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WISER

Water bodies in Europe: Integrative Systems to assess Ecological status and Recovery

DELIVERABLE

Deliverable D4.2-4: Report/manuscript on benthic macroflora indicators for transitional waters, including classification boundaries, definition of reference conditions and uncertainty

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Content

Non-technical summary

This document reports the work conducted in the production of new (or development of already existent) assessment indices in Work Package 4.2 – seagrass and macroalgae in transitional waters (i.e. estuarine and lagoon). The work was conducted within the project WISER under the sponsorship of the European Commission. It presents most of the technical details considered important for the analyses underlying each assessment tool, and provides the necessary information to understand the rationale, approach and underlying assumptions necessary to discuss the results in the scope of the European Water Framework Directive. The focus is therefore to discuss either the developed assessment indices and to produce some recommendations to improve macroalgae/angiosperms-based ecological assessments in transitional waters, mainly estuaries and lagoons. This is perfectly in line with some of the WISER aims, in which the project is expected to assist the WFD implementation. The studied sites represent real environmental concerns and are examples of different environmental situations. These sites were appropriate to test the macroalgae/angiosperms tools developed in WISER and to check their compliance with WFD normative requirements. Furthermore, results of the work package have been shared with relevant Geographical Intercalibration Groups (GIGs) as aimed to support WFD implementation in Europe.

Introduction

The European Water Framework Directive (WFD; Directive 2000/60/EC - European Council 2000) requires classifying the quality status of rivers, lakes, coastal and transitional waters. The ecological status is to be evaluated by biological assessment methods based on the following selected biological quality elements (BQE): phytoplankton, benthic flora, benthic invertebrate fauna and fish fauna. In this sense, the European research project WISER project (Water bodies in Europe – Integrative Systems to assess Ecological status and Recovery) is supposed to support the implementation of the WFD by developing tools for the integrated assessment of the ecological status of European surface waters (with a focus on lakes and coastal/transitional waters), and by evaluating recovery processes in rivers, lakes and coastal/transitional waters under global change constraints. For this, existing data (new and compiled in previous and ongoing projects) covering all water categories, Biological Quality Elements (BQEs) and stressor types were analysed. During the project's duration, specific field-sampling exercises were performed intending to complement the existing information on assessment methodologies, with special focus on how uncertainty affects classification strength. This actions aims to provide information and scientific support to people (e.g., politicians, stakeholders) involved on the decision process when designing future monitoring programs for lakes, coastal and transitional waters.

Worldwide, the deterioration of coastal systems due to the increasing human pressure is clear. The highest population density occurs within the closest 10 km from the coast and around 23% of the global human population presently inhabits not further than 100 km from the sea (Nicholls and Small, 2002). This constitutes a significant anthropogenic pressure into those areas. Natural ecosystems are replaced by urban areas, artificial structures (e.g., harbours, dikes, etc.) and installations to produce and transform resources (e.g., aquaculture farms, desalination plants). Similarly, nutrients, organic matter and other contaminant inputs to the coastal zone have here increased significantly. As a result, there is a widespread deterioration of coastal water quality, evidenced by a decrease of water transparency, increase of nutrient and organic enrichment and coastal eutrophication. As a result, coastal key ecosystems, such as salt marshes and seagrass meadows, are declining at an alarming rate (Duarte et al., 2008).

To combat the present coastal deterioration tendency, priority programs were created in European countries (WFD) and in other regions of the globe (e.g., USA: Clean Water Act (CWA), National Estuary Program (www.epa.gov/nep)), aiming to improve coastal water and ecosystem's quality. In Europe, the implementation of the WFD and the Marine Strategy Framework Directive (MSFD) set a mutual platform and obligations to ensure "good ecological/environmental status" of coastal and marine waters (Borja et al., 2010). On the basis of such quality assessments biological components are included, where angiosperms (seagrass and saltmarsh plants) and macroalgae are BQEs used by both European directives. The high sensitivity of seagrasses to environmental deterioration, as for instance decline of water transparency or coastal eutrophication, together with their widespread geographical distribution, convert them into excellent canaries of coastal deterioration (Orth et al., 2006). The same can be recognised for marine macroalgae, specially the perennial ones, so a wide list of seagrass and macroalgae indicators exist around Europe (see Deliverable D4.2-1), most of them created even before the WFD criteria were defined. The seagrass and macroalgae indicators may focus at different biological organisation levels, aiming the evaluation of plant's chemical composition, individual morphology, meadow abundance and extension, and processes such as growth or population dynamics (e.g. Borum et al., 2004), but some are not WFD compliant. To ensure compliance and to provide valid assessment methods, some tools were transformed or entirely developed during the WISER project, aiming to fulfil the required in the WFD normative definitions. The development process took into account the characteristics of the candidate metrics, the rule used to combine the selected metrics, the response of the tools/metrics against anthropogenic pressure. Moreover, in order to help on future monitoring campaigns/designs, an uncertainty analysis was performed taking into account different situations such as the different number of replicates/samples, different number of sites inside a water body (WB), or the consistency of results obtained throughout consecutive sampling years.

Objectives

The objectives of this Deliverable are: a) to compile the seagrass and macroalgae indices available to assess ecological quality status of European transitional waters (TW), created or developed inside the WISER project; and b) to analyse the uncertainty associated to these classification methods. It aims to clarify the compliance of the presented tools with WFD requirements and to determine which sources of variability (factors) associated with the sampling design of the different indices most greatly influence the ecological status classification of water bodies, providing useful information to European managers about the best practices to adopt in future monitoring programs.

Methods developed or created during the WISER project

During the WISER project (Water bodies in Europe: Integrative Systems to assess Ecological status and Recovery), three assessment tools were developed for TW seagrass and macroalgae (Table 1): the Ecological Evaluation Index continuous (EEI-c), developed in Italy by USALENTO-NAGREF [University of Salento (Italy)-National Agricultural Research Foundation (Greece)]; the Ecological Index (EI), developed in Bulgaria by IO-BAS (Institute of Oceanology, Bulgarian Academy of Sciences) and; the Seagrass Quality Index (SQI), developed in Portugal by IMAR (Institute of Marine Research, University of Coimbra). These indices followed the WFD requirements in the construction, either in terms of parameters they should have included in their structure (e.g., metrics covering the taxonomic composition and abundance), or in relation to the compliance tests they should pass before being considered as acceptable and as validated assessment methods.

WISER also aimed to provide some useful information on the robustness and reliability of the different indices developed by the EU members, addressing all water categories, organism groups and environmental stressor types. These results are presented here, and were achieved mainly through the use of uncertainty analysis, one of the most powerful tools to assess the main weaknesses of biotic indices that allow the identification of the factors contributing to the potential misclassification of the ecological status class of water bodies (Clarke and Hering, 2006; Staniszewski et al., 2006). The estimation of uncertainty is a central element in WFD-compliant assessment methods, since they are based on biological communities that show both spatial and temporal heterogeneity, and because errors will be introduced during sampling and analytical stages (Clarke and Hering, 2006; Carstensen, 2007; Kelly et al., 2009).

If the major sources of variability are known, they can potentially be minimised through the re-design of sampling schemes (additional sampling sites or frequency), through improved training by operating procedures, CEN (European Committee for Standardization) guidance, taxonomic training or through the use of model-based assessment methods (Pont et al., 2009). For this reason, ecological status classification results should always be given in terms of probabilities depending upon the variability associated with these communities over time and space (Hering et al., 2010).

