of the total nitrogen in coastal lagoons. Nitrite, often considered as part of the nitrate compartment since both are quantified in the nitrate analytical procedure, may also be present in the atmospheric deposition as the result of transformations of nitrogen oxides in the atmosphere.

Assessment of the subindicator

The test for determination of nitrite in seawater (and fresh water as well) consists in a standard photometric technique based on the formation of a dye with sulphanilamide and nafthyl-ethylene-diamine. If the procedure starts with the reduction of nitrate, both ions are quantified together. Sometimes, nitrite is determined separately from nitrate although, often, the parameter Nitrate includes Nitrite as well. The precision of this technique is very high; however, concentrations in deeper waters may be near detection level.

Other nitrogen ions and fractions may be analysed, depending on whether the aliquot of water has been previously filtered and/or digested:

- Dissolved inorganic Nitrogen (DIN) as the sum of nitrate, nitrite and ammonium
- Dissolved organic Nitrogen (DON)
- Total Dissolved Nitrogen (DN)
- Particulate organic Nitrogen (PON)

- Total Nitrogen (TN)

From an environmental point of view, the state in which the nutrient is present in the effluent is quite irrelevant, since the transit from one form to another is readily carried out by one or other kind of the omnipresent micro-organisms. Perhaps, it might be noted that the transformation of organic onto inorganic nutrients (also ammonium onto nitrate) are oxygen-consuming processes dealt with in the BOD test and the particulate form will also contribute to turbidity dealt with elsewhere.

Subindicator: Ammonium in transitional, coastal and marine waters

Key message

Ammonium is a chemical entity naturally existing in the environment contributing, as a source of Nitrogen, to the maintenance of the ecosystem. Ammonium is excreted by many organisms, particularly those constituting the zooplankton, and marine plants can take it up even more readily than nitrate or nitrite. Some micro-organisms can transform it onto nitrite, nitrate or even nitrogen gas. Ammonium will, eventually, end up contributing to the production of particulate organic matter (POM) and/or dissolved organic matter (DOM).

In the Mediterranean Sea, like in many other oligotrophic areas, there is always (or nearly) some ammonium. However, a clear pattern of ammonium distribution is not available except in coastal lagoons or when river or effluent waters are discharged into coastal seas constituting a symptom of pollution. The existence of nitrite in other environments (bottom waters, sediment, etc) may be an indication of reducing conditions caused by ammonification carried out by some heterotrophic bacteria and is an early symptom of eutrophication.

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large ammonium loads is detrimental to marine ecosystems. Concentration of ammonium is an excellent indicator for coastal water quality.

Policy context

In 1985, the Contracting Parties to the Barcelona Convention adopted **The Genoa Declaration**. Amongst the targets approved, one of the priorities was the establishment of sewage treatment plants in all cities around the Mediterranean Sea with more than 100,000 inhabitants and appropriate outfalls and appropriate treatment plants for all cities with more than 10,000 inhabitants.

Environmental context

Ammonium is a chemical entity naturally existing in the environment contributing, as a source of Nitrogen, to the maintenance of the ecosystem. Ammonium is excreted by many organisms, particularly those constituting the zooplankton, and marine plants can take it up even more readily than nitrate or nitrite. Some micro-organisms can transform it onto nitrite, nitrate or even nitrogen gas. Ammonium will, eventually, end up contributing to the production of particulate organic matter (POM) and/or dissolved organic matter (DOM).

In the Mediterranean Sea, like in many other oligotrophic areas, there is always (or nearly) some ammonium. However, a clear pattern of ammonium distribution is not available except in coastal lagoons or when river or effluent waters are discharged into coastal seas constituting a symptom of pollution. The existence of nitrite in other environments (bottom

waters, sediment, etc) may be an indication of reducing conditions caused by ammonification carried out by some heterotrophic bacteria and is an early symptom of eutrophication.

Ammonium is relatively stable in seawater under slightly oxidising and/or reducing conditions. Together with other forms of nitrogen (nitrate, nitrite) and other nutrients, particularly phosphorus and silicon, ammonium may contribute to the production of mainly plant material through the photosynthetic process.

If not taken up by plants, ammonium will be readily transformed by bacteria into nitrate (nitrification). Ammonium may be transformed into nitrite and molecular nitrogen (gas) by nitrifying and denitrifying bacteria. These processes take place in eutrophic environments when oxidising/reducing conditions alternate.

Ammonium is not very relevant within the global hydrological cycle (Conveyor Belt). Ammonium concentration in the bulk of oceanic waters is near undetectable levels. However, in oceanic areas with high density of fish and other pelagic organisms, a sizeable part of the nitrogen may be in the form of ammonium.

Local productivity is not significantly affected by ammonium in the open sea. However, it is part of the inorganic nitrogen pool carried out by rivers and effluents onto the coastal areas. It may also be a significant part of the total nitrogen in coastal lagoons.

Ammonium is also present in the atmospheric deposition as the result of emissions from agricultural and other activities.

Assessment of the indicator

The test for determination of ammonium in seawater (and fresh water as well) consists in a standard photometric technique based on the formation of an indophenol dye. The precision of this technique is relatively high; however, concentrations in open sea waters may be near detection level. The technique is subject to laboratory contamination if proper working conditions are not kept.

Other nitrogen ions and fractions may be analysed, depending on whether the aliquot of water has been previously filtered and/or digested:

- Dissolved inorganic Nitrogen (DIN) as the sum of nitrate, nitrite and ammonium
- Dissolved organic Nitrogen (DON)
- Total Dissolved Nitrogen (DN)
- Particulate organic Nitrogen (PON)
- Total Nitrogen (TN)

From an environmental point of view, the state in which the nutrient is present in the effluent is quite irrelevant, since the transit from one form to another is readily carried out by one or other kind of the omnipresent micro-organisms. Perhaps, it might be noted that the transformation of organic onto inorganic nutrients (also ammonium onto nitrate) are oxygen-consuming processes dealt with in the BOD test and the particulate form will also contribute to turbidity dealt with elsewhere.

Subindicator: Total nitrogen in transitional, coastal and marine waters

Key message

Total nitrogen is not a chemical entity but the methodological addition of the nitrogen equivalent of a number of nitrogen-containing substances. Total nitrogen would comprise the ions nitrate, nitrite and ammonium in the dissolved phase (DIN) and the organic forms of nitrogen (mostly proteins and other N-containing substances) existing in biota and other particulate materials (PON) and in dissolved organic matter (DON).

In open sea waters, Total Nitrogen would be in practice equated to DIN, the major fraction of nitrogen. PON may have some significance in the euphotic zone as part of the cycle DIN ? PON. However, in the bulk of the ocean, PON represents a small fraction of Total Nitrogen. The presence of high PON values would be a symptom of land-based pollution except in naturally occurring mesotrophic (or eutrophic) lagoons. On the other hand, a fraction of PON may end up as DON. However, DON is, in general, highly reactive and would not accumulate in the open sea waters.

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large nitrogen loads is detrimental to marine ecosystems. Concentration of total nitrogen is an excellent indicator for coastal water quality.

Policy context

In 1985, the Contracting Parties to the Barcelona Convention adopted **The Genoa Declaration**. Amongst the targets approved, one of the priorities was the establishment of sewage treatment plants in all cities around the Mediterranean Sea with more than 100,000 inhabitants and appropriate outfalls and appropriate treatment plants for all cities with more than 10,000 inhabitants.

Environmental context

Total nitrogen is not a chemical entity but the methodological addition of the nitrogen equivalent of a number of nitrogen-containing substances. Total nitrogen would comprise the ions nitrate, nitrite and ammonium in the dissolved phase (DIN) and the organic forms of nitrogen (mostly proteins and other N-containing substances) existing in biota and other particulate materials (PON) and in dissolved organic matter (DON).

In open seawaters, Total Nitrogen would be in practice equated to DIN, the major fraction of nitrogen, or to Nitrate. PON may have some significance in the euphotic zone as part of the cycle DIN ? PON. However, in the bulk of the ocean, PON represents a small fraction of Total Nitrogen. The presence of high PON values would be a symptom of land-based pollution except in naturally occurring mesotrophic (or eutrophic) lagoons. On the other hand, a fraction of PON may end up as DON. However, DON is, in general, highly reactive and would not accumulate in the open sea waters

Rivers draining large basins and receiving effluents from urban and agricultural areas contribute with significant amounts of Total Nitrogen to the seawater. A surplus of productivity may be given to transitional and coastal waters depending on nutrient availability. Concentrations of TN in river water may be order-of-magnitude higher than those in the surface receiving waters and, in the last years a steady increase in nitrogen concentrations have been obtained in most European rivers through the use of fertilizers in agriculture. This is a controversial issue since nitrogen may also be bound to particles in organic and inorganic forms with the possibility of returning to the dissolved state after transiting in the sediments. Therefore, the N availability cannot be determined only on the

basis of the dissolved inorganic form and also the particulate and organic forms have to be taken into account.

On the other hand, direct discharge of nutrients to the marine environment, through effluents, has the effect of promoting the photosynthetic uptake and the production of organic matter in a limited area. Weather of urban, agricultural or industrial origin effluents always contain important nutrient loads that, unless properly disposed of, may lead to eutrophication with discolouration of waters, reduced transparency, unsightliness and impairing recreation. This is most evident when the effluents discharge into coastal lagoons or embayments with restricted water exchange with the open sea.

The most serious manifestations of eutrophication are appearance of algal blooms (red tides), algal scum, enhanced benthic algal growth and, at times, a massive growth of submersed and floating macrophytes that may choke shallow channels, lagoons and estuaries impairing fishery and navigation. When aging, the organic material produced decays through complex microbial activity, consuming and, in serious cases depleting, the limited oxygen reserve of the water causing an array of secondary problems such as mortality, formation of undesirable substances such as CH_4, H_2S, NH_3, organic acids, toxins, etc, many of which produce intense noxious odour.

Discharge of nutrients to the marine environment may also upset the balanced proportions of naturally occurring nutrients thus creating additional ecological problems like the production of extra cellular materials such as polysaccharides or muco-polysaccharides (mucillagine). The N/P ratio in river and effluent waters is clearly unbalanced towards a greater nitrogen load than that for phosphorus, more in the Eastern than in the Western basin. The same is true of N/P in atmospheric deposition.

Assessment of the indicator

The test for determination of total nitrogen in seawater (and fresh water as well) consists in the digestion of the unfiltered sample followed by Kjeldahl (ammonium) or, after oxidation, by the standard photometric technique used for analysis of nitrate. Alternatively, filtering through glass fiber filters allows the concentration of PN which will be submitted to digestion, while the filtrate would be oxidised and submitted to the nitrate analysis. The precision of these techniques is high; however, concentrations in surface waters may be near detection level.

Other nitrogen ions and fractions may be analysed, depending on whether the aliquot of water has been previously filtered and/or digested:

- Dissolved inorganic Nitrogen (DIN) as the sum of nitrate, nitrite and ammonium
- Dissolved organic Nitrogen (DON)
- Total Dissolved Nitrogen (DN)
- Particulate organic Nitrogen (PON)
- Total Nitrogen (TN)

From an environmental point of view, the state in which the nutrient is present in the effluent is quite irrelevant, since the transit from one form to another is readily carried out by one or other kind of the omnipresent micro-organisms. Perhaps, it might be noted that the transformation of organic onto inorganic nutrients (also ammonium onto nitrate) are oxygen-consuming processes dealt with in the BOD test and the particulate form will also contribute to turbidity dealt with elsewhere.

Data source

- Atmospheric deposition data from UNEP (MTS #133)
- Municipal wastewater treatment from UNEP (MTS #128)

Description of data

- Wastewater treatment, mostly secondary, exists in 79 % of the 101 cities above 100,000 inhabitants.
- Of the nearly 60 million inhabitants "found", 70 % are served by sewerage network and treatment plant; 30 % only by a sewerage network.
- It is estimated that over 8 Million cubic meters of wastewater are treated per day while over 9 Million remain untreated.

Geographical coverage

- With regard to the atmospheric deposition, four locations, two coastal locations in Croatia and another two in Israel with references to other locations in the Mediterranean Sea were included in the research project reported.
- The entire Mediterranean coastal area was covered by the Municipal wastewater survey.

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Further work required

Continuous work in the monitoring of the various fractions of Nitrogen in the marine environment has a dual purpose of better knowing the cycle of matter in the marine ecosystem and also of monitoring pollution from land-based sources. MAP should endeavour to integrate the analysis of all the N fractions in its monitoring programmes.

Marine Pollution Indicator fact sheet

MED POL

Nutrients in effluents discharging to the Mediterranean Sea

Key message

Nutrients, being chemical entities naturally existing in the environment, can only be considered as pollutants when their loads are in excess of the receiving capacity of the systems. Since they are taken up by marine plants and other micro-organisms, they may contribute to the production of particulate organic matter (POM) and, eventually, dissolved organic matter (DOM). Therefore, nutrients may contribute to the production of BOD. If discharged through proper diffusing devices in areas without important circulation restrictions, nutrients should not be of great concern.

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large nutrient loads is detrimental to marine ecosystems.

Policy context

In 1985, the Contracting Parties to the Barcelona Convention adopted **The Genoa Declaration**. Amongst the targets approved, one of the priorities was the establishment of sewage treatment plants in all cities around the Mediterranean Sea with more than 100,000 inhabitants and appropriate outfalls and appropriate treatment plants for all cities with more than 10,000 inhabitants.

Environmental context

Nutrients consist of simple inorganic molecules containing nitrogen, phosphorus, silicon and other minor or trace elements such as iron, molybdenum, etc, indispensable for the production of mainly plant material through the photosynthetic process. Other, mostly carbon-containing, substances utilised by bacteria and other micro-organisms for their heterotrophic development, are also considered nutrients. In the present context, only the inorganic nutrients are considered although they may be constitutive of organic matter either in dissolved (DON, DOP, etc) or particulate (PON, POP) form.

The seawater is a large reservoir of nutrients cycling with the global hydrological cycle (Conveyor Belt). Nutrients alternate between the dissolved inorganic and organic forms by virtue of the two major opposing biogeochemical processes: photosynthesis and metabolic oxidation. Nutrients flow to the illuminated upper layers of the sea (euphotic zone) mainly by advection and turbulent diffusion, i.e. by transport of water and of dissolved/dispersed materials from greater depths by virtue of the existing vertical gradients and the kinetic/turbulent energy-driven motions.

Local productivity is thus the result of a combination of factors that include prevailing meteorological and hydrodynamic conditions, solar radiation and existence of nutrients or

nutrient gradients in the water column, all parameters subject to strong seasonal and regional variability. Part of the organic material resulting from the photosynthetic activity will be metabolically oxidised within a relatively small distance of the place in which it was produced, thus generating a small cycle, often known as the microbial loop, regenerating nutrients that will be re-used within or near the place of “first” utilisation. The remaining of the organic material produced will be metabolised at greater depths entering the global cycle of remineralisation of carbon, nitrogen, phosphorus, etc.

When sources of nutrients external to the marine system add a surplus of productivity, various phenomena may occur depending, among other, on the relative proportion of external to internal nutrient loads and also on the proportion of organic material being regenerated on the spot or exported to greater depths and/or distances. Though the production of supplementary organic material may seem, per se, a favourable process for typically oligotrophic environments such as the Mediterranean Sea in general, the overall assessment will have to take into account the resulting effects on the ecosystem receiving the discharges. It also depends on whether the source is a point or diffuse one.

Atmospheric nutrient deposition to the Mediterranean Sea has been estimated at 0.3–4 $g.m^{-2}.yr^{-1}$ for nitrogen and 0.01– 0.02 $g.m^{-2}.yr^{-1}$ for phosphorus (MTS #133), values comparable to the N and P exported through Gibraltar with the Mediterranean out flowing waters. However, the diffuse atmospheric deposition does not generate any significant increase in fertility except when a heavy rainfall event may trigger the development of short-lived surface phytoplankton blooms.

On the other hand, effluent discharge of nutrients to the marine environment, either directly or through rivers, has the effect of promoting the photosynthetic uptake and the production of organic matter in a limited area thus having a significant effect on the receiving waters.