Table 1. List of tools (and main characteristics) developed or concluded during the WISER project for assessing the ecological quality of TW based on seagrasses and macroalgae

Index	Ecological Evaluation Index (EEI-c)	Ecological Index (EI)	Seagrass Quality Index (SQI)
Coastal/ Transitional	T (soft bottom sediment)	C,T	T
Country of application	Italia (but also in Greek coastal lagoons)	Bulgaria	Portugal
Target species	*Cymodocea nodosa*-ESG IA; *Ruppia cirrhosa*-ESG IA; *Cystoseira barbata*-ESG IB; *Gracilaria bursa-pastoris*-ESG IIA; *Cladophora* spp.-ESG IIB; *Ulva* spp.-ESG IIB	*Cystoseira barbata*-ESGI; *Cystoseira crinita*- ESGI; *Corallina* spp.- ESGI; *Gelidium latifolium*- ESGI; *Zostera noltei*- ESGI; *Zostera marina*- ESGI; *Potamogeton pectinatus*- ESGII; *Ulva* spp.- ESGII; *Cladophora* spp.- ESGII; *Ceramium* spp.- ESGII; *Chaetomorpha* spp.- ESGII; *Polysiphonia* spp.- ESGII	*Zostera noltei*
Metric/s used	Coverage (%) of 5 different Ecological Status Groups clustered hierarchically into two ESG's	Biomass proportion (%) of different macrophyte species classified in 2 main different Ecological Status Groups: sensitive (ESGI) and tolerant (ESGII) These 2 groups are divided to 7 subgroups.	- Taxonomic Composition (TC); - Bed Extent (BE); - Shoot Density (SD)
Definition of reference conditions	Low pressure (Total pressure <6) conditions that support benthic macrophyte communities of relatively low diversity where seagrass/ angiosperm species dominate (mean % coverage >60%) all the year around. Opportunistic macroalgae (epiphytes or not) abundance remains low (mean % coverage <30%) all the year around.	Expert knowledge, Historical data, reference site (Maslen Nos). The reference conditions were estimated according to the parameters of specific surface, EI index, biomass of the phytocoenoses and the biomass of Cystoseira spp. A higher biodiversity of macrophyte species – 102 sp. was previously established in literature in that region, and high biomass of the oligosaprobic indicator *Cystoseira* spp. was estimated. Reference sites have been identified according to the low pressures and impacts they receive in accordance with Annex V of WFD. Criteria used: -population density: no settlement with more than 1000 in/km2 in the next 15 km and/or more than 100 habitats/km2 no in the next 3 km within that area (winter population). -no more than 10% of artificial coastline -no harbour (more than 100 boats) in 3 km -no beach regeneration within 1 km -no industries within the 3 km -no fish farms within the 1 km -no desalination plants within 1 km -no evidence of *Cystoseira* forest regression due to other unconsidered impacts	Reference conditions estimated from historical data, expert judgment, healthy sites: - the maximum no. of taxa ever recorded in the system (one species for the Mondego estuary); - the largest registered area occupied by the meadows with coverage density higher than 5% or 5% from the available intertidal (15 ha for the Mondego) and; - the percentile 0.90 of the no. of shoots per m2 registered in samples collected randomly inside healthy meadows (12,000 for the Mondego). (the available intertidal is considered as the intertidal area minus the saltmarsh or other occupied areas)
EQR calculation	$p(x,y) = a + b^*(x/100) + c^*(x/100)^2 + d^*(y/100) + e^*(y/100)^2 + f^*(x/100)\ ^*(y/100)$ where x is the score in ESG I, y is the score in ESG II and a, …, f are the coefficients of the hyperbola: a = 0.4680, b = 1.2088, c = - 0.3583 d = -1.1289, e = 0.5129, f = -0.1869	EQR = [%ESGI / (%ESGI+%ESGII)] / (10* Ref. Value) EI method – EQR is calculated according to the formula 1.EI-EQR high-moderate =[(ESGI%/ESGI%+ESGII%)/10]/Ref value; EI-EQR poor = 0,05*[ESGI%/ESGI%+ESGII%]+2;/ref.value; EI-EQR bad (1-2)=0,01* [ESGII(A+B)/ESGII]%+1/ref.value, when ESGI=0; EI-EQR bad (0-1) = 0,01* [ESGIICa/ESGII]%/ref. value, when ESGI=0, ESGII(A+B)=0,where ESGI (biomass of sensitive species) is the score of 3 sensitivity subgroups and ESGII (tolerant species) is a score of 4 subgroups* Ref. value is equal to 10. This differentiation is done, because in most cases in bad status we have not sensitive species. ESGII(A+B) are more sensitive species from tolerant species and ESGIIC(a+b) are less sensitive from tolerant species.	EQR = 0.2*TC + 0.3*BE + 0.5*SD where: - TC: score for the taxonomic composition, from 1 to 5 (maximum possible number of species = 5, and downgrades one score-point every time a species is lost. When no species remain in the system = 1, independently of the maximum possible number of species set as reference for the system); - BE: calculated as the ratio between the measured Bed Extent and the reference conditions estimated for the Bed Extent; - SD: calculated as the ratio between the measured Shoot Density and the reference conditions values estimated for the Shoot Density
Boundaries	0.04 / 0.25 / 0.48 / 0.76	0,2/0,4/0,6/0,8 Expert judgment on biological criteria- EI=8, (EQR=0,8): Equidistant division at 20% ; EI=6 (EQR=0.6): Equidistant division at 40% and so on. Boundaries are set according to biotic index (EI) and to community structure. The dominance of the late-successional species of the genera *Cystoseira* form communities indicative of pristine state, which is characterized by low nutrient and clear water conditions, whilst the dominance of opportunistic seaweeds as *Ulva*, *Cladophora*, *Ceramium*, Cyanobacteria form communities indicative of degraded state, which is characterized by high nutrients, and turbid conditions. The coexistence of the late-successional like *Cystoseira*, *Corallina* species with opportunistic like *Ulva*, *Cladophora*, *Ceramium*, Cyanobacteria, Bacillariophyta species form communities that are indicative intermediate (moderate) conditions. Equidistant division of the EI and EQR .	0.2 / 0.4 / 0.6 / 0.8
Response to pressure (assessed/not assessed)	Yes to total (sum of) Pressure based on expert judgement (see below) -Non-point sources (Agricultural diffuse inputs, Freshwater input) -Pollution (Domestic discharges, Domestic/industrial discharges, Industrial discharges) -Habitat loss (Land-claim) -Industry (Industrial area, Water abstraction, Power generation) -Ports (Port activity, Navigation, Dredging) -Fisheries (Fin-Fisheries, Shell-fisheries) -Physico-chemical (Chlorophyll, Nutrients DIN, Nutrients P, Oxygen, Turbidity)	Yes Varna bay-lake study case-2009. Significant linear correlations Pressures-Agricultural diffuse inputs, domestic discharge, industrial discharge, port activity, turbidity, chlorophyl, TOC in sediments.	Yes. Tested inside WFD IC2, based on Aubry & Elliott, 2006 proposal)
Developed during WISER (yes/no)	No	Yes	Yes (partially)
References	Orfanidis et al., 2011	Dencheva *in press*	Neto et al., *submitted* (to SI Hydrobiologia)

Uncertainty analysis

The here presented uncertainty analyses based both on WISER's official and non-official EQR datasets from the different indices that include some of the key sources of variability associated with the design and implementation of a regional scale bio-monitoring program (e.g. spatial scales of sampling, the temporal scale of sampling, the human-associated source of error). Although the same procedure could be followed for all indices, the number and the nature of factors examined, that potentially contribute to the uncertainty of the EQR estimations of coastal water bodies, differ among the indices, especially due to differences in both the metrics used and their sampling designs. First of all, the total variance and variance of components associated to each factor were estimated for all indices using a linear mixed effects model in the lme4 package of R (Bates, 2005; 2007; Version 2.10.1, R_Development_Core_Team 2009). Afterwards, the uncertainty in ecological status classification was estimated using WISERBUGS (WISER Bioassessment Uncertainty Guidance Software®; Clarke, 2010). WISERBUGS helps to determine whether or not an observed ecological status classification is indeed the most probable classification for a particular site, given the inherent sources of variability. Since the current study was interested in the uncertainty in classification generated by a particular factor (rather than the probability of misclassifying individual sites), the probability of misclassification for each factor was determined along the full range of possible observed EQR values (0 - 1).

Ecological Evaluation Index (EEI-c)

The Ecological Evaluation Index, inspired by the "alternative stable stages" theory (Holling, 1973), is based on the well-known pattern where anthropogenic stress, for example eutrophication and heavy metal pollution, shifts the ecosystem from being pristine, where late-successional species are dominant, to a degraded state, where opportunistic, nitrophilous species are dominant. Human-induced shifts are assessed by classifying benthic macrophytes in two functional groups that respond differently to environmental disturbance: the late-successional group with low growth rates and long life histories (Ecological Status Group I, mostly K-selection) and the opportunistic group with high growth rates and short life histories (ESG II, mostly r-selection) (Orfanidis et al., 2003, 2011). During WISER project the EEI-c methodology has been improved to assess the ecological status of Mediterranean coastal lagoons inhabited by benthic macrophyte communities with fresh water affinities.

Materials and methods

Biological data

Sampling in Lesina Lagoon (Figure 1) was undertaken between 21st and 23rd September 2009. A 0.0225 m^2 Ekman grab was used to sample at 0.6 to 1.2 m depth (infralittoral zone). Four random grab-samples were collected and merged into one replicate; 3 replicates were sampled in total within a site. Overall, 54 samples with vegetation were sent to SO laboratory after formalin fixation. In the laboratory, the formalin preserved samples were first washed in tap water for a few seconds, passed through a sieve of 500 µm and then transferred to sea water. Benthic macrophytes were very carefully sorted out and species were identified to functional group level and as much as possible to species level using a stereoscope and a binocular microscope. Taxonomically difficult taxa were consistently summarized to genus level as spp. No detailed taxonomic analysis of Cyanobacteria colonies was undertaken.

In order to estimate % coverage, a transparent double bottom square PVC container, filled with sea water and having at its bottom a square 15x15 cm matrix divided in 100 squares was used. The surface covered by each sorted taxon in vertical projection floating in sea water was quantified as % of coverage (2.25 cm^2 = 1% sampling surface). The % coverage of epiphytes on seagrass leaves was roughly assessed without removal of the epiphytes from the host plants. The total coverage often exceeded 100% due to the presence of different layers at the vegetation i.e. mainly canopy and understory layers. For species present with insignificant abundance a coverage value of 0.01 % was allocated. From each sample, voucher specimens of taxonomically difficult taxa were fixed in 3-5% formalin sea water, which were deposited in the Fisheries Research Institute for future study. Taxa with a higher than 2% coverage were dried for a while on a filter paper and weighted (fresh weight). Then, they were dried up to constant weight in an oven (50°C) and weighted (dry weight). Taxonomy was standardized using Algae base: http://www.algaebase.org.

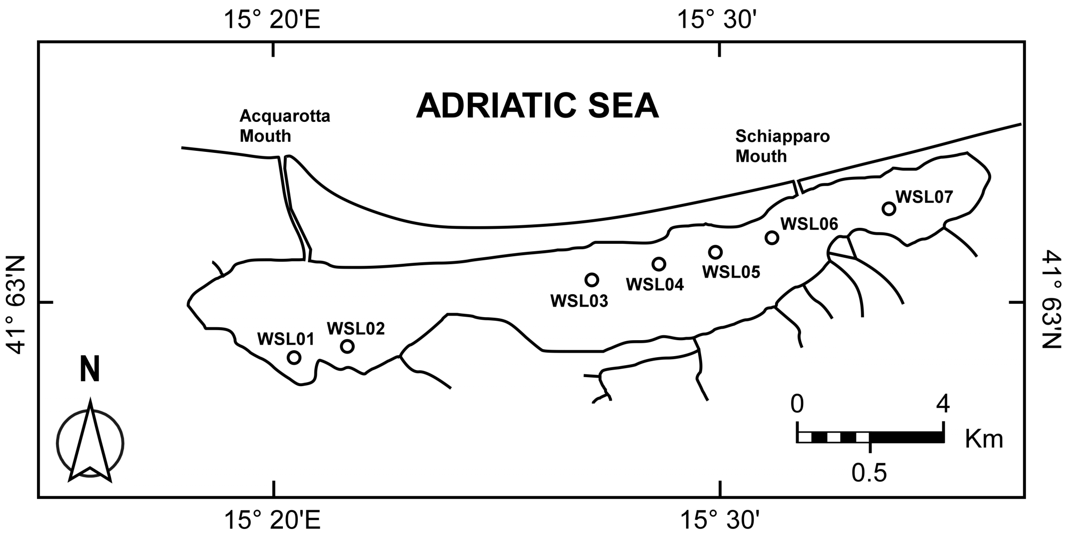

Figure 1. Map of the studied sites in Lesina Lagoon.

Anthropogenic pressures

Pressures were quantified (1: low, 2: medium and 3: high) for each location and sampling station, as partial pressure, total pressure and as a pressure index, following an approach close to that proposed by Aubry and Elliott (2006), based upon best professional judgment. The total pressure is the sum of partial pressures, and the pressure index was calculated as an average value of the pressures (Borja et al., 2011).

When available, quantitative data used for determining pressures were obtained from the systems' time-series. Physico-chemical parameters correspond to averaged monthly measurements (surface and/or bottom).

Metrics and quality assessment method (EEI-c)

The abundance (% coverage) of the two Ecological Status Groups (ESG I, ESG II) and the Ecological Evaluation Index (EEI-c) for each site were calculated according to Orfanidis et al. (2011). The calculation of EEI-c in site 7 was modified by introducing a new group (ESG IIC) that includes species of fresh water affinity such as *Stuckenia (Potamogeton)* sp. These species are valued similarly to opportunistic species (ESG IIB) since their existence in transitional waters is explained by low salinity (close to 10 PSU) that may prohibit the growth of opportunistic species with seawater affinity.

Statistical treatment

Multivariate analyses were based on mean coverage data after a 4th-root transformation. The similarity of the sites was investigated using non-parametric multidimensional scaling analysis based on the Bray-Curtis similarity index. The ANOSIM test was used in order to verify the statistical significance of the ordination analysis. Species contributing mostly to the dissimilarity among the ordination clusters of sites were investigated using the SIMPER analysis (Carr, 1997). Linear relationships were estimated using "Statistica v. 7 and 7.1" software package.

Results

Abiotic factors

The total pressures (TP) and the main environmental characteristics of each sampled site can be seen in Tables 2 and 3, respectively.

Table 2. Pressures determined at each location and sampling site (see Figure 1), showing the pressure gradient in the total value. Values: 1- low pressure; 2- moderate pressure; 3- high pressure.

Type of pressures	Non-point sources		Pollution			Habitat loss	Industry			Ports			Fisheries		Physico-chemical					TOTAL
	Agricultural diffuse inputs	Freshwater input	Domestic discharges	Domestic/industrial discharges	Industrial discharges	Land-claim	Industrial area	Water abstraction	Power generation	Port activity	Navigation	Dredging	Fin-Fisheries	Shell-fisheries	Chlorophyll	Nutrients DIN	Nutrients P	Oxygen	Turbidity	
Lesina lagoon																				
L01	2		2		3								3							10
L02	2		2		2								3							9
L06	2		1		1								3							7
L07	1		1		1								3							6
L03	1												3							4
L04	1												3							4
L05	1												3							4

Table 3. Key environmental characteristics of the sampling sites.