One of the most striking rules applying to the oceanic nutrient concentrations in their cycling is the Redfield principle by which N-Nitrate and P-Phosphate concentrations maintain a precise ratio of about 16 when expressed in molar form. This ratio seems to be controlled, in the biogeochemical sense, by the average composition of the marine organisms though this is subject to space and time variations at scales below those of the oceanic cycles. By extension, a similar ratio is also applied to other elements such as Carbon and Oxygen.

Weather of urban, agricultural or industrial origin effluents always contain important nutrient loads that, unless properly disposed of, may lead to eutrophication with discolouration of waters, reduced transparency, unsightliness and impairing recreation. The most serious manifestations of this phenomenon are appearance of algal blooms (red tides), algal scum, enhanced benthic algal growth and, at times, a massive growth of submersed and floating macrophytes that may choke shallow channels, lagoons and estuaries impairing fishery and navigation.

When aging, the organic material produced as a consequence of nutrient discharges decays through complex microbial activity, consuming and, in serious cases depleting, the limited oxygen reserve of the water causing an array of secondary problems such as mortality, formation of undesirable substances such as CH4, H2S, NH3, organic acids, toxins, etc, many of which produce intense noxious odour.

Discharge of nutrients to the marine environment may also upset the balanced proportions of naturally occurring nutrients thus creating additional ecological problems like the production of extra cellular materials such as polysaccharides or muco-polysaccharides (mucilage). The N/P ratio in atmospheric deposition is clearly unbalanced towards a greater N load than required by the P deposition thus favouring the high N/P ratio observed in Mediterranean

Sea waters, more in the Eastern than in the Western basin. The same is true of N/P in river and in effluent waters.
Assessment of the indicator

The test for determination of nutrients consists in analysing, usually by standard specific photometric techniques, nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$), ammonium (NH_4^+), orthophosphate (HPO_4^{3+}), orthosilicic acid ($Si(OH)_4$). Trace elements also considered sometimes as nutrients (Fe, Mo, etc) are not included in this list since there is no evidence that they may contribute in any degree to increase productivity causing negative effects on the ecosystem.

Various fractions may be analysed, depending on whether the aliquot of water has been previously filtered and/or digested:

- Dissolved inorganic nutrients (DIN, DIP):
- Dissolved organic nutrients (DON, DOP)
- Particulate inorganic nutrients (PIN, PIP)
- Total Dissolved nutrients (DN, DP)
- Total nutrient contents (TN, TP)

From an environmental point of view, the state in which the nutrient is present in the effluent is quite irrelevant, since the transit from one form to another is readily carried out by one or other kind of the omnipresent micro-organisms. Perhaps, it might be noted that the transformation of organic onto inorganic nutrients will be an oxygen-consuming process dealt with in the BOD test and the particulate form will also contribute to turbidity dealt with elsewhere.

From a purely technical point of view, it should be stressed that all analytical procedures and techniques should be subject to inter-calibration and quality control protocols.

Data source

- Atmospheric deposition data from UNEP (MTS #133)
- Municipal wastewater treatment from UNEP (MTS #128)

Description of data

- Wastewater treatment, mostly secondary, exists in 79 % of the 101 cities above 100,000 inhabitants.
- Of the nearly 60 million inhabitant “found”, 70 % are served by sewerage network and treatment plant; 30 % only by a sewerage network.
- It is estimated that over 8 Million cubic meters of wastewater are treated per day while over 9 Million remain untreated.

Geographical coverage

- With regard to the atmospheric deposition, four locations, two coastal locations in Croatia and another two in Israel with references to other locations in the Mediterranean Sea were included in the research project reported.
- The entire Mediterranean coastal area was covered by the Municipal wastewater survey.

Quality information

All these measurements suffer from difficulties in the frequency of sampling and correlation with water flow.

Marine Pollution Indicator fact sheet

MED POL

Orthophosphate in transitional, coastal and marine waters of the Mediterranean Sea

Key message

Orthophosphate is a chemical entity naturally existing in the environment of great importance for the maintenance of the ecosystem since it is required by marine plants and other microorganisms for the production of particulate organic matter (POM) and, eventually, dissolved organic matter (DOM). The Mediterranean Sea has very peculiar conditions when compared to the world's oceans for having one order of magnitude lower concentrations in the intermediate and deep waters. The Mediterranean Sea also has a higher than normal N/P ratio which makes this sea possibly P-limited instead of N-limited as is considered normal for most oceanic waters.

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large phosphate loads is detrimental to marine ecosystems since they promote the appearance of eutrophication phenomena.

Policy context

In 1985, the Contracting Parties to the Barcelona Convention adopted **The Genoa Declaration**. Amongst the targets approved, one of the priorities was the establishment of sewage treatment plants in all cities around the Mediterranean Sea with more than 100,000 inhabitants and appropriate outfalls and appropriate treatment plants for all cities with more than 10,000 inhabitants.

Environmental context

Orthophosphate is one of the chemical forms possible for phosphorus in seawater and largely the most abundant one in the open sea. Together with other nutrients, particularly nitrogen and silicon, orthophosphate is indispensable for the production of mainly plant material through the photosynthetic process. Bacteria and other micro-organisms also take up orthophosphate to allow them the breakdown of P-depleted organic matter in their heterotrophic development often competing for P with photosynthetic organisms. Phosphorus may also be constitutive of organic matter either in dissolved (DOP) or particulate (POP) form. However, in all the matrices, the usual chemical state is either orthophosphate or its organic esters.

Seawater is a large reservoir of nutrients cycling with the global hydrological cycle (Conveyor Belt). Nutrients alternate between the dissolved inorganic and organic forms by virtue of the two major opposing biogeochemical processes: photosynthesis and metabolic oxidation. Nutrients flow to the illuminated upper layers of the sea (euphotic zone) mainly by advection and turbulent diffusion, i.e. by transport of water and of dissolved/dispersed materials from greater depths due to existing vertical gradients and kinetic/turbulent energy-driven motions.

Local productivity is thus the result of a combination of factors that include prevailing meteorological and hydrodynamic conditions, solar radiation and existence of nutrients or nutrient gradients in the water column, all parameters subject to strong seasonal and regional variability. Part of the organic material resulting from the photosynthetic activity will be metabolically oxidised regenerating inorganic nutrients that will be re-used within or near the place of "first" utilisation thus generating a small cycle, often known as the microbial loop. The remaining of the organic material produced will be metabolised at greater depths entering the global cycle of remineralisation of carbon, nitrogen, phosphorus, etc.

The Mediterranean Sea, like all other parts of the world's ocean system, has a pool of nutrients albeit a reduced one, since the concentrations of orthophosphate in the deep and intermediate waters of the Mediterranean Sea are 5 to 10 times lower than those in the oceans. This is due to the particular hydrological conditions of the Mediterranean Sea at the outflow in Gibraltar where intermediate and deep water flowing out carry in solution a large amount of nutrients compensated by the combined inflows at Gibraltar and from land-based sources through the rivers, effluents and the atmosphere. The loss of orthophosphate to the ocean through the Straits of Gibraltar may be estimated at not less than 14 Kg of phosphorus per second (or 450,000 tm per year).

Another specific characteristic of the Mediterranean Sea is the high N/P ratio, the proportion of nitrate to orthophosphate (the so-called Redfield ratio), with values that range from 21 to 27 when expressed in molar form and even higher compared to a ratio of about 16 considered normal for all the oceans. This ratio seems to be controlled, in the biogeochemical sense, by the average composition of the marine organisms and these high values point out to a limitation by orthophosphate of biological productivity. This issue is, however, to be demonstrated since so far only circumstantial evidence has been provided except in some coastal and estuarine areas where the ratio is even larger.

Sources of nutrients external to the marine system all have large nitrogen relative to phosphorus loads. Atmospheric phosphorus deposition to the Mediterranean Sea has been estimated at 0.01 – 0.02 $g.m^{-2}.yr^{-1}$ for phosphorus (MTS #133), values comparable to the P exported through Gibraltar with the Mediterranean out flowing waters. However, the diffuse atmospheric deposition does not generate any significant increase in fertility except when a heavy rainfall event may trigger the development of short-lived surface phytoplankton blooms.

Also rivers draining large basins and receiving effluents from urban and agricultural areas contribute with significant amounts of phosphorus to the seawater. A surplus of productivity may be given to transitional and coastal waters depending on the most limiting factor, orthophosphate. Concentrations of orthophosphate in river water may be order-of-magnitude higher than those in the surface receiving waters. However, in the last years a steady decrease in phosphorus concentrations has been obtained in most European rivers through the implementation of water quality regulations. This is a controversial issue since phosphorus may also be bound to particles in organic and inorganic forms with the possibility of returning to the dissolved state after transiting in the sediments. Therefore, the P availability cannot be determined only on the basis of the dissolved inorganic form and also the particulate and organic forms have to be taken into account.

On the other hand, direct discharge of nutrients to the marine environment, through effluents, has the effect of promoting the photosynthetic uptake and the production of organic matter in a limited area. Weather of urban, agricultural or industrial origin effluents always contain important nutrient loads that, unless properly disposed of, may lead to eutrophication with discolouration of waters, reduced transparency, unsightliness and impairing recreation. This

is most evident when the effluents discharge into coastal lagoons or embayments with restricted water exchange with the open sea.

The most serious manifestations of eutrophication are appearance of algal blooms (red tides), algal scum, enhanced benthic algal growth and, at times, a massive growth of submersed and floating macrophytes that may choke shallow channels, lagoons and estuaries impairing fishery and navigation. When aging, the organic material produced decays through complex microbial activity, consuming and, in serious cases depleting, the limited oxygen reserve of the water causing an array of secondary problems such as mortality, formation of undesirable substances such as CH4, H2S, NH3, organic acids, toxins, etc, many of which produce intense noxious odour.

Discharge of nutrients to the marine environment may also upset the balanced proportions of naturally occurring nutrients thus creating additional ecological problems like the production of extra cellular materials such as polysaccharides or muco-polysaccharides (mucillagine). The N/P ratio in atmospheric deposition is clearly unbalanced towards a greater N load than required by the P deposition favouring the high N/P ratio observed in Mediterranean Sea waters, more in the Eastern than in the Western basin. The same is true of N/P in river and in effluent waters.

Assessment of the indicator

The test for determination of orthophosphate in seawater (and fresh water as well) consists in a standard specific photometric technique based on the reduction of molybdate to molybdenum blue. The precision of this technique is very high, however, concentrations in surface waters are near its detection level.

Various fractions may be analysed, depending on whether the aliquot of water has been previously filtered and/or digested:

- Dissolved inorganic Phosphorus (DIP)
- Dissolved organic Phosphorus (DOP)
- Particulate inorganic Phosphorus (PIP)
- Total Dissolved Phosphorus (DP)
- Total Phosphorus (TP)

From an environmental point of view, the state in which the nutrient is present in the effluent is quite irrelevant, since the transit from one form to another is readily carried out by one or other kind of the omnipresent micro-organisms. Perhaps, it might be noted that the transformation of organic onto inorganic nutrients will be an oxygen-consuming process dealt with in the BOD test and the particulate form will also contribute to turbidity dealt with elsewhere.

From a purely technical point of view, it should be stressed that all analytical procedures and techniques should be subject to inter-calibration and quality control protocols.

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Further work required

More work should be undertaken in the framework of the MAP monitoring activities with regard to the discharges of nutrients (nitrogen and phosphorus) through rivers and direct outfalls particularly in enclosed bays and coastal lagoons since eutrophication phenomena may cause serious impacts to the coastal and marine ecosystem.

Marine Pollution Indicator fact sheet

MED POL

Orthosilicic acid in transitional, coastal and marine waters of the Mediterranean Sea

Key message

Orthosilicic acid is a chemical entity naturally existing in the environment of great importancefor the maintenance of the ecosystem since it is required by a number of marine organisms (diatoms, silicoflagellates, radiolarians, etc) for the production of shells.

The Mediterranean Sea has much lower concentrations in the intermediate and deep waters than those in the world's oceans. Yet, the abundance of diatoms in ordinary Mediterranean phytoplankton is large (around 50 %) all through the year, at least in the Western Mediterranean. Surface waters of the Mediterranean Sea tend to be depleted of orthosilicic acid but often not at as low levels as happens with other nutrients.

River and effluent waters contain concentrations of orthosilicic acid relatively low as compared to nitrate, particularly in the Mediterranean region where much of the soil and rocks are carbonated. This can explain the observed depletion of orthosilicic acid in surface waters at the neighbourhood of coastal runoff.

Policy relevance

The objective of indicators is to co nvey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. No detrimental effect of large silicate loads has been reported.

Policy context

No specific mention to silicate is made by the Barcelona Convention and its Protocols.

Environmental context

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Together with other nutrients, particularly phosphorus and nitrate, orthosilicic acid is indispensable for an equilibrated production of phytoplankton. It is often stated that planktonic systems based on diatoms are healthier than those based on dinoflagellates. Although little evidence exists, such a hypothesis would prone the use of soluble silicate detergents as opposite to phosphorus-based ones.

Seawater is a large reservoir of nutrients cycling with the global hydrological cycle (Conveyor Belt). Nutrients alternate between the dissolved inorganic and organic forms by virtue of the two major opposing biogeochemical processes: photosynthesis and metabolic oxidation. Nutrients flow to the illuminated upper layers of the sea (euphotic zone) mainly by advection and turbulent diffusion, i.e. by transport of water and of dissolved/dispersed materials from greater depths due to existing vertical gradients and kinetic/turbulent energy-driven motions.

Local productivity is thus the result of a combination of factors that include prevailing meteorological and hydrodynamic conditions, solar radiation and existence of nutrients or nutrient gradients in the water column, all parameters subject to strong seasonal and regional variability. Part of the organic material resulting from the photosynthetic activity will be metabolically oxidised regenerating inorganic nutrients that will be re-used within or near the place of “first” utilisation thus generating a small cycle, often known as the microbial loop. The remaining of the organic material produced will be metabolised at greater depths entering the global cycle of remineralisation of carbon, nitrogen, silicon, phosphorus, etc.

The Mediterranean Sea, like all other parts of the world’s ocean system, has a pool of nutrients albeit a reduced one, since the concentrations of orthosilicic acid in the deep and intermediate waters of the Mediterranean Sea are up to 15 times lower than those in the deep ocean. This is due to the particular hydrological conditions of the Mediterranean Sea at the outflow in Gibraltar where intermediate and deep water flowing out carry in solution a large amount of nutrients that have to be compensated by the combined inflows at Gibraltar and from land-based sources through the rivers, effluents and the atmosphere. The loss of orthosilicic acid to the ocean through the Straits of Gibraltar may be estimated at not less than 300 Kg of Silicon per second (or 10 Million tonnes per year).

Rivers draining large basins with forest or agricultural areas contribute with significant amounts of orthosilicic acid to the seawater. However, the carbonated nature of Mediterranean soils and rocks make orthosilicic acid less abundant in fresh waters than it would be in other regions of the world. Direct discharge of nutrients other than orthosilicic acid to the marine environment, through effluents, has the effect of promoting the growth of non-diatom phytoplankton and this may have some effect on the health of the coastal ecosystem as well as on the fate of carbon, oxygen and other elements.

Discharge of other nutrients to the marine environment may also upset the balanced proportions of naturally occurring nutrients thus creating additional ecological problems like the production of extra cellular materials such as polysaccharides or muco-polysaccharides (mucillagine). Like the N/P ratio in atmospheric deposition is clearly unbalanced towards a greater N load than required by the P deposition favouring the high N/P ratio observed in Mediterranean Sea waters, more in the Eastern than in the Western basin, the Si/N ratio might be greater than that in fresh water and effluents.

Assessment of the indicator

The distribution and cycling of silicate in the marine environment being important from a purely scientific point of view, it has little or no relevance for pollution studies.

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Further work required

No pursuance should be given to the Silicate as an indicator of marine pollution.

Marine Pollution Indicator fact sheet

MED POL

pH in transitional, coastal and marine waters of the Mediterranean Sea

Key message

pH is related to the highly buffered carbonate system and is only very slightly modified upon cooling or warming or when bringing deep seawater to the atmospheric pressure.