Country and water type (name of the site)	Site	Depth (m)	Distance to the pressure (km)	Temperature (°C)	Salinity PSU	Oxygen Saturation (%)	Turbidity (Secchi disk) (cm)	Redox potential (mV)	Gravel (%)	Sand (%)	Mud (%)	Organic Content (%)
Italian lagoon	L01	-1	0.60	24	18,06	89.3	70	-429	6.1	55.4	38.5	4.7
(Lesina)	L02	-1	0.98	25	18.46	92	100	-384	4.3	46.1	49.6	5.6
	L03	-1.1	7.76	25.8	17.36	84.7	110	-382	1.9	58.4	39.8	10.4
	L04	-1.1	9.30	24.6	17.02	117.7	110	-360	4.9	66.7	28.4	9.4
	L05	-1.2	11.27	24.4	17.28	73.5	120	-393	3.6	63.2	33.2	14.0
	L06	-1.05	12.79	24.4	16.55	114.1	105	-384	4.4	70.0	25.5	8.7
	L07	-0.6	15.88	24.4	13.06	150	60	-333	0.3	63.5	36.3	9.8

Description of the macroalgal communities

The angiosperms *Ruppia cirrhosa* and *Zostera noltei* and the Chlorophyta *Cladophora vadorum* dominated at Lesina sites. Bray-Curtis similarity cluster analysis and multidimensional scaling ordination of the studied sites are shown in Figure 2. At 45% similarity, four clusters were present at the Lesina Lagoon: 1) L01 and L02 (group A), 2) L03 and L04 (group B), 3) L05 (group C) and 4) L07 (group D). The ANOSIM test showed that these groups are significantly different at level 0.1% (global R equals 0.821). An analysis of the contribution of each species to the average Bray-Curtis similarity between the groups using the SIMPER analysis showed that differences were mainly due to the species *Ruppia cirrhosa*, *Zostera noltei*, *Stuckenia pectinata*, and *Cladophora vadorum* (Table 4).

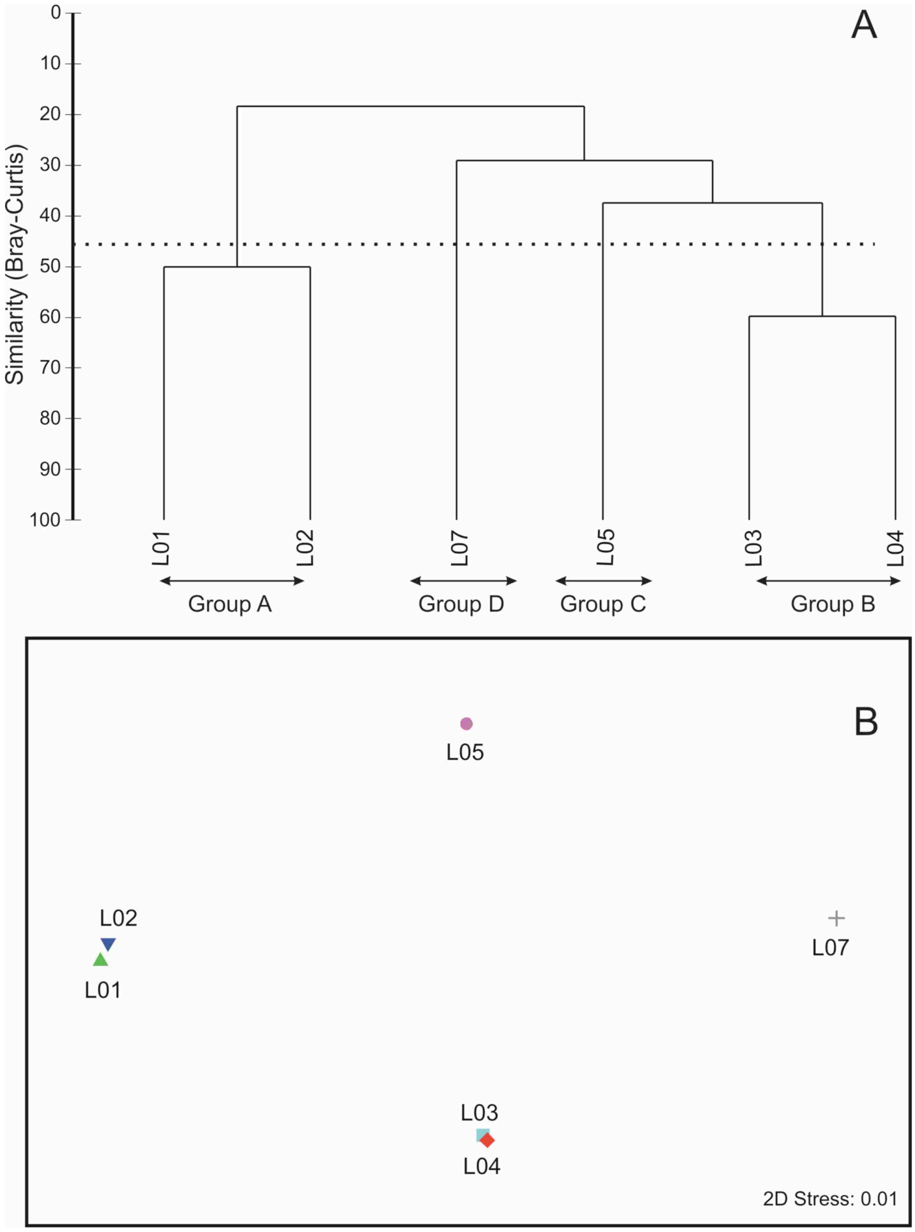

Figure 2. Similarity clusters and 2-d multidimensional scaling plot of the studied sites in Lesina Lagoon.

Table 4. SIMPER analysis of the Lesina lagoon benthic macrophyte multivariate groups. AD=average dissimilarity; Contribution (%): relative contribution to total dissimilarity.

Groups a & b (AD = 96.91)	***Group a***	***Group b***	
Species	***Average Abundance***	***Average Abundance***	***Contribution (%)***
Ruppia cirrhosa	0	81.25	41.95
Cladophora vadorum	8.35	38.19	22.63
Zostera noltei	0	5.83	13.1
Groups a & c (AD = 99.89)	***Group a***	***Group c***	
Species	***Average Abundance***	***Average Abundance***	***Contribution (%)***
Zostera noltei	0	64.58	61.86
Groups b & c (AD= 90.93)	***Group b***	***Group c***	
Species	***Average Abundance***	***Average Abundance***	***Contribution (%)***
Zostera noltei	5.83	64.58	40.68
Ruppia cirrhosa	81.25	0	30.35
Groups a & d (AD= 100.00)	***Group a***	***Group d***	
Species	***Average Abundance***	***Average Abundance***	***Contribution (%)***
Ruppia cirrhosa	0	56.42	59.92
Groups b & d (AD = 77.84)	***Group b***	***Group d***	
Species	***Average Abundance***	***Average Abundance***	***Contribution (%)***
Ruppia cirrhosa	81.25	56.42	56.09
Stuckenia pectinata	0	21.58	19.15
Groups c & d (AD = 99.93)	***Group c***	***Group d***	
Species	***Average Abundance***	***Average Abundance***	***Contribution (%)***
Zostera noltei	64.58	0	37.02
Ruppia cirrhosa	0	56.42	35.96

Abiotic and biotic metric relationships

Figure 3 shows linear relationships between key abiotic data and EEI-c index. Statistical significant relationships ($p<0.05$) were identified between EEI-c and total pressure, distance to pressure, organic matter and sand.

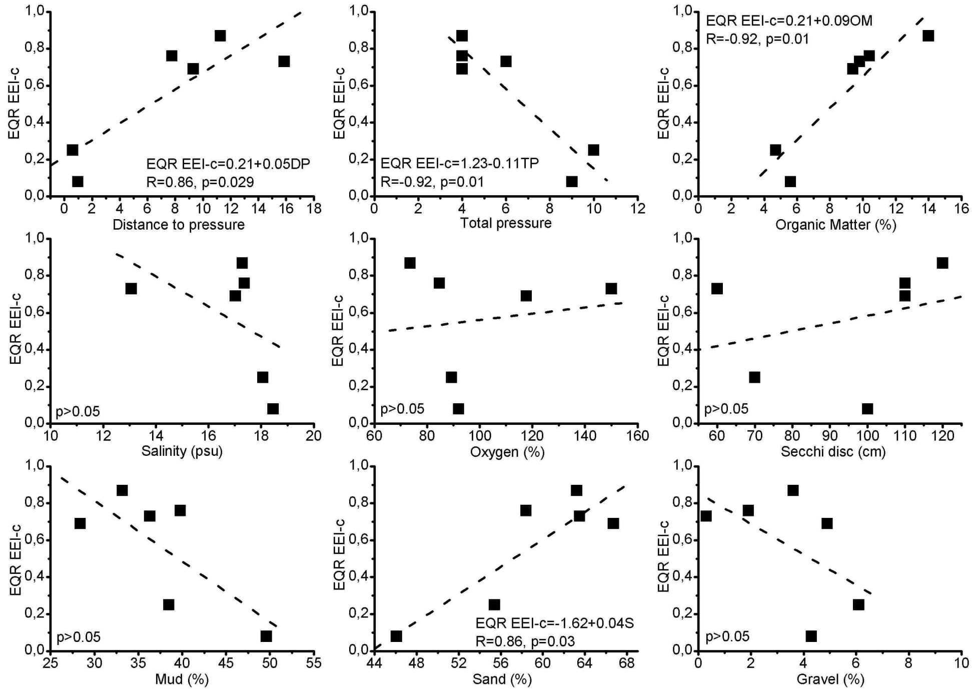

Figure 3. Linear relationships between EEI-c and key abiotic factors.