Changes may also take place when part of the ions involved in the carbon system are taken up (photosynthesis) or released (respiration) by organisms. In practice, another indicator (Total Alkalinity) is better suited to follow these processes and is notsensitive to changes of temperature or pressure.

Gross pH differences exist between the well oxygenated open sea waters and hypoxic or even anoxic lagoons or basins. pH in sulphur-reducing environments is much lower than in normal oxygen-based systems.

Policy relevance

The GESAMP definition of marine pollution includes the discharge of substances, heat and other forms of energy into the marine environment detrimental to the legitimate uses of the sea.

Policy context

No policy or management principles have been included in the Barcelona Convention and Protocols regarding the discharge of high acidity or alkalinity water into the coastal areas.

Environmental context

Although of apparent simplicity, pH measurement in seawater is one of the most complicated operations in chemical oceanography. A magnitude that depends on the equilibrium amongst the various acids and bases (carbonate, borate, phosphate, silicate, nitrogen systems –see below-) as well as the equilibrium with atmospheric gases (CO_2), its measurement is rendered very imprecise unless very careful working precautions are taken.

Small changes in pH occur in seawater upon cooling or warming or when bringing deep seawater to the atmospheric pressure. Changes may also take place when part of the ions involved in the carbon system are taken up (photosynthesis) or released (respiration) by organisms. In practice, another indicator (Total Alkalinity) is better suited to follow these processes and is not sensitive to changes of temperature or pressure.

Gross pH differences exist between open seas, well oxygenated waters, and hypoxic or even anoxic lagoons or basins. pH in sulphur-reducing environments is much lower than in normal oxygen-based systems. Much more than this cannot be said about this Indicator.

Assessment of the indicator

pH is a measure of the concentration of H^+ ions in solution expressed as pH, - log [H^+] and, in seawater, is part of the CO_2 system. Various magnitudes are defined to represent the concentration of the components of this system (CO_2, HCO_3^-, CO_3^{2-}, H^+, OH^-) but, in practice, four measurable parameters are normally used: TCO_2 (i.e., the sum of the dissolved CO_2, the carbonate, and the bicarbonate), TA, pH, and either fCO_2 or pCO_2.

$TCO_2 = [CO_2] + [HCO_3] + [CO_3]$

Alkalinity is given by (Dickson, 1981):

$TA = [HCO_3] + 2[CO_3] + [B(OH)_4] + [OH] + [HPO_4] + 2[PO_4] + [SiO(OH)_3] + [HS] + 2[S] + [NH_3] - [H] - [HSO_4] - [HF] - [H_3PO_4]$,

pCO_2 is the partial pressure of gaseous CO_2 in wet (100% water-saturated) air which is in equilibrium with seawater and is proportional to the dissolved gas CO_2. fCO_2 is the fugacity of gaseous CO_2 (a thermodynamically defined property representing the non ideality of CO_2 - about 0.3% to 0.4% lower than the partial pressure over the range of interest-).

The knowledge of any two of these parameters, along with the temperature, salinity, pressure and the relevant equilibrium constants, allows the determination of the other two parameters. Unfortunately, this is not as easy as it sounds. Total alkalinity (TA) and total inorganic carbon (TCO_2) are independent of temperature and pressure; pCO_2, fCO_2 and pH are not. The two definitions of alkalinity in current usage differ in how minor species are treated. Four different pH scales [total, seawater, free, and NBS (National Bureau of Standards, now the National Institute of Standards and Technology)] are in current usage although not always specified in the literature.

The situation with the equilibrium constants is potentially more confusing: There are several different formulations of K_1 and K_2 (the first and second dissociation constants of carbonic acid in seawater) and also several formulations for the other dissociation constants of interest, on various pH and concentration scales.

Many of these differences are slight, but their importance is in direct proportion to the desired precision of the calculated values. The difference in the definitions of alkalinity consists mainly in the treatment of phosphate. This difference may seem minor, but a modest phosphate concentration, such as 3 micro-moles per kilogram of seawater (µmol/kg-SW), can result in a difference in fCO_2 (or pCO_2) of 20 micro-atmospheres (µatm) or more, when calculated from TA and TCO_2, depending on the definition of alkalinity. This difference, therefore, is quite significant.

Sub-Indicator:

pH in open sea waters of the Mediterranean Sea

Key message

pH in open sea water is related to the highly buffered carbonate system and is only very slightly modified upon cooling or warming or when bringing deep seawater to the atmospheric pressure.

Changes in pH may also take place when part of the ions involved in the carbon system are taken up (photosynthesis) or released (respiration) by organisms. In practice, another indicator (Total Alkalinity) is better suited to follow these processes and is not sensitive to changes of temperature or pressure.

Assessment of the sub-indicator

PH in open seawaters has only a theoretical interest not relevant to pollution studies.

Sub-Indicator:

pH in coastal waters of the Mediterranean region

Key message

pH in open sea water is related to the highly buffered carbonate system and is only very slightly modified upon cooling or warming or when bringing deep seawater to the atmospheric pressure.

Changes in pH may also take place when part of the ions involved in the carbon system are taken up (photosynthesis) or released (respiration) by organisms. In practice, another indicator (Total Alkalinity) is better suited to follow these processes and is not sensitive to changes of temperature or pressure.

Assessment of the sub-indicator

There is no concern about changes of pH due to waste water discharges. However, highly acidic or alkaline industrial effluents may harm the ecosystem, particularly the benthic communities. However, provided diffusing devices are used, no detrimental effects due to the pH should appear. Other effects due to antifouling substances are doubtless more important.

Sub-Indicator:

pH in coastal lagoons and estuaries of Medite rranean states

Key message

Gross pH differences exist between the well-oxygenated open sea waters and hypoxic or even anoxic lagoons or basins. pH in sulphur-reducing environments is much lower than in normal oxygen-based systems.

Assessment of the sub-indicator

The pH of coastal lagoons receiving large amounts of nutrients may experience significant changes due to alternating oxidising and reducing conditions even in absence of acidic and alkaline wastewater discharges. This is a sign of eutrophication and can only be neutralized by aeration thus avoiding highly reducing conditions.

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Further work required

There is not much interest in monitoring the pH in coastal or open seawaters. It may, however be relevant when acidic or alkaline effluents are discharged thus harming the local ecosystems.

Marine Pollution Indicator fact sheet

MED POL

Salinity in transitional, coastal and marine waters of the Mediterranean Sea

Key message

The average salinity of the Mediterranean Sea is higher than the average in the global ocean. This high salinity is a reflection of the evaporative losses of water in the basin that outweigh the precipitation plus river runoff. A large flux of less saline North Atlantic water enters the Mediterranean Sea through the Straits of Gibraltar while a nearly equal flux of higher salinity Mediterranean water flows out to the ocean.

Due to river-water management, including the rivers discharging to the Black Sea, and other factors among which possibly the effects of Global Change, the deep and intermediate waters of the Mediterranean Sea may be significantly increasing the average salinity of the Eastern Mediterranean. This may cause noticeable changes in the global water cycle and even on the climate of the hemisphere if these changes were sufficient to affect the formation of North Atlantic Deep Water.

Policy relevance

The GESAMP definition of marine pollution includes the discharge of substances, heat and other forms of energy into the marine environment detrimental to the legitimate uses of the sea.

Policy context

No policy or management principles have been included in the Barcelona Convention and Protocols regarding the discharge of high salinity water into the coastal areas such as happens to be the case in desalination plants and other evaporative systems along the shoreline.

Environmental context

The average salinity of the Mediterranean Sea is higher than the average in the global ocean. This high salinity is a reflection of the evaporative losses of water in the basin that outweigh the precipitation plus river runoff. A large flux of less saline North Atlantic water enters the Mediterranean Sea through the Straits of Gibraltar while a nearly equal flux of Mediterranean water (the difference is the equivalent to the net loss) is being lost to the ocean with higher salinity.

The inflowing modified North Atlantic water, following a complex pattern, is distributed all over the Mediterranean Sea entering processes of mixing and evaporation that lead to its integration in the deep and intermediate waters that finally constitute the Mediterranean outflow water. Changes in these processes may lead to the modification of the in/out flow of

water and, indirectly to the circulation pattern, not only of the Mediterranean Sea but of the Atlantic Ocean and the global ocean as well.

Salinity is one of the factors controlling the distribution of organisms in the marine system. Although most of them are capable of standing wide ranging salinities, populations are very often associated to water masses and fronts. Salinity and temperature determine water circulation to a significant degree, through their control of density. Therefore, thermohaline fronts at the boundaries of water masses have associated vertical motions that modify the internal fertilization processes generating special heterogeneity in biological production.

In coastal areas, salinity is mostly controlled by rivers and effluents discharging fresh water. Although Mediterranean rivers are highly seasonal, a number of them, particularly those with greater flow rates, have permanent discharges affecting stretches of the coastal zone. Partly due to the absence of important tides, most Mediterranean rivers flow to the sea through deltas and coastal plains of different sizes. Large deltas are those of the Nile, Rhone, Po and Ebro rivers but small deltas, sometimes not detectable intrusions of the coastal plain into the sea, are common in seasonal rivers all around the Mediterranean Sea.

River plumes are a normal feature of the interaction of fresh and sea water in the coastal seas. Fresh water, less dense than sea water, experiences a buoyancy effect that generates small and meso-scale dynamics. Salinity is the major parameter controlling the mixing of the fresh and sea-water. Large plumes may extend considerably along the coast as is the case of the Po river plume affecting hundreds of kilometres of the coastal strip of the Emilia-Romagna and Marche regions and further to the south in the Adriatic Sea. The Rhone river plume may reach the Cap Creus to the southwest and further. Smaller plumes may only be visible at times of especially intense raining events.

In highly seasonal rivers, sea water may enter the river channel when fresh water flow is below average, forming salt water wedges that eventually become anoxic when the continuous downward flux of organic matter is not compensated by frequent replenishment of the sea water after wash out caused by floods. This is the case of the Ebro river in which the salt water wedge enters up the river channel distances from 15 to 30 Km, depending on the fresh water flow. This wedge becomes first hypoxic and, if the low-flow conditions persist, anoxic and P-laden since the particulate organic matter carried out within the fresh water layer falls into the deep salt water layer where it is metabolically degraded with consumption of oxygen. Nitrogen does not accumulate in the salt-water layer since the prevailing red-ox conditions are very adequate for denitrification.

River water management, including that of rivers discharging to the Black Sea, and other factors, among which possibly the effects of Global Change, is causing a significant increase in the average salinity of the Eastern Mediterranean and changes in circulation patterns, particularly in intermediate and deep waters, therefore, affecting the pattern of distribution of biological productivity. Disruption of the long-term equilibrium between river runoff and evaporation may cause significant changes in the global water cycle with a potential impact on the climate of the hemisphere through participation of high-salinity Mediterranean water in the formation of North Atlantic Deep Water.

Large scale desalination, may also contribute to the general increase in Mediterranean salinity although an important fraction of the extracted fresh water ends up into the sea. Local impacts of desalination are caused by the discharge of brines and of pollutants used as antifouling in desalination plants.

Assessment of the indicator

Water salinity in the Mediterranean Sea is of little concern as far as the protection of the marine and coastal environment is concerned. Only indirect effects of high salinity water

discharges may be relevant if carried out in semi-enclosed parts of the basin where wind energy may be relatively low at times thus not dissipating the excess salt introduced.

Sub-Indicator:

Salinity in open sea waters of the Mediterranean Sea

Key message

Salinity in open sea waters of the Mediterranean Sea can hardly be subject to any kind of effect due to the discharges of high-salinity water. However, river water management resulting in reduced freshwater inflow in the Mediterranean basin may continue affecting the salinity of the intermediate and deep waters.
Assessment of the sub-indicator

The combination of reduced freshwater inflow by river water management and discharges of high-salinity effluents from desalination plants may upset the overall water balance of the Mediterranean Basin thus affecting the exchanges in Gibraltar and, eventually, the general circulation not only of the Mediterranean Sea itself but of the oceans as well.

Sub-Indicator:

Salinity in coastal waters of the Mediterranean region
Key message

Salinity in coastal waters of the Mediterranean Sea can be subject to the effects of the discharges of high-salinity water from desalination plants. Proper control of the conditions should be maintained to avoid toxic or detrimental effects to the marine ecosystem.
Assessment of the sub-indicator

There is a growing concern about the effects of desalination plants on the ecosystem, particularly the benthic communities. However, provided diffusing devices are used, no detrimental effects due to the high salinity should appear. Other effects due to antifouling substances are doubtless more important.

Sub-Indicator:

Salinity in coastal lagoons and estuaries of Mediterranean states

Key message
Coastal lagoons, with high evaporation rates, may be naturally high-salinity environments sensitive to further increases in salinity. Discharge of high-salinity waters should be avoided in order not to exert detrimental effects on the fragile ecosystems.
Assessment of the sub-indicator

Even with diffusing devices, high-salinity waters from desalination plants should not be discharged onto lagoons and other enclosed water bodies.
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Further work required

Salinity so far cannot be remotely sensed (though the SMOS sensor should be placed on board a satellite in the coming months). However, a number of ARGO floats and similar are wandering around in the Mediterranean Sea measuring Salinity among other variables. Data on real time are being stored and made available to the scientific community and the end-users through the web page address: http://doga.ogs.trieste.it/WP4/real_time.html. MAP, as an end-user, should be able to access such data.

MED POL

Marine Pollution Indicator fact sheet

Temperature in transitional, coastal and marine waters of the Mediterranean Sea

Key message

High seasonality due to the strong solar radiation produces an intense thermocline all over the basin in summer while, in winter, the water column is mostly vertically homogeneous. Some cold waters in coastal and offshore up welling areas have been identified although general circulation in the Mediterranean Sea is contrary to coastal up welling processes.

Restricted coastal areas and lagoons tend to become warmer than open sea waters although local processes (night cooling, wind stirring, etc) regulate summer and winter temperatures. In spite of the large number of industries and power plants located around the Mediterranean Sea no reports of important impacts due to thermal pollution have been made available.

Temperature control of metabolic oxygen consumption may lead specific coastal areas and lagoons to hypoxia and anoxia events within hours of calm winds. However, these systems recover also fast as soon as wind stirring brings oxygen down to the bottom layers.

Policy relevance

The GESAMP definition of marine pollution includes the discharge of heat and other forms of energy into the marine environment detrimental to the legitimate uses of the sea. However, it is not very common to see Temperature listed as an indicator of pollution (see for e xample http://themes.eea.eu.int/indicators/).

Policy context

No policy or management principles have been included in the Barcelona Convention and Protocols regarding the discharge of hot water into t he coastal areas such as happens to be the case in power plants and other cooling systems along the shoreline.

Environmental context

Sea water temperature is a major environmental factor resulting from the equilibrium between solar radiation and back-radiation, air-sea heat and water exchanges and internal hydrodynamics.

Between the latitudes 30 and 45 ºN, the Mediterranean Sea, the dryness and a marked seasonality is the main feature of the Mediterranean climate that changes from a temperate climate in the northwest with hot and dry summers but cold and rainy winters to a sub-tropical climate in the east consisting in mild winters and extremely hot summers. Deep water temperatures are very homogeneous and constant, increasing from west (13 ºC) to east (15 ºC). Small temperature differences from 12.75 ºC to 13.5 ºC in the western basin may be attributed to water masses of different origins.

High seasonality is shown in the surface waters of the Mediterranean Sea with changes in the west that range between 13 and 26 ºC and in the east ranging from 15 to 29ºC. A sharp

thermocline is formed at the end of March and beginning of April lasting until mid October. Only shy sea surface cold spots appear in areas of wind-induced coastal up welling (Gulf of Lions) or by offshore up welling around current jets (Alboran Sea fronts). At the end of the summer, the thermocline is eroded by storms showing surface cooling while it becomes thicker until the water column becomes homogeneous and highly unstable.

Slightly higher temperatures may be shown by inshore waters with restricted exchange with the open sea although local processes (night cooling, wind, etc) maintain an upper limit to their temperature.