Uncertainty analysis

In this index, variability among sites was negligible, for which the risk of misclassification associated to this factor was 0% along the whole EQR range (Figure 4). This indicates that the spatial heterogeneity displayed by these biological communities was properly captured in the corresponding sampling designs. Although a considerable uncertainty is still linked to the replication factor, this is specially true for lower quality classes where its wide is smaller and the high variable seaweed communities dominated. The risk of misclassifying increases here and an higher replication should be implemented in sites where lower environmental quality is present. In contrast, the residual variance in mean EQR values was high, accounting for 30.5% of total variance and determining high levels of uncertainty that remained ≥50% almost along the whole EQR range (Figure 4). This could indicate that other unknown sources of variability may influence the water body classification obtained with the use of the EEI-c, for which further data needs to be included in the analysis (e.g., zone, sample, year) in order to minimize the risk of misclassification. The increasing width of the status classes along the EQR range (from 0 to 1) promoted that the general risk of misclassification decreased from "poor" to "high" status.

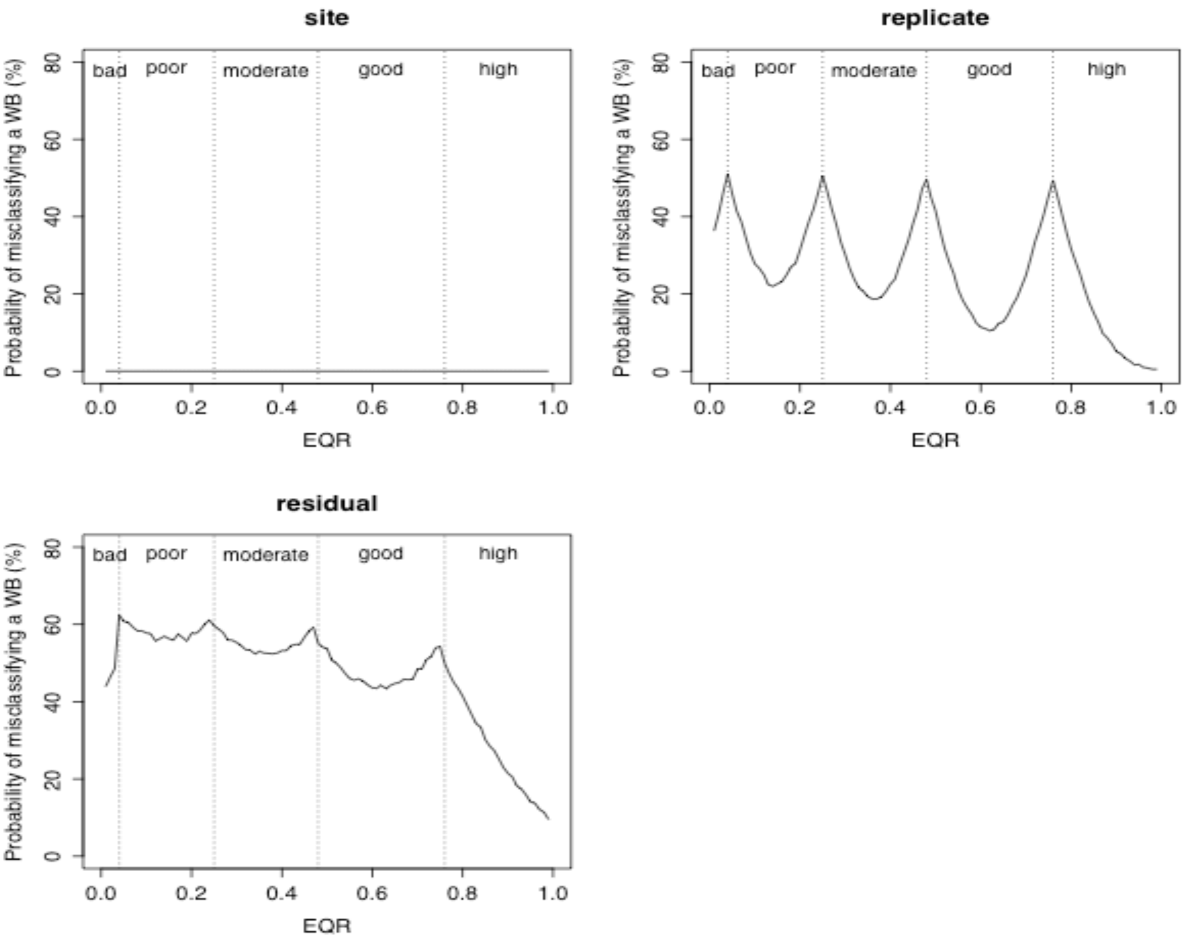

Figure 4. Probability of misclassifying the ecological status associated to the different factors analysed for the EEI-c. Vertical dashed lines represent the boundaries of each status class. Bad = EQR values from 0 – 0.04; Poor = 0.041 – 0.25; Moderate = 0.26 – 0.48; Good = 0.49-0.76 and High = 0.77 – 1.

Ecological Index (EI).

Similarly to the Ecological Evaluation Index (EEI), several ecological groups were differentiated in the Black Sea taking into account the peculiarities of species structure. Low temporal stability of the environment and physical disturbances explain the lower complexity of benthic macrophyte communities in the Black Sea region. Biotic interactions play a minor role in controlling benthic communities and the community dynamics is mainly due to abiotic forcing.

Eutrophication is ranked among the most serious threats to species diversity. In high eutrophication conditions, macrophytobenthic communities obtain very simplified patchy structure, with monospecific character and prevailing of tolerant species.

Species are classified according to literature information and our experience.

Main criteria in differentiating the species into sensitivity groups was eutrophication gradient.

ESG IA: *Cystoseira barbata*, *Cystoseira crinita*, *Zostera marina*

ESG IB *Corallina officinallis*, *Polysiphonia elongata* (thick branches form), *Laurencia* spp., *Osmundea pinnatiffida*, *Zostera noltei*, *Zannichelia major*. *Peisonellia* spp., *Dermatoliton*

ESG IC *Gelidium latifolium*, *Lomentharia*

ESG IICa , *Callithamnion corymbosum*, *Porphyra leucosticta*, *Ceramium rubrum*

ESG IICb -*Ulva intestinalis*, *Cladophora vagabunda*, *Cladophora albida*, *Ulva rigida*, *Ulva compressa*, *Ulva flexuosa*, Bacillariophyta, Cyanobacteria

ESG IIB *Chaetomorpha linum*, *Ulva linza*, *Polysiphonia denudata*

ESG IIA *Gelidium crinale*, *Polysiphonia subulifera*, *Polysiphonia opaca*

Similarly to Ecological Evaluation Index (EEI-c) (Orfanidis, 2011) the mean group biomass proportion is estimated as follows:

$ESG\ I\ =[(IA*1)+(IB*0.8)+(IC*0.6)]$

$ESG\ II\ =[(IIA*0.6)+(IIB*0,8)+(IIC(a+b)(1)]$

The biomass proportion of sensitive species is estimated as biomass value of sensitive representatives divided by total biomass of a given transect. The same way of calculation is about biomass proportion of tolerant species.