Although a number of industrial and power plants are established a long the Mediterranean coastline, high thermal-pollution effects have not been reported.

Temperature plays an important role in determining the velocity of biochemical reactions and thus, metabolic oxygen utilisation may be extremely fast at temperatures of up to 25 ºC and higher). Eutrophication-prone areas may experience sudden decreases in dissolved oxygen concentration and anoxia may develop in a matter of days if not hours in coastal lagoons and coastal areas with very high productivity and restricted circulation, particularly in the vertical direction. However, wind-induced stirring of the surface layer, will supply the oxygen required at depths of 15 m and more.

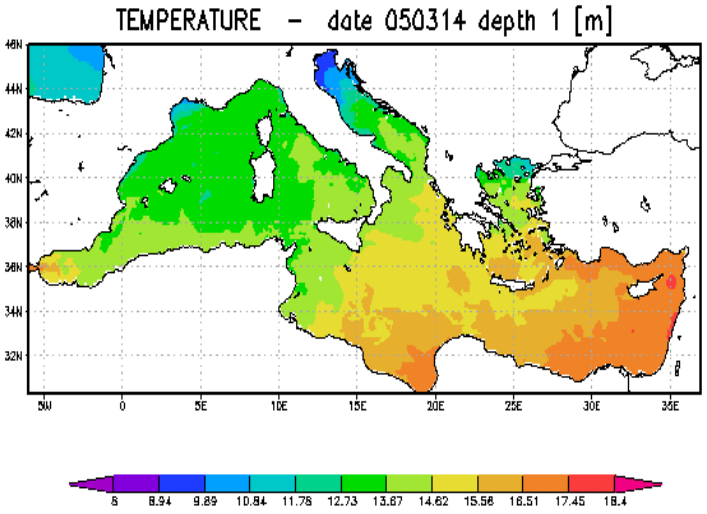

Fig. 1. Sea Surface Temperature of the Mediterranean Sea in march (from MFSTEP prediction system).

Assessment of the indicator

With existing knowledge and available technology, the Temperature of the Mediterranean Sea may be computed from remote sensing data and numerical simulations not only for the sea surface but also for any given depth (Fig. 1). However, water temperature in the Mediterranean Sea is of little concern as far as the protection of the marine and coastal environment is concerned. Only indirect effects of thermal pollution may be relevant and only in semi-enclosed parts of the basin where wind energy may be relatively low at times not dissipating the excess heat introduced.

Sub-Indicator

Temperature in open sea waters of the Mediterranean Sea

Key message

Between the latitudes 30 and 45 ºN, the Mediterranean Sea, the dryness and a marked seasonality is the main feature of the Mediterranean climate that changes from a temperate

climate in the northwest with hot and dry summers but cold and rainy winters to a sub-tropical climate in the east consisting in mild winters and extremely hot summers. Deep water temperatures are very homogeneous and constant increasing from west (13 ºC) to east (15 ºC). Small temperature differences from 12.75 ºC to 13.5 ºC in the western basin may be attributed to water masses of different origins.

High seasonality is shown in the surface waters of the Mediterranean Sea with changes in the west that range between 13 and 26 ºC and in the east ranging from 15 to 29ºC. A sharp thermocline is formed at the end of March and beginning of April lasting until mid October. Only shy sea surface cold spots appear in areas of wind-induced coastal up welling (Gulf of Lions) or by offshore up welling around current jets (Alboran Sea fronts). At the end of the summer, the thermocline is eroded by storms showing surface cooling while it becomes thicker until the water column becomes homogeneous and highly unstable.

Assessment of the sub-indicator

Open waters of the Mediterranean Sea are not subject to thermal pollution although a slight but steady rise in temperature has been reported in the last few decades probably due to changes in the freshwater inputs in the Mediterranean Sea proper and in the Black Sea as a result of river water management.

Sub-Indicator:

Temperature in coastal waters of the Mediterranean region
Key message

Slightly higher temperatures may be shown by inshore waters with restricted exchange with the open sea although local processes (night cooling, wind, etc) maintain an upper limit to their temperature. Although a number of industrial and power plants are established along the Mediterranean coastline, high thermal-pollution effects have not been reported.

Temperature plays an important role in determining the velocity of biochemical reactions and thus, metabolic oxygen utilisation may be extremely fast at temperatures of up to 25 ºC and higher). Eutrophication-prone areas may experience sudden decreases in dissolved oxygen concentration and anoxia may develop in a matter of days if not hours in coastal lagoons and coastal areas with very high productivity and restricted circulation, particularly in the vertical direction. However, wind-induced stirring of the surface layer, will supply the oxygen required at depths of 15 m and more.

Assessment of the sub-indicator

Coastal waters of the Mediterranean Sea are not subject to thermal pollution although some power plants and other cooling systems exist along the coastline, particularly on the northern shores. Local heating may be of some importance although natural dissipation processes should easily take care of keeping the temperatures within levels non-lethal to marine biota.

Sub-Indicator:

Temperature in coastal lagoons and estuaries of Mediterranean states

Key message

Temperature plays an important role in determining the velocity of biochemical reactions and thus, metabolic oxygen utilisation may be extremely fast at temperatures of up to 25 ºC and higher). Eutrophication-prone areas may experience sudden decreases in dissolved oxygen

concentration and anoxia may develop in a matter of days if not hours in coastal lagoons and coastal areas with very high productivity and restricted circulation, particularly in the vertical direction. However, wind-induced stirring of the surface layer, will supply the oxygen required at depths of 15 m and more.

Assessment of the sub-indicator

Eutrophication-prone areas such as coastal lagoons and estuaries should not be submitted to thermal pollution sources since increased surface temperature may give rise to a more intense thermocline restricting the transfer of oxygen from surface to bottom.

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Further work required

Remotely sensed temperature measurements are carried out routinely by several satellites and the data are made available in real time or near real time. Efforts are being made through various EU funded projects (MFSTEP, MERSEA, etc) to make available near-real-time Sea Surface Temperature (SST) data for the entire Mediterranean Sea (see for example, the web at the address: http://gos.ifa.rm.cnr.it/index.php). The MAP should be able, as an end-user, to access such data.

MED POL

Marine Pollution Indicator fact sheet

Transparency in transitional, coastal and marine waters of the Mediterranean Sea

Key message

Transparency of the seawater is an asset of the Mediterranean Sea. Great depths and narrow shelves are common, thus resuspension of sediments is highly unlikely in most of the basin.

The small number of rivers discharging and their highly seasonal regime make suspended sediments in coastal areas only episodic.

The typical blue hue of pure water is a characteristic of the highly oligotrophic Mediterranean Sea.

Policy relevance

The GESAMP definition of marine pollution includes the discharge of matter into the marine environment detrimental to the transparency affecting the legitimate uses of the sea.

Policy context

No policy or management principles have been included in the Barcelona Convention and Protocols regarding the discharge of particulate matter affecting the transparency of the sea.

Environmental context

Transparency of the seawater is an asset of the Mediterranean Sea. Great depths and narrow shelves are common, thus resuspension of sediments is highly unlikely in most of the basin. On the other hand, the small number of rivers discharging and their highly seasonal regime make suspended sediments in coastal areas only episodic. If the oligotrophic character of the Mediterranean Sea is added, the typical blue hue of pure water is a characteristic of the Mediterranean Sea.

The highest Secchi disk readings on record, a measure of turbidity, may have been gauged in the eastern Mediterranean Sea waters due to their extremely low productivity. Low nutrient concentrations, particularly of orthophosphate, makes these waters highly oligotrophic thus with very low turbidity.

Highly turbid waters due to the presence of suspended sediments occur in coastal areas within river plumes at high-flow regimes. Highly turbid waters may also appear in shallow coastal areas when wave action brings into suspension the finer sediments deposited over the bottom at depths generally less than about 15 m.

Discoloured waters may appear when algal blooms develop as a result of intense fertilisation events. Blue-green algae (cyanophyceae or cyanobacteria) are present in the Mediterranean

waters and, although capable of carrying out nitrogen fixation, do not develop the high-density blooms typical of other seas like the Baltic, for example. Red tides, mostly produced by high-density swarms of the mixotrophic dinoflagellate of the genus Noctilucca may appear in costal areas, particularly in areas under the influence of river plumes. Medium density blooms of light-reflecting coccolithophorid organisms have also been reported.

Other water discolouring blooms are rare in the Mediterranean Sea and have not been reported. However, surface slicks and floating mucilage has been reported occasionally in the northern and medium Adriatic Sea.

Assessment of the indicator

Transparency of the sea water is an asset of the Mediterranean Sea. Resuspension of sediments is episodic in coastal areas. The oligotrophic character of the Mediterranean Sea ensures the typical blue hue of pure water characteristic of the Mediterranean Sea.

Turbid waters due to the presence of suspended sediments occur in coastal areas within river plumes at high-flow regimes and in shallow coastal areas when wave action brings in suspension the finer sediments deposited over the bottom.

High Secchi disk readings have been gauged in the eastern Mediterranean Sea waters due to their extremely low productivity. However, discoloured waters may appear when algal blooms develop as a result of intense fertilisation events.

Other water discolouring blooms are rare in the Mediterranean Sea and have not been reported. However, surface slicks and floating mucilage has been reported occasionally in the northern and medium Adriatic Sea.

Sub-Indicator

Transparency in open sea waters of the Mediterranean Sea

Key message
Transparency of the open sea waters in the Mediterranean Sea is due to its oligotrophic character that ensures the characteristic blue hue of pure water.

Assessment of the sub-indicator

Open sea waters in the Mediterranean Sea are always highly transparent due to the lack of sediments in suspension and of dense phytoplankton populations.

Sub-Indicator

Transparency in coastal waters of the Mediterranean region

Key message

Turbid waters due to the presence of suspended sediments occur in coastal areas within river plumes at high-flow regimes.

Highly turbid waters may also appear in shallow coastal areas when wave action brings in suspension the finer sediments deposited over the bottom at depths generally less than about 15 m.

Assessment of the sub-indicator

Turbidity is enhanced by river flow after heavy rainfall in the coastal zone. Intense wave action may also bring into suspension fine sediments deposited at depths not larger than about 15 m. Such events should not last more than a few days and recovery of the normal meteorological situation should bring back the low turbidity conditions.

Sub-Indicator

Transparency in coastal lagoons and estuaries of Mediterranean states

Key message

Transparency in coastal lagoons may be quite reduced by wind stirring but also by development of dense phytoplankton communities. Control of nutrients should help keeping the transparency within limits.

Estuaries in the Mediterranean Sea, usually forming deltas with outside river plumes, may have a surface layer of turbid water and maintain the fine sediments in suspension near shore.

Assessment of the sub-indicator

Coastal lagoons may have reduced transparency as a consequence of natural conditions but also due to high nutrient loads causing eutrophication.

Estuaries are zones naturally affected by high sediment resuspension and thus turbid particularly during flood events and wind stirring.

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Further work required

Transparency/turbidity should be monitored in Coastal lagoons and coastal areas with river and/or effluent out flowing waters. Building along the shoreline is an important source of suspended matter as is the erosion of coastal areas around build-up zones. Excess suspended matter may produce loss of biodiversity such as in the Posidonia beds. Such areas should be the subject of transparency monitoring.

UNEP

Marine Pollution Indicator fact sheet

MAP

MED POL

Halogenated hydrocarbons in biota of the Mediterranean Sea

Key message

Halogenated hydrocarbons are amongst the most toxic and persistent substances reaching the marine and coastal environment through point and diffuse sources.

Although levels of DDT, PCBs and congeners are decreasing worldwide, scarcity of data do not allow a proper assessment to be made.

Policy relevance

The objective of this indicator is to assess the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large loads of halogenated hydrocarbons, especially 20-30 years ago, may have been detrimental to marine ecosystems. Concentrations in blue mussels and fish constitute time integrating state indicators for coastal water quality. An advantage to using biota concentrations as indicators as opposed to using water or sediment is that they are of direct ecological importance as well as relevant to human health and economical factors due to consumption. Mussels are attached to the shallow-water surfaces, thus reflecting exposure at fixed points. The disadvantage of this aspect is that they are restricted to the coastal zone. Fish are exposed to pollution over wider areas and can, in some cases, reflect offshore conditions.

Policy context

Measures to reduce fluvial inputs, direct discharges and atmospheric deposition of halogenated hydrocarbons and to protect the marine environment from these hazardous substances are being taken as a result of various initiatives taken on different levels.

The UN Global Programme of Action for the Protection of the Marine Environment against Land-Based Activities and the Convention for the Protection of the Mediterranean Sea against Pollution have identified contaminants or groups of contaminants whose dumping or discharges from land-based sources are prohibited or limited (Barcelona Convention and Protocols). Mediterranean regional policies aim at reducing the inputs of hazardous substances, among which halogenated hydrocarbons, and improving the state of the marine and coastal environment. However, emission sources and emission patterns differ between various hazardous substances. Thus, in determining the results of abatement policies, each substance should be considered separately.

In particular, the Strategic Action Programme, elaborated and adopted by the Contracting Parties to the Barcelona Convention, proposed:

By the year 2010 to phase out inputs of 9 pesticides and PCBs and reduce to the fullest possible extent inputs of unwanted contaminants.

By the year 2005, to reduce 50% of the inputs of the priority 12 POPs.
By the year 2005, to collect and dispose of all PCB waste in a safe and sound manner.

Halogenated hydrocarbons are also on the EU's list of priority substances (2455/2001/EC (EU, 2001a)). The Water Framework Directive (2000/60/EU), the Dangerous Substances

Directive (76/464/EEC); the Waste Management directives (91/157/EEC, 93/86/EEC and 98/101/EC), limit values for discharges and concentration in foodstuffs (90/642/EC, EC466/2002) and, among others, aim at a substantial reduction of the input of halogenated hydrocarbons to coastal waters, thereby improving the biological state. However, for the time being there is no specific management target for this indicator.

Environmental context

PCBs are a group of synthetic organic chemicals that can cause a number of different harmful effects. There are no known natural sources of PCBs in the environment. Because they don't burn easily and are good insulating materials, PCBs were used widely as coolants and lubricants in transformers, capacitors, and other electrical equipment. PCBs are either oily liquids or solids and are colorless to light yellow. Some PCBs are volatile and may exist as a vapor in air. They have no known smell or taste. PCBs enter the environment as mixtures containing a variety of individual chlorinated biphenyl components, known as congeners, as well as impurities. Most of the information in this toxicological profile is about PCB mixtures that were commercially produced. Some commercial PCB mixtures are known by their industrial trade name, Aroclor. For example, the name Aroclor 1254 means that the mixture contains approximately 54% chlorine by weight, as indicated by the second two digits in the name. The manufacture of PCBs stopped in many developed countries around 1977 because there was evidence that PCBs build up in the environment and may cause harmful effects. Consumer products that may contain PCBs include old fluorescent lighting fixtures, electrical devices or appliances containing PCB capacitors made before PCB use was stopped, old microscope oil, and old hydraulic oil.

Before the ban, PCBs entered the air, water, and soil during their manufacture and use. Wastes that contained PCBs were generated at that time, and these wastes were often placed in landfills. PCBs also entered the environment from accidental spills and leaks during the transport of the chemicals, or from leaks or fires in transformers, capacitors, or other products containing PCBs. Today, PCBs can still be released into the environment from poorly maintained hazardous waste sites that contain PCBs; illegal or improper dumping of PCB wastes, such as old transformer fluids; leaks or releases from electrical transformers containing PCBs; and disposal of PCB-containing consumer products into municipal or other landfills not designed to handle hazardous waste. PCBs may be released into the environment by the burning of some wastes in municipal and industrial incinerators.

Once in the environment, PCBs do not readily break down and therefore may remain for very long periods of time. They can easily cycle between air, water, and soil. For example, PCBs can enter the air by evaporation from both soil and water. In air, PCBs can be carried long distances and have been found in snow and sea water in areas far away from where they were released into the environment, such as in the Arctic. As a consequence, PCBs are found all over the world.

In general, the lighter the type of PCBs, the further they may be transported from the source of contamination. PCBs are present as solid particles or as a vapor in the atmosphere. They will eventually return to land and water by settling as dust or in rain and snow. In water, PCBs may be transported by currents, attach to bottom sediment or particles in the water, and evaporate into air. Heavy kinds of PCBs are more likely to settle into sediments while lighter PCBs are more likely to evaporate to air. Sediments that contain PCBs can also release the PCBs into the surrounding water. PCBs stick strongly to soil and will not usually be carried deep into the soil with rainwater. They do not readily break down in soil and may stay in the soil for months or years; generally, the more chlorine atoms the PCBs contain, the more slowly they break down. Evaporation appears to be an important way by which the lighter PCBs leave the soil.

As a gas, PCBs can accumulate in the leaves and above-ground parts of plants and food crops. PCBs are taken up into the bodies of small organisms and fish in water. They are also taken up by other animals that eat these aquatic animals as food. PCBs especially accumulate in fish and marine mammals (such as seals and whales) reaching levels that may be many thousands of times higher than in water. PCB levels are highest in animals high up in the food chain.

DDT or 2,2-bis(*p*-chlorophenyl)-1,1,1,-trichloroethane is a chlorinated hydrocarbon compound used as an insecticide. First introduced during the 1940s, it killed insects that spread disease and feed on crops. Swiss scientist Paul Muller was awarded the 1948 Nobel Prize in Physiology or Medicine for discovering (1939) DDT's insecticidal properties. DDT, however, is toxic to many animals, including humans, it is not easily degraded into non-poisonous substances and can remain in the environment and the food chain for prolonged periods. By the 1960s its harmful effects on the reproductive systems of fish and birds were apparent after the insecticide had been heavily used for agricultural purposes. After the United States banned its use in 1972, the wildlife population returned, particularly the bald eagle and the osprey. Nevertheless, DDT use continues in parts of the world, particularly in tropical regions, to control the mosquitoes that spread malaria. In 2001 the Stockholm Convention on Persistent Organic Pollutants called for the phasing out of DDT once a cost-effective alternative becomes available.
Assessment of the indicator

Polychlorinated biphenyls (PCBs). The PCBs have been used in a wide variety of manufacturing processes, especially as plasticizers, insulators and fire retardants. They are widely distributed in the environment through inappropriate handling of waste material, leakage from large condensers or hydraulic systems, and other sources. No natural sources are known. Their toxic effects are well documented.

The number of possible PCB congeners is 209, having one to ten chlorine atoms. Twenty of these congeners have non-ortho chlorine substitutions, and so can attain a co-planar structure, similar to the highly toxic polychlorinated dibenzo-p-dioxins and dibenzofurans. Metabolites like methylsulfonyl-PCB have also been detected.

Shifting the analytical technique from packed to capillary column GC permits analysis of specific congeners. As the relative percentage of these congeners remains reasonably constant, two of the main components, CB-138 and CB- 153, and the congener CB-118, which contains one chlorine atom in ortho-position implying a planar character, were selected by ICES to represent the PCBs.

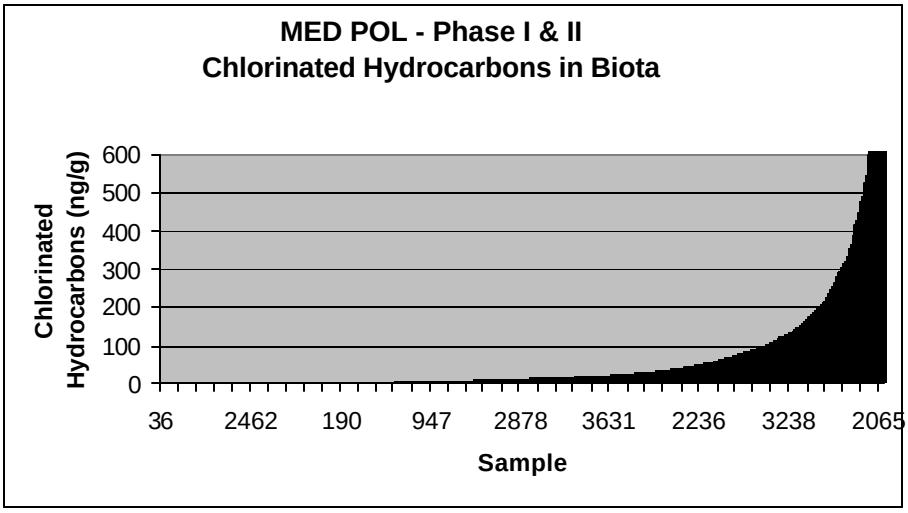
Dichloro-diphenyl-trichloro-ethane and its metabolites and derivatives (DDTs). DDT is a mixture of mainly p,p´-(4,4) and some o,p´-(2,4) dichloro-diphenyl-trichloro-ethane used as insecticide with a large geographical distribution. The world-wide production in 1963 was estimated at 100,000 t, of which the USA accounted for 63,000 t. The annual use of DDT dropped between 1973 and 1983 from 1,000 to 7 t. The use of DDT is now banned in most countries. However, it is still used to a great extent in tropical regions. Over a long time period, the DDTs have been shown to decrease after the regulations and bans in the developed countries.

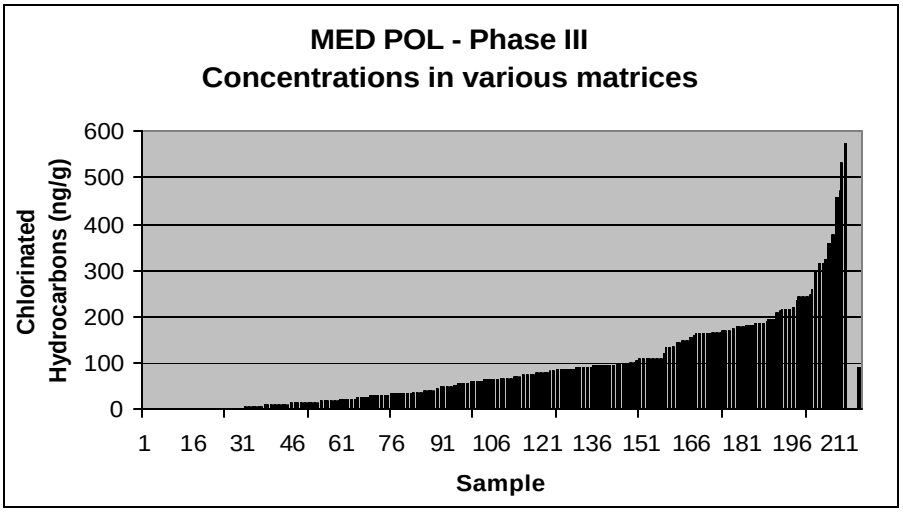
Hexachlorocyclo-hexanes (HCHs). Technical-grade hexachloro-cyclohexane (HCH) is a manufactured chemical that exists in eight isomers. HCH came into use in1950 as an insecticide and typically contained 10-15% ?-HCH (commonly called lindane practically having all the insecticidal properties) as well as the alpha (a), beta (ß), delta (d), and epsilon (e) forms of HCH. Since 1980, the use of lindane in Europe has been allowed only as an insecticide used on fruit, vegetables, and forest crops and as lotion, cream, or shampoo to treat head and body lice, and scabies.

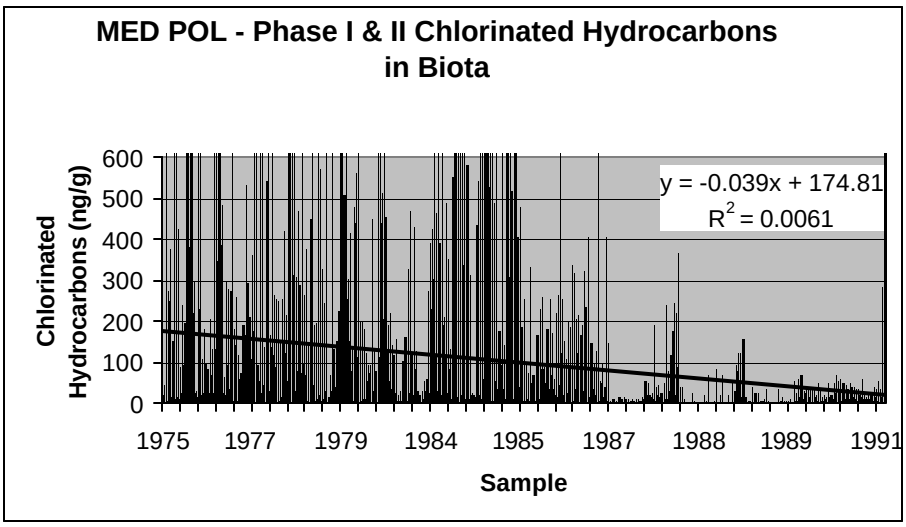
The HCH isomers have been found in soil and surface waters near hazardous waste sites. In the air, the different forms of HCH can exist as a vapour or attached to small particles such as soil and dust. The particles may be removed from the air by rain or degraded by other compounds in the atmosphere. HCH can remain in the air for long periods of time and travel great distances. In soil, sediments, and water, HCH is broken down to less toxic substances by algae, fungi, and bacteria, but this process can take a long time. HCH can accumulate in the fatty tissue of fish.

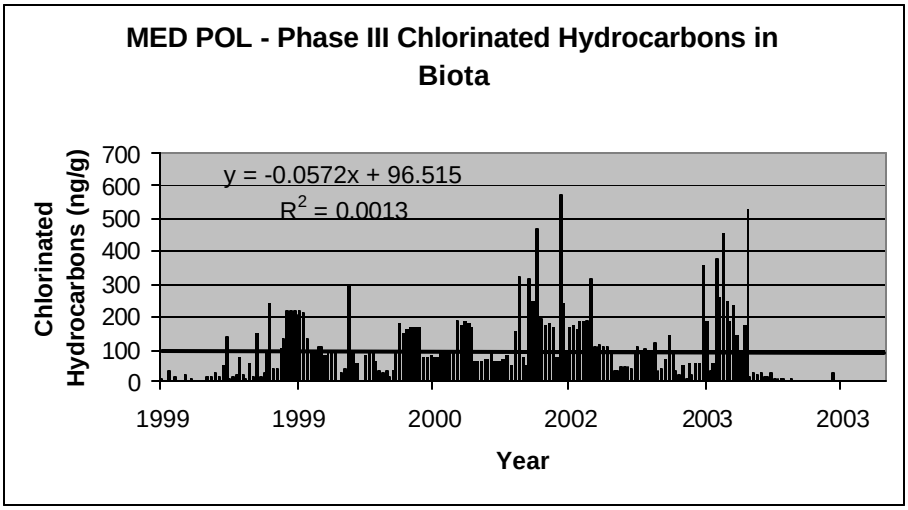
Hexachloro-benzene (HCB). The use of the highly persistent HCB as a fungicide is banned in many developed countries. Although it may still reach the environment as a by-product of many chlorinating processes, e.g., from the pentachlorophenol and vinyl chloride monomer production, there are reasons to expect a decrease in various environmental matrices.

Polybrominated diphenyl ethers (PBDEs). Production of PBDEs, used in brominated aromatic flame retardants, has dramatically increased in the last years having been detected in various levels of the ecosystem. The presence in samples from remote areas indicates a world-wide distribution. Higher levels of PBDEs in predators compared to those in their prey indicate bio magnification of these substances.









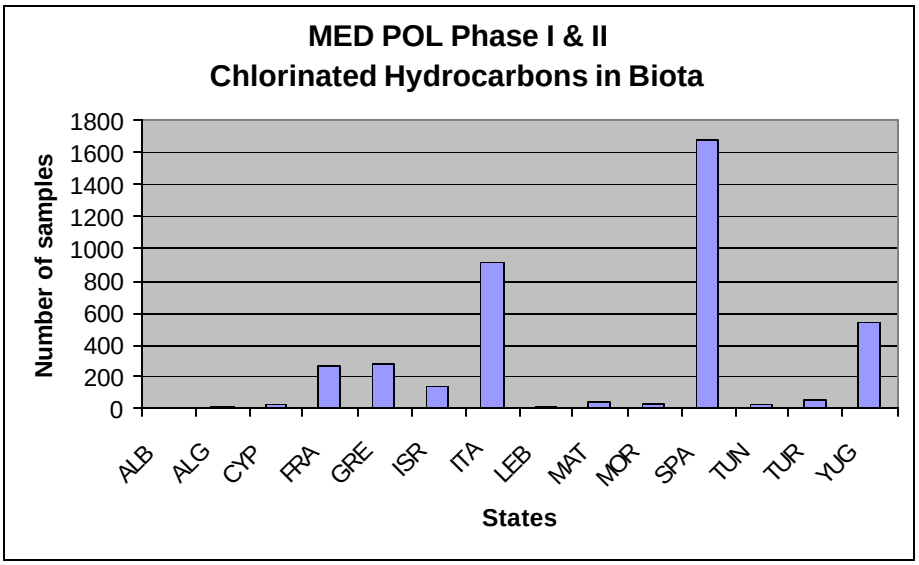
During the Phases II, 2 % of the samples and I analysed showed total concentrations greater than 600 ng/g, a value never reached in the Phase III. On the other hand, a continuously decreasing trend, consistent though not very significant, was appreciated since the early stages of the programme with a slope of -0.039 ng/g per year in the first period and of -0.057 ng/g per year during the latest years.

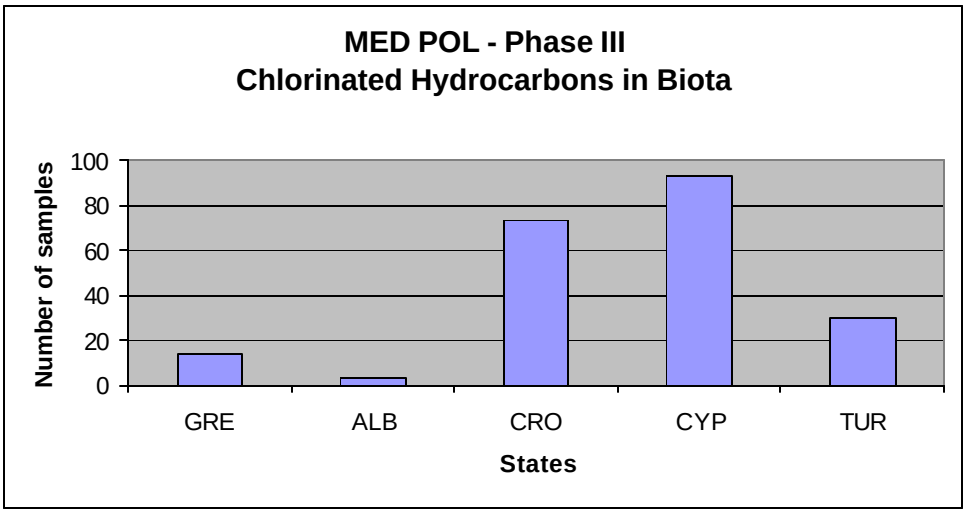
Data source

The data used for the assessment of the halogenated hydrocarbons in biota was obtained mostly from the MED POL database and included the Phase I and II and the Phase III files. Data and other information from the European Environmental Agency was also used, in particular, the Environmental Indicators as listed in the following web address http://themes.eea.eu.int/indicators/ were consulted.

Description of data

The MED POL Phase I and II data set contains data produced between 1975 and 1991 by the laboratories participating in the relevant component of the Programme (Phase I) and in the National Monitoring Programmes of fourteen Member States to the Barcelona Convention (Phase II) (see Figure 1a). The MED POL Phase III data set refers only to five member states (see Figure 1b).