Brief discussion

There are some differences in classification of macrophytobenthic communities from that proposed by Orfanidis et al., 2011. *Cystoseira barbata* is spread along more sheltered places and its rare distribution in poor conditions is maybe not only due to anthropogenic pressure, but due to some natural conditions (e.g., higher temperature and less intensive wave action and water exchange). *Cystoseira crinita* is found in open waters, with good and high conditions. Because of this, we considered both species in one ESG, which together with *Phyllophora crispa* are noun as the most sensitive to pollution in Black Sea. Other species, such as *Polysiphonia elongata*, are known as species with very high phenotypic plasticity and adaptation to different levels of eutrophication. It has several morphological forms with thick branches and with thin branches which differ with their surface area /weight ratio values. Thick branched *Polysiphonia elongata* is distributed in high, good and moderate conditions, while thin branched form could be spread in poor and bad conditions. For that reason thick branched form of *Polysiphonia elongata* are assigned into ESG IB group and the thin branched form in ESG IIC. *Laurentia* and *Osmundea* spp. are distributed in high and good conditions and are defined as olygotrophic species in the Black Sea and they present low surface area/weight ratio values. As before *Nemalion* spp. and *Cladostefus* spp. are also categorized at ESG IB. *Gelidium latifolium* and *Lomentharia* are present in high, good and moderate conditions and sometimes in poor ecological status, but in our opinion they are more sensitive to pollution than the other tolerant species. Besides that, they have lower surface area/weight values than the other tolerant species, so they assigned to ESG IC. The surface area/weight ratio values are known to correlate well with functional characteristics such as productivity, photosynthesis, and nutrient uptake, reason why they are taken into account when categorizing all the species into ESG groups. Less tolerant species are *Gelidium crinale*, *Polysiphonia subulifera*, *Polysiphonia opaca*. They are distributed in high and good conditions and also in moderate conditions together with *Cystoseira* populations. Their lower values of surface area/weight distinguish them from the other tolerant species.

Groups of tolerant species (ESG IIA, ESG IIB and ESG IIC) are representatives well known from literature as filamentous and foliose species, predominant in low conditions with high eutrophication level. In ESG IIC one can find macrophytes and microphytes known to grow in the most polluted areas.

There are included also some angiosperms' species, which were examined together with macroalgae communities. We would like to pay special attention to *Potamogeton pectinatus*, which is classified in ESG IIB group. Our arguments in supporting this fact are that this species can be found in abundance in polluted areas in bad and poor conditions both with lower salinity and higher salinity (15-17 PSU).

All these classification improvements followed the method of Orfanidis et al. (2011) for Ecological Evaluation index and its philosophy and were possible within the frames of WISER project. The methodology have been used and validated for coastal waters in intercalibration phase 2.

Material and methods

Biological data

Varna bay and lake were sampled on 8th -10th September 2009. Five sampling sites were selected (Figure 5), specifically taking into account the environmental gradient of nutrients, whose concentrations decrease from the lake to the bay. The main current from lake to the south part of the bay which carries contaminants in this direction is one of the main reasons for worse environmental conditions in this part in comparison with the north part. At each transect (sampling site) different number (from 7 to 42) square frame (0.01 m^2) samples and an additional sample with corer, for the sediment analysis were collected. Samples were taken from 0-2 m depth by scuba diving. Totally, 123 samples were processed. Visual assessment of total percent cover of the communities of every depth layer was carried out.

Macrophyte samples were frozen for preservation to -20°C till the processing for biomass estimation, without damage of cellular structure. In laboratory conditions all benthic macrophyte samples were washed and sieved to remove sediments. Macrophytes were sorted and identified to the lowest possible taxonomic level (under microscope when needed). Species were dried for a while on a filter paper and weighed (fresh weight). Taxonomy was standardized using Algae base: http: //www.algabase.org

Sediment samples were processed at the Institute of Oceanology-Varna. Particle size distribution was determined for fractions less than 2 mm and dry sieving through a nest of sieves for coarser particles. To determine the organic content, the samples are dried at room temperature. Chemical oxidation of organic Carbon (C org) was carried out by "wet ashing" of the dried sediments using sulphuric acid mixture of dichromate at high temperature followed by photometric measurement. Organic content was expressed as TOC and all data were incorporated into a database.

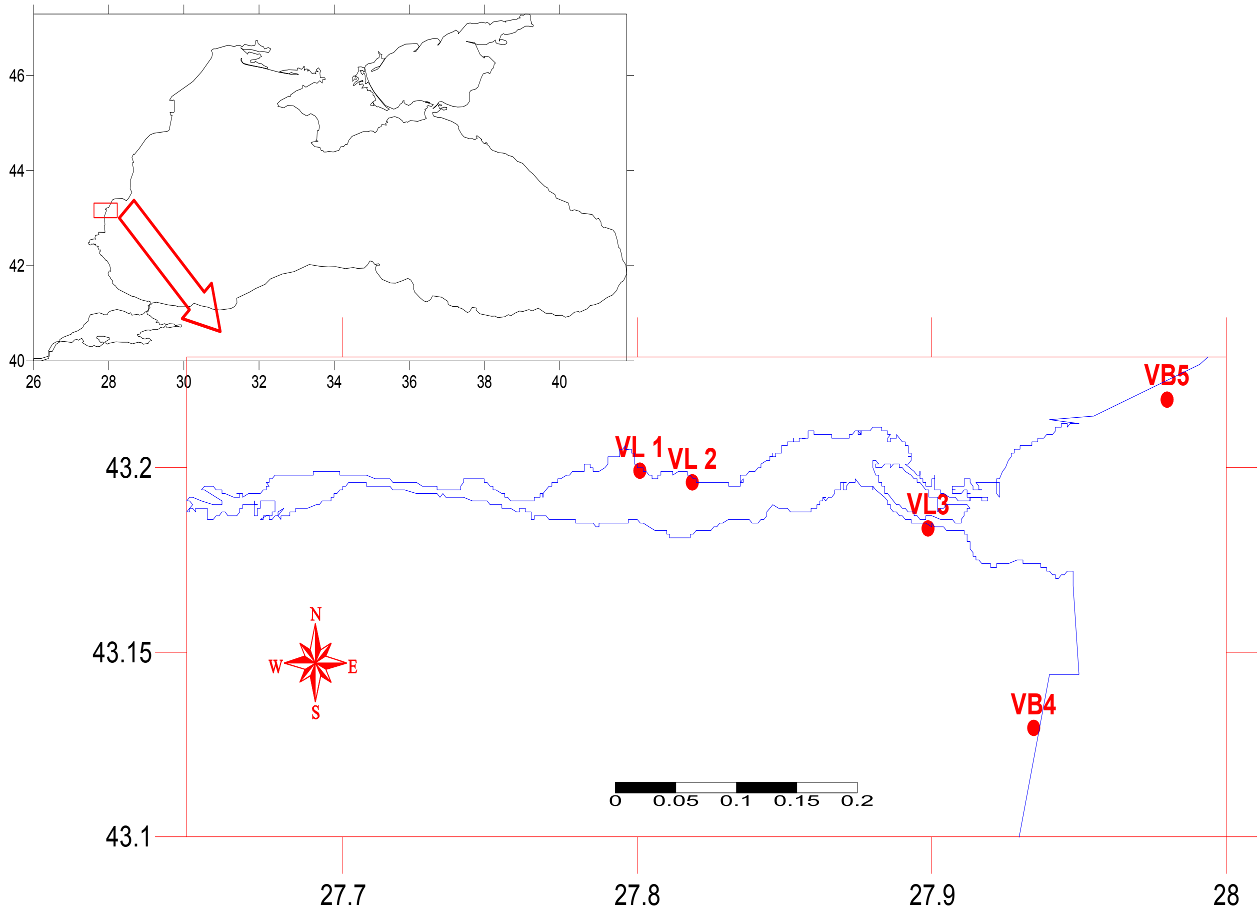

Figure 5. Map of investigated transects.VL1,VL2, VL3-Varna lake;VB4, VB5-Varna bay.