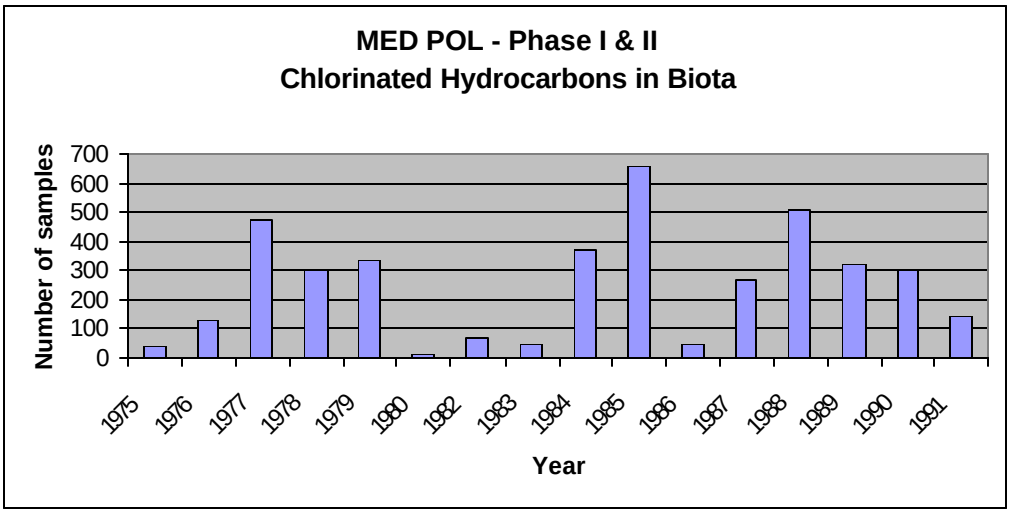


Geographical coverage

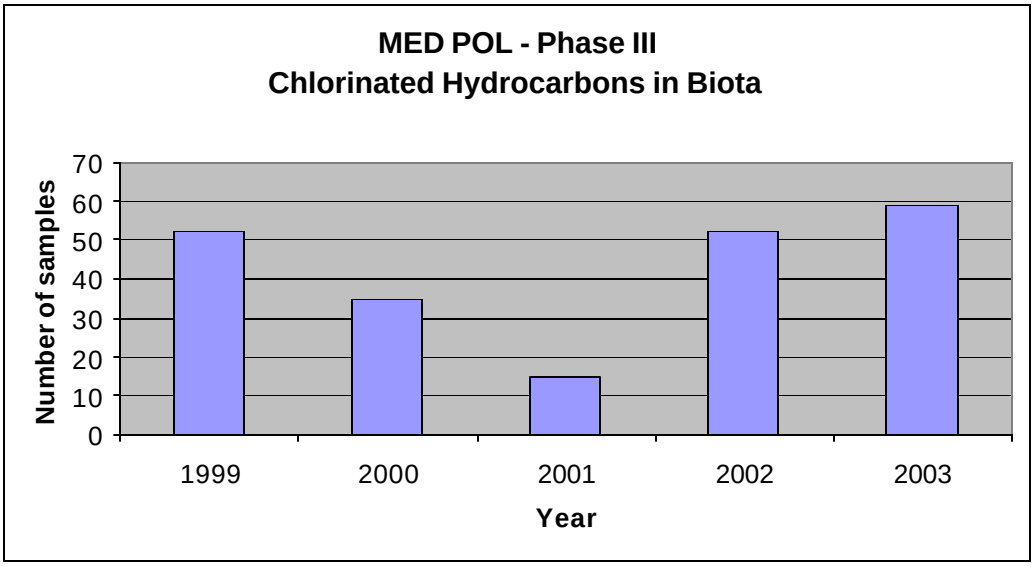
The data from MED POL Phase I and II covered most of the Mediterranean Sea (yellow dots in Figure 2) while Phase III was limited to a smaller set of coastal stations (purple dots in Figure 2).

Temporal coverage

The MED POL Phase I lasted from 1975 to 1981, while the Phase II was progressively implemented between 1982 and 1985 and lasted until 1991 (see Figure 3a).

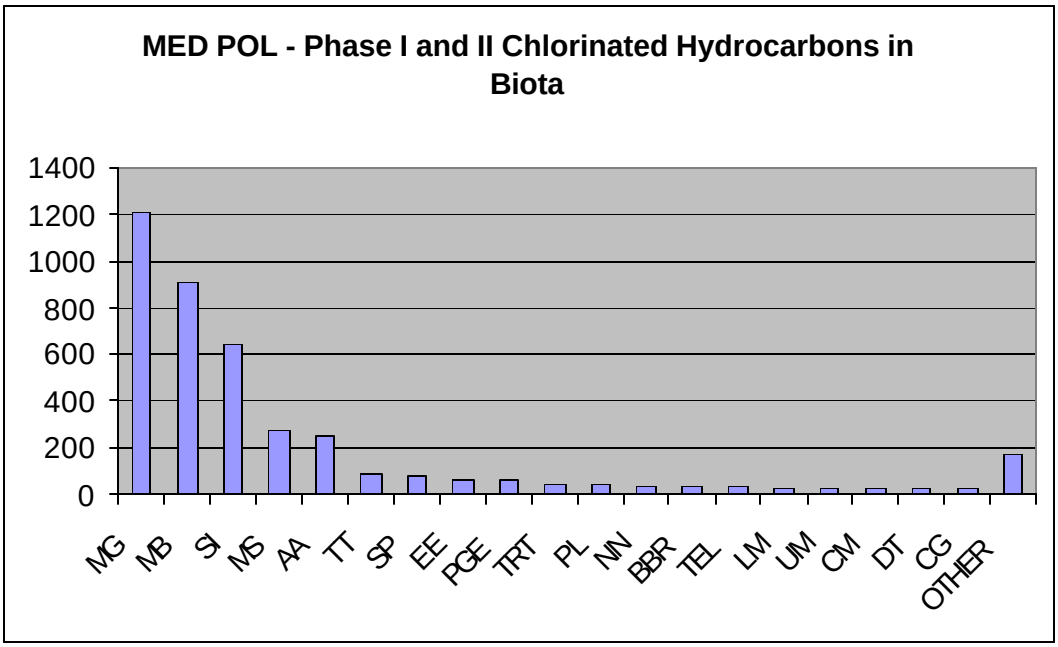


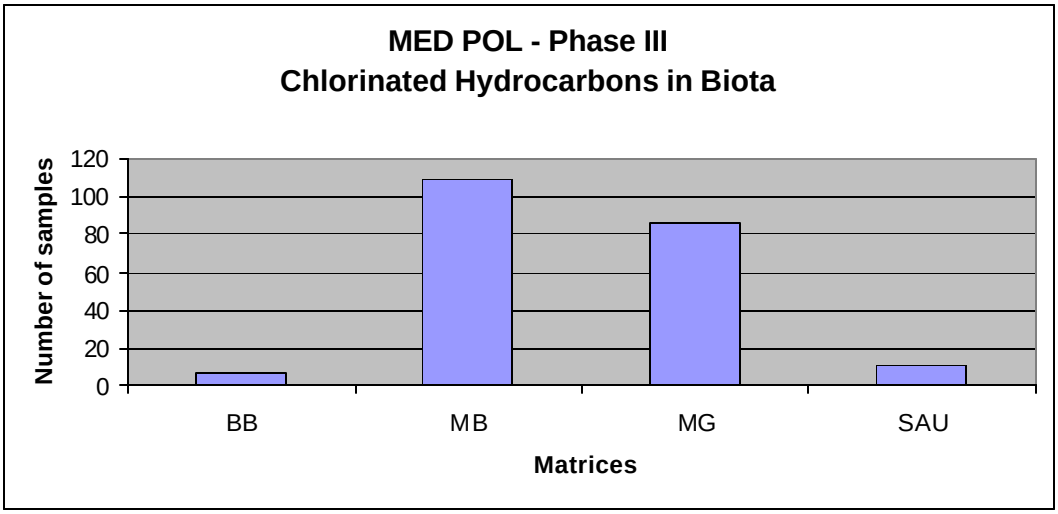
The Phase III lasted from 1999 up to 2003 (see Figure 3.b)



Methodology and frequency of data collection

The samples were always collected following specified protocols both with respect to the location and frequency. The proper Reference Method was followed (RM # 1??). The matrices sampled (species) were also chosen according to their respective ecological/trophic position in each area studied. Figure 4a shows the matrices sampled during Phase I and II, namely *Mytilus Galloprovincialis* and *Mullus Barbatus*. Figure 4b shows the matrices sampled during Phase III..





Methodology of data manipulation

The analytical techniques followed by the various participants were set in the proper Reference Methods (RF #?????). The data were submitted to the central Data Bank in the MAP UNEP Office by the participating laboratories. Various assessments were made on the basis of these data.

Quality information

The analytical quality of the participating laboratories was checked with periodic intercalibration exercises, coordinated by IAEA's Marine Radioactivity Laboratory on the basis of samples prepared and distributed by them to all laboratories around the world and, in particular, in the Mediterranean region.

The data were assessed by external experts and checked by the laboratories and their national coordinating authorities for completion of the complementary data. A system of codes is attached to every data set witnessing the degree of data quality that can be attributed to them.

Further work required

Collection of the large of analytical data existing in the various administrations of the member states belonging to the EU and in the Commission ought to be consolidated and made comparable to the large database existing in the MED POL data bank.

Marine Pollution Indicator fact sheet

MED POL

Halogenated hydrocarbons in effluents discharging to the Mediterranean Sea

Key message

Halogenated hydrocarbons are amongst the most toxic and persistent substances reaching the marine and coastal environment through point and diffuse sources.

Although levels of DDT, PCBs and congeners are decreasing worldwide, scarcity of data do not allow a proper assessment to be made.

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large loads of halogenated hydrocarbons, especially 20-30 years ago, may have been detrimental to marine ecosystems. Concentrations in blue mussels and fish constitute time integrating state indicators for coastal water quality. An advantage to using biota concentrations as indicators as opposed to using water or sediment is that they are of direct ecological importance as well as relevant to human health and economical factors due to consumption. Mussels are attached to the shallow-water surfaces, thus reflecting exposure at fixed point. The disadvantage of this aspect is that they are restricted to the coastal zone. Fish are exposed to pollution over wider areas and can in some cases reflect offshore conditions.

Policy context

Measures to reduce fluvial inputs, direct discharges and atmospheric deposition of halogenated hydrocarbons and to protect the marine environment from this hazardous substances are being taken as a result of various initiatives taken on different levels.

The UN Global Programme of Action for the Protection of the Marine Environment against Land-Based Activities and the Convention for the Protection of the Mediterranean Sea against Pollution have identified contaminants or groups of contaminants whose dumping or land-based discharges are prohibited or limited (Barcelona Convention and Protocols). Mediterranean regional policies aim at reducing the inputs of hazardous substances, among which cadmium, and improving the state of the marine and coastal environment. However, emission sources and emission patterns differ between various hazardous substances. Thus, in determining the results of abatement policies, each substance should be considered separately.

In particular, the Strategic Action Programme, elaborated and adopted by the Contracting Parties to the Barcelona Convention, proposed:

- By the year 2010 to phase out inputs of 9 pesticides and PCBs and reduce to the

fullest possible extent inputs of unwanted contaminants.
- By the year 2005, to reduce 50% of the inputs of the priority 12 POPs.
- By the year 2005, to collect and dispose of all PCB waste in a safe and environmentally sound manner.

Halogenated hydrocarbons are also on the EU's list of priority substances (2455/2001/EC (EU, 2001a)). The Water Framework Directive (2000/60/EU), the Dangerous Substances Directive (76/464/EEC); the Waste Management directives (91/157/EEC, 93/86/EEC and 98/101/EC), limit values for discharges and concentration in foodstuffs (90/642/EC, EC466/2002) and, among others, aim at a substantial reduction of the input of halogenated hydrocarbons to coastal waters, thereby improving the biological state. However, for the time being there is no specific management target for this indicator.

Environmental context

PCBs are a group of synthetic organic chemicals that can cause a number of different harmful effects. There are no known natural sources of PCBs in the environment. Because they don't burn easily and are good insulating materials, PCBs were used widely as coolants and lubricants in transformers, capacitors, and other electrical equipment. P CBs are either oily liquids or solids and are colourless to light yellow. Some PCBs are volatile and may exist as a vapour in air. They have no known smell or taste. PCBs enter the environment as mixtures containing a variety of individual chlorinated biphenyl components, known as congeners, as well as impurities. Most of the information in this toxicological profile is about PCB mixtures that were commercially produced. Some commercial PCB mixtures are known by their industrial trade name, Aroclor. For example, the name Aroclor 1254 means that the mixture contains approximately 54% chlorine by weight, as indicated by the second two digits in the name. The manufacture of PCBs stopped in many developed countries around 1977 because there was evidence that PCBs build up in the environment and may cause harmful effects. Consumer products that may contain PCBs include old fluorescent lighting fixtures, electrical devices or appliances containing PCB capacitors made before PCB use was stopped, old microscope oil, and old hydraulic oil.

Before the ban, PCBs entered the air, water, and soil during their manufacture and use. Wastes that contained PCBs were generated at that time, and these wastes were often placed in landfills. PCBs also entered the environment from accidental spills and leaks during the transport of the chemicals, or from leaks or fires in transformers, capacitors, or other products containing PCBs. Today, PCBs can still be released into the environment from poorly maintained hazardous waste sites that contain PCBs; illegal or improper dumping of PCB wastes, such as old transformer fluids; leaks or releases from electrical transformers containing PCBs; and disposal of PCB-containing consumer products into municipal or other landfills not designed to handle hazardous waste. PCBs may be released into the environment by the burning of some wastes in municipal and industrial incinerators.

Once in the environment, PCBs do not readily break down and therefore may remain for very long periods of time. They can easily cycle between air, water, and soil. For example, PCBs can enter the air by evaporation from both soil and water. In air, PCBs can be carried long distances and have been found in snow and sea water in areas far away from where they were released into the environment, such as in the arctic. As a consequence, PCBs are found all over the world.

In general, the lighter the type of PCBs, the further they may be transported from the source of contamination. PCBs are present as solid particles or as a vapor in the atmosphere. They will eventually return to land and water by settling as dust or in rain and snow. In water, PCBs may be transported by currents, attach to bottom sediment or particles in the water, and evaporate into air. Heavy kinds of PCBs are more likely to settle into sediments while

lighter PCBs are more likely to evaporate to air. Sediments that contain PCBs can also release the PCBs into the surrounding water. PCBs stick strongly to soil and will not usually be carried deep into the soil with rainwater. They do not readily break down in soil and may stay in the soil for months or years; generally, the more chlorine atoms that the PCBs contain, the more slowly they break down. Evaporation appears to be an important way by which the lighter PCBs leave soil.

As a gas, PCBs can accumulate in the leaves and above-ground parts of plants and food crops. PCBs are taken up into the bodies of small organisms and fish in water. They are also taken up by other animals that eat these aquatic animals as food. PCBs especially accumulate in fish and marine mammals (such as seals and whales) reaching levels that may be many thousands of times higher than in water. PCB levels are highest in animals high up in the food chain.

DDT or 2,2-bis(*p*-chlorophenyl)-1,1,1,-trichloroethane, a chlorinated hydrocarbon compound used as an insecticide. First introduced during the 1940s, it killed insects that spread disease and feed on crops. Swiss scientist Paul Muller was awarded the 1948 Nobel Prize in Physiology or Medicine for discovering (1939) DDT's insecticidal properties. DDT, however, is toxic to many animals, including humans, and it is not easily degraded into non-poisonous substances and can remain in the environment and the food chain for prolonged periods. By the 1960s its harmful effects on the reproductive systems of fish and birds were apparent after the insecticide had been heavily used for agricultural purposes. After the United States banned its use in 1972, the wildlife population returned, particularly the bald eagle and the osprey. Nevertheless, DDT use continues in parts of the world, particularly in tropical regions, to control the mosquitoes that spread malaria. In 2001 the Stockholm Convention on Persistent Organic Pollutants called for the phasing out of DDT once a cost-effective alternative becomes available.

Assessment of the indicator

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The number of possible PCB congeners is 209, having one to ten chlorine atoms. Twenty of these congeners have non-ortho chlorine substitutions, and so can attain a co-planar structure, similar to the highly toxic polychlorinated dibenzo-p-dioxins and dibenzofurans. Metabolites like methylsulfonyl-PCB have also been detected.