Anthropogenic pressures

Pressures were quantified (low, medium and high) for each location and sampling sites, as partial pressure, total pressure (see WISER 4.3.1 Deliverable). The total pressure is the sum of partial pressures. When available, quantitative data used for defining pressures were obtained from the systems' time-series. Physico-chemical parameters correspond to averaged monthly measurements (surface). Other types of pressures were defined based on expert judgement (Table 5).

Table 5. Pressures determined at each location and sampling station (see Figure 1), showing the pressure gradient in the total value. Values: 1- low pressure; 2- moderate pressure; 3- high pressure.

Type of pressures	Non-point sources		Pollution			Habitat loss	Industry			Ports			Fisheries		Physico-chemical					
Pressures	Agricultural diffuse inputs	Freshwater input	Domestic discharges	Domestic/industrial discharges	Industrial discharges	Land-claim	Industrial area	Water abstraction	Power generation	Port activity	Navigation	Dredging	Fin-Fisheries	Shell-fisheries	Chlorophyll	Nutrients DIN	Nutrients P	Oxygen	Turbidity	TOTAL
Varna bay and lake																				
VL1	1		3		3		3		3							3	3	3	3	25
VL2	1		3		1											3	3	3	3	18
VL3			2		2				1	1						3	3	3	3	17
VB4	2		1													2	2	2	2	11
VB5			2													1	1	1	1	6

Varna Bay is the second largest bay along the Bulgarian coast, widely open to the east. It is 4.5 km long and 8 km wide. Occupies 20 km^2 area, 18.6 m deep. Varna bay is connected through two channels to Varna and Beloslav lakes. Due to its geographical characteristics, shoaled bottom, unlimited water exchange with the sea and a connection with Varna Lake, the bay is characterised by specific hydrological and hydrophysical regimes reflecting on the biota. Main sources of pollution are port activity, industry, tourism, concentration of population.

Varna Lake is one of the important Black Sea coastal lakes affected by human activities (industry, agriculture, transport and urbanisation). Varna Lake is situated in NE part of Bulgaria and it is one of the biggest coastal lakes with average depth 9.5 m. It covers about 17 km^2 area. Hydrochemical peculiarities of Varna Lake are determined by its connections with Beloslav Lake and Varna Bay. The first one is very important for the recent state of the lake water quality (WQ) because Beloslav Lake receives contaminated industrial and domestic waste waters. The other main factors for water contamination are agriculture and port activities, maritime transport shipping.

The lake eutrophication which is a significant and increasing problem has been well documented (Rozhdestvensky, 1992; Stoyanov, 1991; Shtereva et al., 2004; Dencheva, 2010). The drivers of enhanced eutrophication in the lake are nutrient inputs from point sources and non-point sources namely agriculture and urban activities. In fact the load of nutrients to the lake system has greatly increased with time through human activity (Rozhdestvensky, 1992; Stoyanov, 1991) with increase especially in nitrogen (N) and phosphorus (P). The Chemical Industrial complex plays a major role in emission of nutrients and other pollutants. The pollution of both rivers Devnya River and Provadijska River flowing into the near lake (Beloslav) is one of the major problems in the lakes area; Two Waste Water Treatment Plants (WWTPs) with mechanical and biological treatment discharge into the lake; Termal Power Station (TPS) and several small ports are located in the lake banks.

Biodiversity changes as well as changes in plankton and benthos population structures were provoked by long-term pollution. An increase not only of nitrogen and phosphorus content is responsible for eutrophication but a significant pollution with metals and petroleum hydrocarbons (TPH) are recorded during the 80-s (Stoyanov, 1991; Shtereva et. al, 2004). The events of hypoxia/anoxia and fish mortality as consequences of water contamination and eutrophication have been reported (Rozhdestvensky, 1992). The described changes result in dramatic alterations in the chemical and biological regime. The large amount of particulate and suspended organic matter, pesticides and other pollutants contribute also to the eutrophication processes and the ecosystem was identified as highly disturbed. Hydromorphological alterations, due to permanent dredging of the channel, connecting Varna lake with sea, also contribute to worsening state of the system. An estimation of water quality (WQ) and anthropogenic impact on the lake is of the first importance for a sustainable management and for establishment of lake-sea interactions.

Metrics and quality assessment method (EI)

The Bulgarian ecological index was modified from the EEI index (Orfanidis et al., 2011), in conformity with the Black Sea peculiarities. Its value is calculated as biomass percent of sensitive species divided by biomass of sensitive and tolerant species. The total cover of the depth layer sampled was multiplied by the percent biomass values of ecological state groups in order to correct (corrective coefficient) the obtained values. The percent biomass values of sensitive taxa are presented as continuous numerical values from 0 to 10. For example: 80% sensitive species biomass is 8 EI; 65% sensitive macrophyte biomass is a 6,5 EI value. EQR value has been calculated as current obtained EI value divided by reference value (10) (see Orfanidis et al., 2003). EQR values for different ecological state classes are the following: 0-0.2 bad; 0.2-0.4 poor, 0.4-0.6 moderate, 0.6-0.8 good and 0.8-1 high.

Due to the low diversity of Bulgarian Black sea coast, the ecological quality has been calculated on the basis of the transect level. In this study case, for the same reasons we tried to unite samples at depth level and to estimate obtained depth index values as replicates of the transect, but in most cases, the obtained values were equal to "0". That was the reason, for which we made some improvement of the index, when there are not registered sensitive species in bad conditions, values to be calculated on the base of percent cover of tolerant representatives of the community. In this case one can avoid to obtain always 0, when sensitive macrophytes are not present. Unfortunately, the distribution of plant communities of this water system was in most cases monocoenotic, with low presented polycoenotic communities.

The single metrics total cover, average biomass, number of *taxa*, the Ecological State Groups (ESG) (sensitive to tolerant species with more ecological state groups, defined according to specific ecological conditions of Black sea coastal area), were calculated.

Statistical treatment

Within each of the five locations, sampling stations were ordered in an increasing pressure gradient, according to a preliminary classification based on professional judgement (Table 1). The response of single metrics and assessment methods to the pressure gradient was evaluated using Spearman rank correlation coefficients (ρ). Overall, Pearson correlation was used to determine relationships between metrics and methods and between these and environmental variables. All statistical analyses were undertaken using "Statistica 7" for Varna bay and lake study case

Results

The main environmental characteristics of each sampled station (transect) can be seen in Table 6. The data show distinct environments and water types, in terms of depth, salinity, grain size, etc., including a lake and, a bay, and coastal waters in Black Sea ecoregion.

Table 6. Environmental characteristics of the sampling sites. Stations are ordered according to the distance from the pressure source, from the closest to the farthest. TOC- total organic carbon

Country And water type (name of the site)	Site	Depth (m)	Distance to the pressure (km)	Temperature (°C)	Salinity PSU	Oxygen Saturation (%)	Turbidity (Secchi disk) (cm)	Redox potential (mV)	Gravel (%)	Sand (%)	Mud (%)	TOC (%)
Bulgarian bay and lake(Varna)	VI1	-0.5	1.31	24.9	14.4	76	50	na	57.6	41.7	0.6	05
	VI2	-1	2.09	24.5	15.2	83	100	na	55.9	43.2	0.9	0.43
	VI3	-1.5	9.68	24.3	15.1	89	150	na	0	55	45	0.4
	VB4	-2	17.4	24.8	16.9	98	180	na	26.4	72.7	0.9	0.1
	VB5	-2	19.18	24.1	16.1	118	250	na	4.6	94.4	1	0.05

Key biotic parameters of macrophytobenthic communities are established in table 7.

Table 7. Biotic characteristics of the benthic macrophyte communties. Sites are ordered according to the distance from the pressure source, from the closest to the farthest.