Shifting the analytical technique from packed to capillary column GC permits analysis of specific congeners. As the relative percentage of these congeners remains reasonably constant, two of the main components, CB-138 and CB- 153, and the congener CB-118, which contains one chlorine atom in ortho-position implying a planar character, were selected by ICES to represent the PCBs.

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Hexachlorocyclo-hexanes (HCHs). Technical-grade hexachloro-cyclohexane (HCH) is a manufactured chemical that exists in eight isomers. HCH came into use in1950 as an insecticide and typically contained 10-15% ?-HCH (commonly called lindane practically having all the insecticidal properties) as well as the alpha (a), beta (ß), delta (d), and epsilon (e) forms of HCH. Since 1980, the use of lindane in Europe has been allowed only as an insecticide used on fruit, vegetables, and forest crops and as lotion, cream, or shampoo to treat head and body lice, and scabies.

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Hexachloro-benzene (HCB). The use of the highly persistent HCB as a fungicide is banned in many developed countries. Although it may still reach the environment as a by-product of many chlorinating processes, e.g., from the pentachlorophenol and vinyl chloride monomer production, there are reasons to expect a decrease in various environmental matrices.

Polybrominated diphenyl ethers (PBDEs). Production of PBDEs, used in brominated aromatic flame-retardants, has dramatically increased in the last years having been detected in various levels of the ecosystem. The presence in samples from remote areas indicates a worldwide distribution. Higher levels of PBDEs in predators compared to those in their prey indicate bio magnification of these substances.

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Marine Pollution Indicator fact sheet

Heavy metals in effluents discharging to the Mediterranean Sea

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large heavy metal loads, especially 20-30 years ago, may have been detrimental to marine ecosystems. Concentrations of metals in blue mussels and fish constitute time integrating state indicators for coastal water quality. An advantage to using biota concentrations as indicators as opposed to using water or sediment is that they are of direct ecological importance as well as relevant to human health and economical factors due to consumption. Mussels are attached to the shallow-water surfaces, thus reflecting exposure at fixed point. The disadvantage of this aspect is that they are restricted to the coastal zone. Fish are exposed to pollution over wider areas and can in some cases reflect offshore conditions.

Policy context

Measures to reduce fluvial inputs, direct discharges and atmospheric deposition of heavy metals and to protect the marine environment from these hazardous substances are being taken as a result of various initiatives taken on different levels.

The UN Global Programme of Action for the Protection of the Marine Environment against Land-Based Activities and the Convention for the Protection of the Mediterranean Sea against Pollution have identified contaminants or groups of contaminants whose dumping or land-based discharges are prohibited or limited (Barcelona Convention and Protocols). Mediterranean regional policies aim at reducing the inputs of hazardous substances, and improving the state of the marine and coastal environment. However, emission sources and emission patterns differ between various hazardous substances. Thus, in determining the results of abatement policies, each substance should be considered separately.

Most heavy metals are also on the EU's list of priority substances (2455/2001/EC (EU, 2001a)). The Water Framework Directive (2000/60/EU), the Dangerous Substances Directive (76/464/EEC); the Waste Management directives concerning disposal of batteries (91/157/EEC, 93/86/EEC and 98/101/EC), limit values for discharges (83/513/EEC), concentration in foodstuffs (90/642/EC, EC466/2002) and, among others, aim at a substantial reduction of the input of heavy metals to coastal waters, thereby improving the biological state. However, for the time being there is no specific management target for this indicator.

Environmental context

Heavy metals are a general collective term applying to the group of metals and metalloids with an atomic density greater than 6 g/cm3. Although it is only a loosely defined term it is widely recognized and usually applied to the elements such as Cd, Cr, Co, Cu, Fe, Hg, Ni, Pb and Zn which are commonly associated with pollution and toxicity problems.

An alternative, theoretically more acceptable, name for this group of elements is “Trace Metals” but is not as widely used. In addition, a number of other lighter elements such as aluminium (Al) arsenic (As) and selenium (Se) have most frequently been associated with toxicity from environmental exposures.

Unlike other pollutants, all the above metals are ubiquitous in the environment and occur naturally in rock-forming and ore minerals. Consequently there is a range of normal background concentrations of these elements in soils, sediments, waters and living organisms.

A great number of them, like copper, iron, zinc and possibly aluminium and selenium are essential for life however, they reach the marine environment from an array of anthropogenic sources as well as from natural geochemical processes like land erosion and volcanic activity.

A number of elements in this group are required by most living organisms in small but critical concentrations for normally healthy growth. These essential metals include Al, Co, Cr, Cu, Fe, I, Mn, See and Zn. Under certain conditions, however, they can bio accumulate to toxic concentrations and cause ecological damage.

These elements are consistently present in the environment. The background concentrations though, meaning the concentrations of metals that occur in the environment in situations that have not been influenced by anthropogenic emissions or by unusual natural exposures, differ between elements.

Arsenic usually occurs as compounds with sulphur either alone or in combination with metals. The toxicity of arsenic depends very much upon the nature of the compound it forms and particularly its valence.

Cadmium is highly toxic and accumulates in the mammalian kidney causing kidney dysfunction. Cd is closely related to zinc and will be found wherever zinc is found in nature. Zinc is an essential metal for most life forms thus it is probable that no naturally occurring material will be completely free from cadmium.

Lead is a cumulative toxin in the mammalian body and toxic concentrations can accumulate in the bone marrow, where red blood corpuscle formation occurs. Like Hg, lead is a powerful neurotoxin and a range of pathological conditions are associated with acute Pb poisoning, most characteristic of which is cerebral oedema. Zn has a relatively low toxicity to animals and humans. Arsenic, cobalt, mercury, lead and selenium can be methylated in the environment through the action of enzymes secreted by micro organisms and also by abiotic chemical reactions.

However, consideration of total quantity of a metal in the organisms’ tissues gives little information about its potential toxicity.

Copper is plentiful in the environment and essential for the normal growth and metabolism of all living organisms. Despite the existence of a number of detoxifying and storage systems for copper, it is the most toxic metal after mercury and silver to a wide spectrum of marine life. It often accumulates and could cause irreversible harm to some species at concentration just above levels required for growth and reproduction. Copper levels can increase markedly in coastal areas where there is runoff from the land.

Bioavailability and toxicity of copper to aquatic organisms depends on the total concentration of copper and its speciation. Elevated concentrations of copper interfere with oxygen transport and energy metabolism. In animals, copper interacts with essential trace elements

such as iron, zinc, molybdenum, manganese, nickel and selenium and also with nonessential elements like silver, cadmium, mercury and lead. These interactions could be either beneficial or harmful to the organism.

Zinc is a commonly occurring trace-metal and is essential to living organisms for enzymatic functions. High levels of zinc are found in coastal areas but biota, dispersion and diffusion can rapidly remove zinc.

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Marine Pollution Indicator fact sheet

Total Cadmium in Biota of the Mediterranean Sea

Key message

- There seems to be a clear decrease of cadmium levels, mostly as a result of emission reductions achieved over Europe, in the last decade.
- Air pollution abatement policies in many European countries are resulting in reductions of atmospheric inputs to the sea.
- The levels of Cadmium in biota vary widely with an average of 160 ppb and 96 % of the samples with concentrations below 1000 ppb in the period 1998 - 2003.
- Estimations by EEA indicate an inconsistent but decreasing trend for cadmium in mussels from both the Mediterranean Sea and the northeast Atlantic.
- However, the data existing in the MED POL Database shows the opposite, a slightly increasing trend (0.2 ng/g per year).

Policy relevance

The objective of indicators is to convey the levels and trends of hazardous substances inputs and concentrations in the Mediterranean Sea. The effect of large cadmium load, especially 20-30 years ago, may have been detrimental to marine ecosystems. Concentrations of cadmium in blue mussels and fish constitute time integrating state indicators for coastal water quality. An advantage to using biota concentrations as indicators as opposed to using water or sediment is that they are of direct ecological importance as well as relevant to human health and economical factors due to consumption. Mussels are attached to the shallow-water surfaces, thus reflecting exposure at fixed point. The disadvantage of this aspect is that they are restricted to the coastal zone. Fish are exposed to pollution over wider areas and can in some cases reflect offshore conditions.

Policy context

Measures to reduce fluvial inputs, direct discharges and atmospheric deposition of cadmium and to protect the marine environment from this hazardous substance are being impemented as a result of various initiatives taken on different levels.

The UN Global Programme of Action for the Protection of the Marine Environment against Land-Based Activities and the Convention for the Protection of the Mediterranean Sea against Pollution have identified contaminants or groups of contaminants whose dumping or land-based discharges are prohibited or limited (Barcelona Convention and Protocols). Mediterranean regional policies aim at reducing the inputs of hazardous substances, among which cadmium, and improving the state of the marine and coastal environment. However, emission sources and emission patterns differ between various hazardous substances. Thus, in determining the results of abatement policies, each substance should be considered separately.

Cadmium is also on the EU's list of priority substances (2455/2001/EC (EU, 2001a)). The Water Framework Directive (2000/60/EU), the Dangerous Substances Directive

(76/464/EEC); the Waste Management directives concerning disposal of batteries (91/157/EEC, 93/86/EEC and 98/101/EC), limit values for discharges (83/513/EEC), concentration in foodstuffs (90/642/EC, EC466/2002) and, among others, aim at a substantial reduction of the input of cadmium to coastal waters, thereby improving the biological state. However, for the time being there is no specific management target for this indicator.

Environmental context

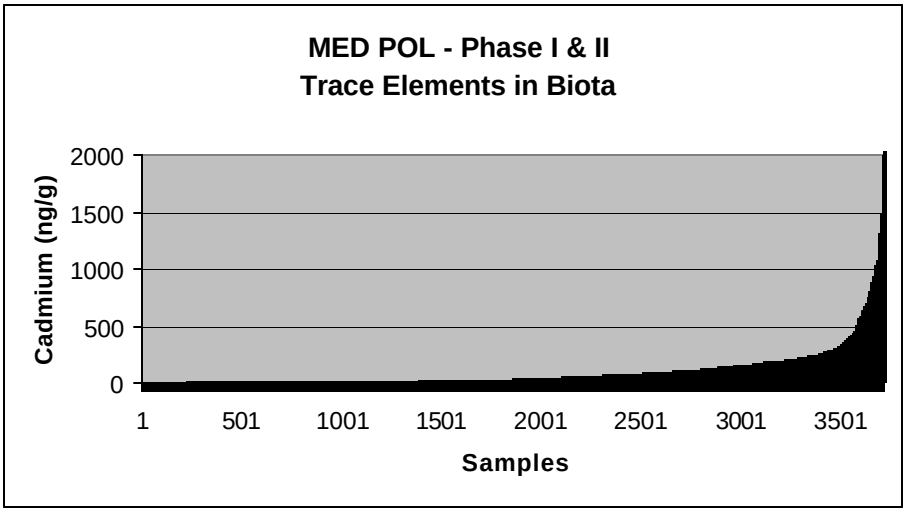
Cadmium is naturally occurring in crust material and, at low levels, is always present in marine waters and sediments. Many marine organisms accumulate cadmium even in areas remote from point sources. Cadmium appears to be unnecessary for any organism and may be toxic. In humans, long-term exposure or consumption of contaminated seafoods can be detrimental.

The main sources of anthropogenic cadmium to the environment (NSC, 2002) include mining, metal industry (including steel industry), coating/electroplating industry, production and disposal of batteries, burning of fossil fuels, the use of phosphate fertilisers, waste incineration, leaching from waste deposits and, finally the use of cadmium salts as stabiliser and/or colouring agent. Therefore, there are many diffuse sources of metal in addition to readily identifiable point sources. EEA has recently published a thorough description of the cadmium sources and dangers to the environment (EEA 2003a).

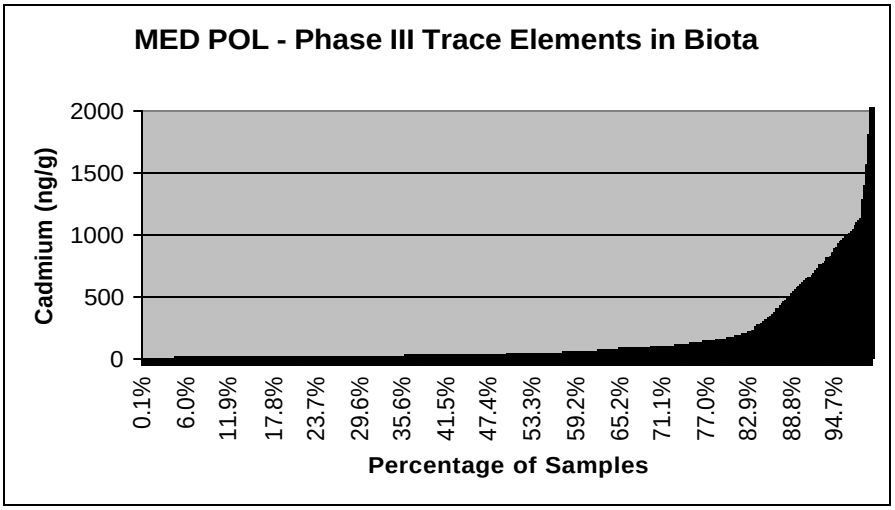
Inputs and atmospheric deposition of cadmium constitute a pressure indicator for marine and coastal water quality. The increased inputs and concentrations of all hazardous substances in coastal waters, bays, estuaries and lagoons represent a threat to the well-being of both biota and humans and had a potentially negative effect on the quality of the ecosystem. Inputs of cadmium into the marine and coastal areas, particularly around the Mediterranean Sea, increased due to human activities especially in the 1970s and 1980s. However, in general, the concentrations of metals in Mediterra nean rivers are lower than in most western European rivers (EEA/UNEP 1999) probably due to retention of metals within them.

Assessment of the Indicator

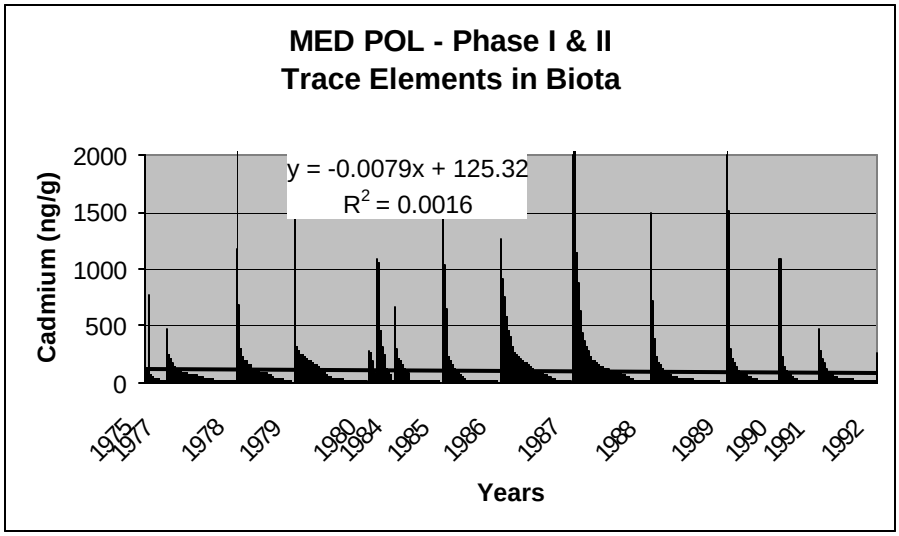
Cadmium is naturally occurring in crust material and, at low levels, is always present in marine waters and sediments. Many marine organisms accumulate cadmium even in areas remote from point sources. Cadmium is unnecessary for any organism and is toxic. In humans, long-term exposure or consumption to contaminated sea foods may be detrimental.

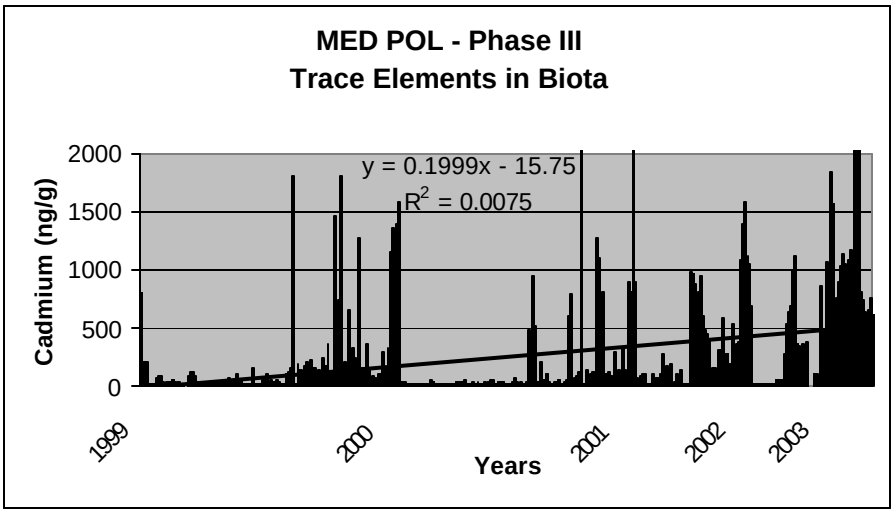


In the last decade, cadmium concentrations in some traditionally contaminated areas such as the Po estuary appear to be decreasing (EEA, 2004) and there also appears to be a general decreasing trend in the French Mediterranean coast. One exception is the Marseilles area possibly with influence from the river Rhone.



Increasing trends found in other Mediterranean areas as well as elsewhere in Europe appear to be associated with harbour areas. The Figure shows the trend of Cadmium data in the fish *Mullus Barbatus*. However, the increasing trend of Cadmium in this indicator species during MED POL – Phase III was entirely due to a set of samples reported in 2003 by one country.





Meta data

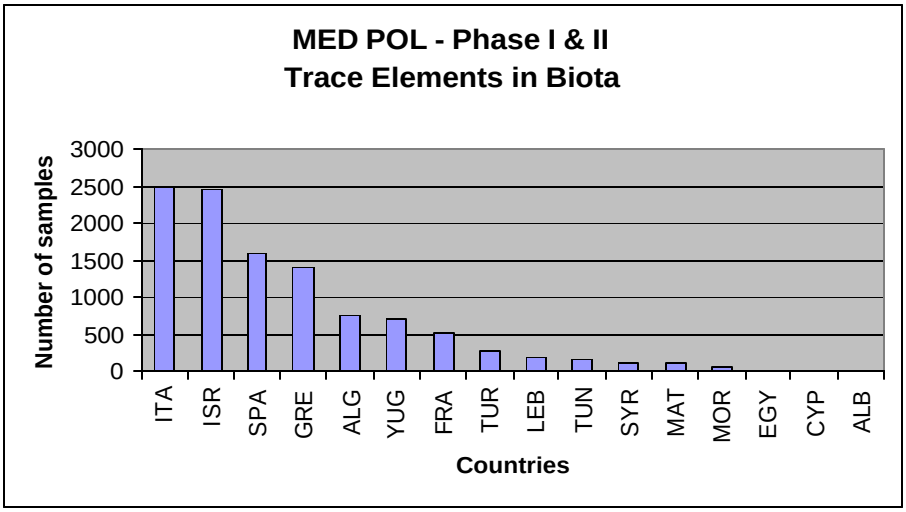
Technical information

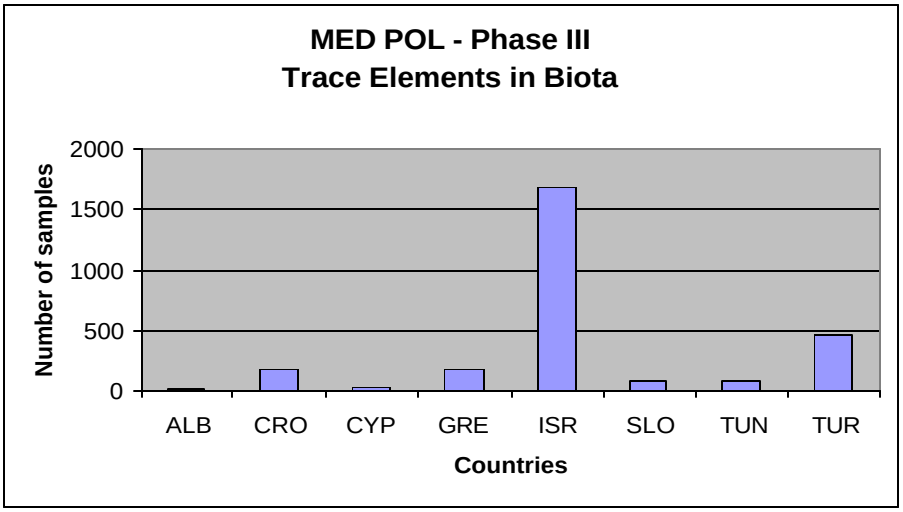
Data source

The data used for the assessment of the trace elements, particularly Cadmium, in biota was obtained mostly from the MED POL database and included the Phase I and II and the Phase III files. Data and other information from the European Environmental Agency was also used, in particular, the Environmental Indicators as listed in the following web address http://themes.eea.eu.int/indicators/ were consulted.

Description of data

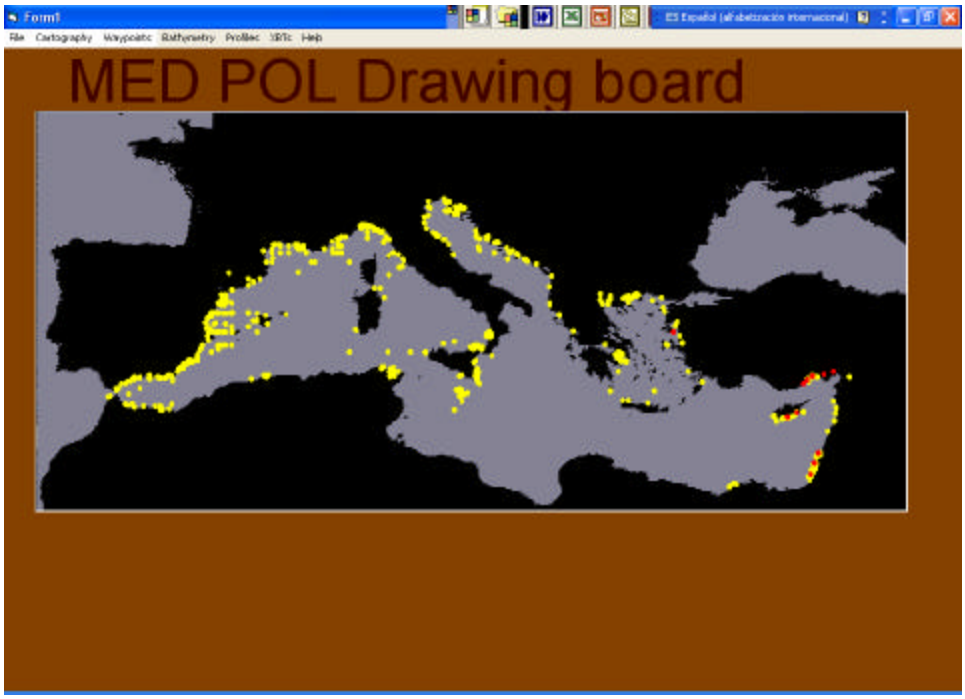
The MED POL Phase I and II data set contains data produced between 1975 and 1991 by the laboratories participating in the relevant component of the Programme (Phase I) and in the National Monitoring Programmes of sixteen Member States to the Barcelona Convention (Phase II) (see Figure 1a). The MED POL Phase III data set refers only to five member states (see Figure 1b). For more information related to these data sets, refer to the web http://195.97.36.231/medpol/.





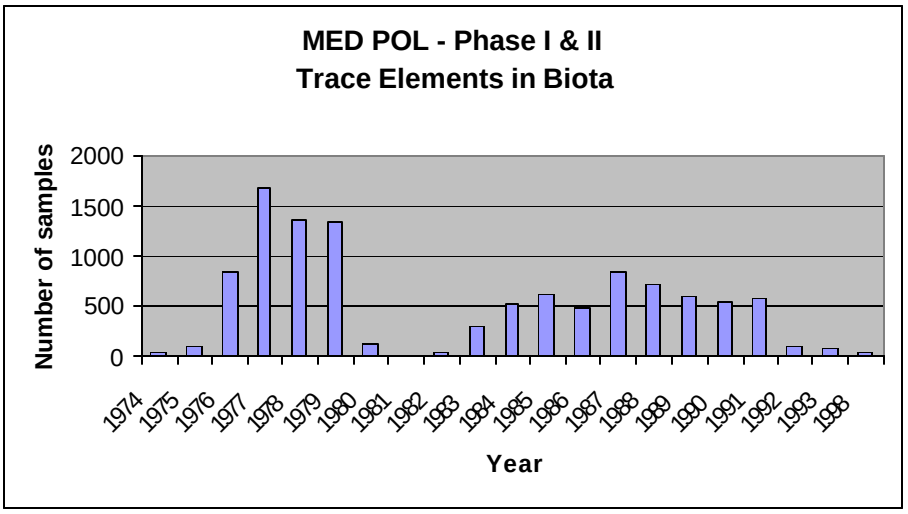
Geographical coverage

The data from MED POL Phase I and II covered most of the Mediterranean Sea (yellow dots in Figure 2) while Phase III was limited to a smaller set of coastal stations (purple dots in Figure 2).

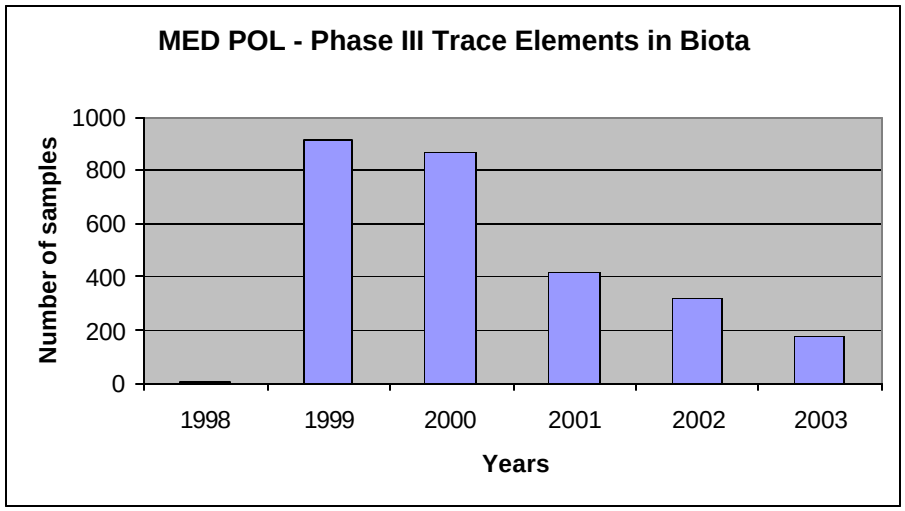


Temporal coverage

The MED POL Phase I lasted from 1975 to 1981, while the Phase II was progressively implemented between 1982 and 1985 and lasted until 1991 (see Figure 3a).

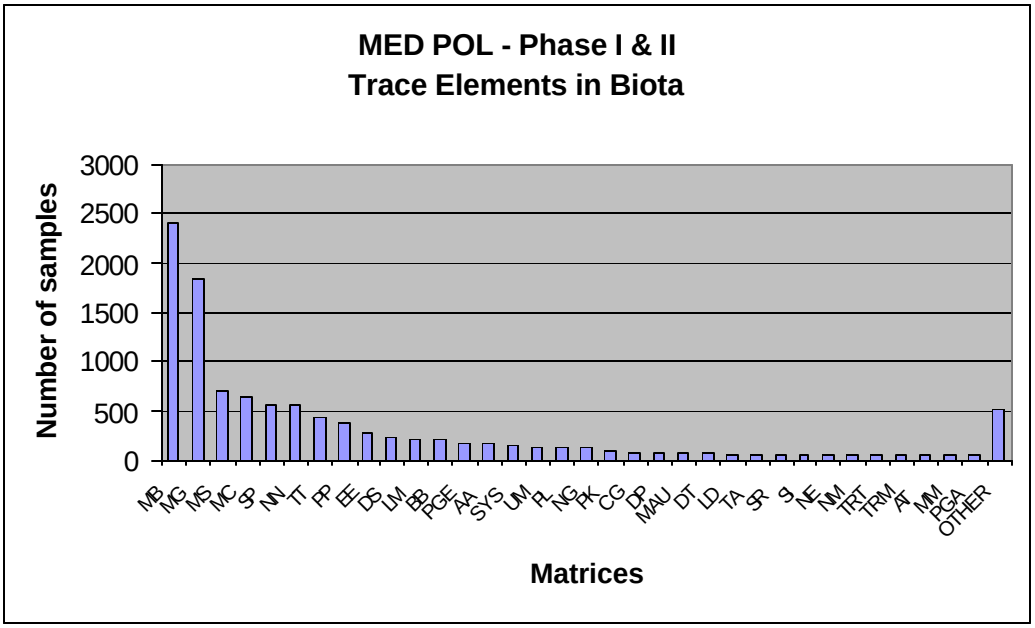


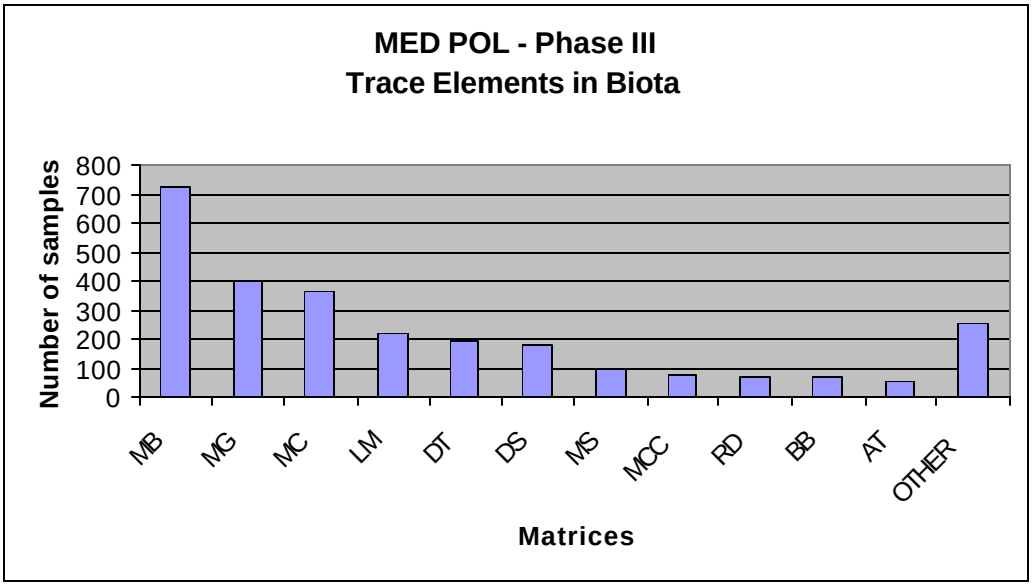
The Phase III lasted from 1999 up to 2003 (see Figure 3.b)



Methodology and frequency of data collection

The samples were always collected following specified protocols both with respect to the location and frequency. The proper Reference Method was followed (RM # 6). The matrices sampled (species) were also chosen according to their respective ecological/ trophic position in each area studied. Figure 4a shows the matrices sampled during Phase I and II, namely *Mytilus Galloprovincialis* and *Mullus Barbatus*. During Phase III only *Mullus barbatus* was reported.





Methodology of data manipulation

Sub-sampling and analysis of biota followed the recommendations made on UNEP's Reference Methods (RF # 6, 7 and 12). Trend monitoring for the Phase III was carried out to detect a minimum linear trend of 10% per year in 10 years with 90% power. Figure shows a linear trend line fitted in EXCEL on sorted data by year.

Quality information

Sub-sampling and analysis of biota followed the description made on UNEP's Reference Methods (RF # 6, 7, 12 and 57). Quality check/coding of data was made in the MEDPOL Data Base in cooperation with MED POL laboratories.