Country and water type (name of the site)	Site	Total Coverage (%)	Biomass (g/m²)	Species No	J'	H'	ESG I (%)	ESG II (%)	EQR EI_{EEI}	Dominant species
Bulgarian bay and lake (Varna)	VIL1	70	*843.7	5	0.74	1.75	0	70	0.07	*Cladophora vagabunda*
	VL2	67.5	*1159.56	5	0.63	1.12	0	67.5	0.07	*Cladophora vagabunda*
	VL3	65	*775.45	7	0.66	1.39	0	65	0.07	*Cladophora vagabunda*
	VB4	61.67	*808.77	13	0.89	2.39	7	55	0.1	*Ulva rigida*
	VB5	68	*1789.27	12	0.73	2.19	40	31	0.4	*Cystoseira barbata*

** fresh weigt*

Dominant from sensitive species in VB5 site is *Cystoseira barbata* (43% from all species biomass) and it is found only in this site from whole investigated area. From tolerant representatives, *Ulva rigida* (18%) is with highest biomass percent. In VB4, *Gelidium latifolium* is only the sensitive species (7%), present in this site. From tolerant species *Ulva rigida* (20%) and *Cladophora vagabunda* (14%) differ with highest biomass percent. In VL1, VL2and VL3, sensitive species were not registered. Among tolerant species *Cladophora vagabunda* dominates, respectively in VL1 it is 46%, VL2-49% and VL3-36%.

In figures 6 and 7 is delineated trend of changes in hydrochemical parameters (TP, TOC) and biotic index quality ratio. In direction Varna lake-Veteran-Trakata, the ecological quality ratio increases and hydrochemical indicators decrease. That is in conformity with high eutrophication processes (natural and anthropogenic induced) and pressures in lake and Veteran (south part of the bay) and better quality in Trakata site. Varna lake and Veteran are classified as bad ecological status, Trakata-moderate.

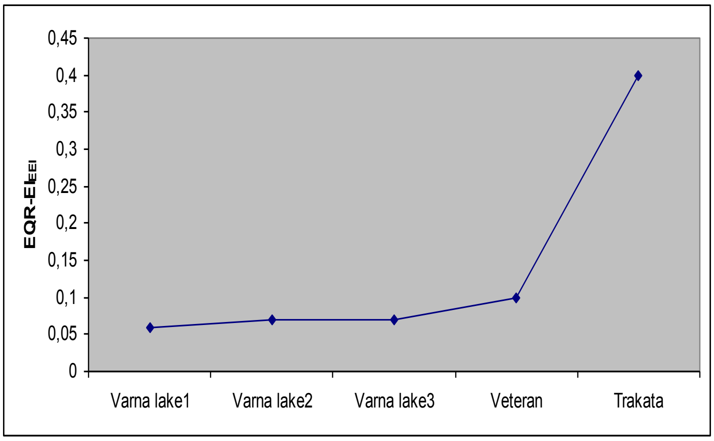

Figure 6. Distribution of ecological index quality ratio(EQR-EI$_{EEI}$) along the investigated transects.

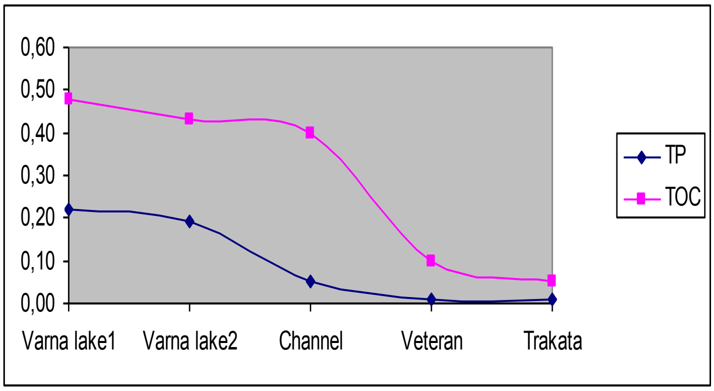

Figure 7. Distribution of total phosphorus (TP) and total organic carbon (TOC) in sediments of investigated transects.

The comparative analysis of biotic parameters and EI index with abiotic environmental parameters and total pressure are presented in table 8.

Table 8. Pearson correlations between key abiotic (Table 6) and biotic (Table 7) parameters in Varna bay and lake. Values in gray show significant correlation for $p<0.05$. ESG: Ecological Status Group.

	Total Coverage (%)	**Biomass (g/m^2)**	**Species No**	**ESG I (%)**	**ESG II (%)**	**EQR EI**
Depth (m)	0.682	-0.465	**-0.917**	-0.572	0.740	-0.520
Distance to the pressure (km)	-0.561	0.549	**0.968**	0.679	-0.824	0.627
Temperature (°C)	-0.080	-0.646	-0.221	-0.672	0.652	-0.699
Salinity (PSU)	-0.755	0.349	**0.932**	0.401	-0.590	0.330
Oxygen Saturation (%)	-0.241	0.829	0.842	**0.901**	**-0.974**	0.871
Turbidity (Secchi disk) (cm)	-0.405	0.708	0.858	0.799	**-0.906**	0.765
Gravel (%)	0.418	-0.332	-0.590	-0.512	0.608	-0.499
Sand (%)	-0.290	0.777	**0.902**	0.872	**-0.958**	0.834
Mud(%)	-0.254	-0.414	-0.199	-0.273	0.237	-0.249
Organic Content(%)	0.482	-0.657	**-0.973**	-0.731	0.862	-0.675
Total pressure	0.442	-0.719	**-0.879**	-0.772	**0.889**	-0.731

It is obvious in this table that there is strong significant correlation between ESG II group of tolerant species with environmental parameters - oxygen saturation (-0.974), turbidity (-0.906), and total pressure (0.889)-. It is very reasonable, in these bad conditions to prevail tolerant species and the connection between them. Species number decrease with increase of organic content, total pressure, and increase with distance to the pressure. With depth, species richness is lower. The correlation of ESG I with oxygen saturation (0.901) is understandable. With increase of oxygen, there are more sensitive representatives as indicators of more good ecosystem conditions.

Other approach than Pearson correlation was also followed by using the nonparametric Spearman rank correlation (table 9).

Table 9. Spearman rank correlation coefficient within Varna bay and lake: correlation between key abiotic (Table 6) and biotic (Table 7) parameters in Varna bay and lake. Values in gray show significant correlation for $p<0.05$. ESG: Ecological Status Group.

	Biomass (g/m^2)	Species No	ESG I (%)	ESG II (%)	EQR EI
Depth (m)	-0.821	-0.632	-0.860	0.667	-0.860
Distance to the pressure (km)	0.500	0.872	0.894	-1.000	0.894
Temperature (^oC)	-0.400	-0.308	-0.447	0.700	-0.447
Salinity (PSU)	0.600	0.821	0.783	-0.800	0.783
Oxygen Saturation (%)	0.500	0.872	0.894	-1.000	0.894
Turbidity (Secchi disk) (cm)	0.500	0.872	0.894	-1.000	0.894
Gravel (%)	0.100	-0.564	-0.335	0.700	-0.335
Sand (%)	0.500	0.872	0.894	-1.000	0.894
Mud(%)	-0.051	0.395	0.229	-0.616	0.229
Organic Content (%)	-0.500	-0.872	-0.894	1.000	-0.894
Total pressure	-0.500	-0.872	-0.894	1.000	-0.894

ESG I, ESG II and EQR-EI correlate significantly with distance to the pressure (0.894:1:0.894), oxygen saturation (0.894:1:0.894), turbidity (0.894:1:0.894), organic content (0.894:1:0.894), total pressure (0.894:1:0.894).

Uncertainty analysis

In this index, temporal variability presented relatively low levels of uncertainty, indicating that variability among years is properly captured in the monitoring program (Figure 8). In contrast, high levels of variability were observed in the mean EQR scores among sites and among depths, explaining 25% and 37% of total variance respectively. Their corresponding probability of misclassification was extremely high, with levels between 60 to 70% along the EQR range (Figure 8). These results suggest that a greater sampling effort must be assigned within sites and that depth should remain fixed in the monitoring programs in order to reduce the high levels of uncertainty associated to these two factors. Finally, the residual variance was low, representing only 3% of the total variance and accounting for a risk of misclassification that was also relatively low, indicating that all sources of uncertainty are represented in the monitoring program.

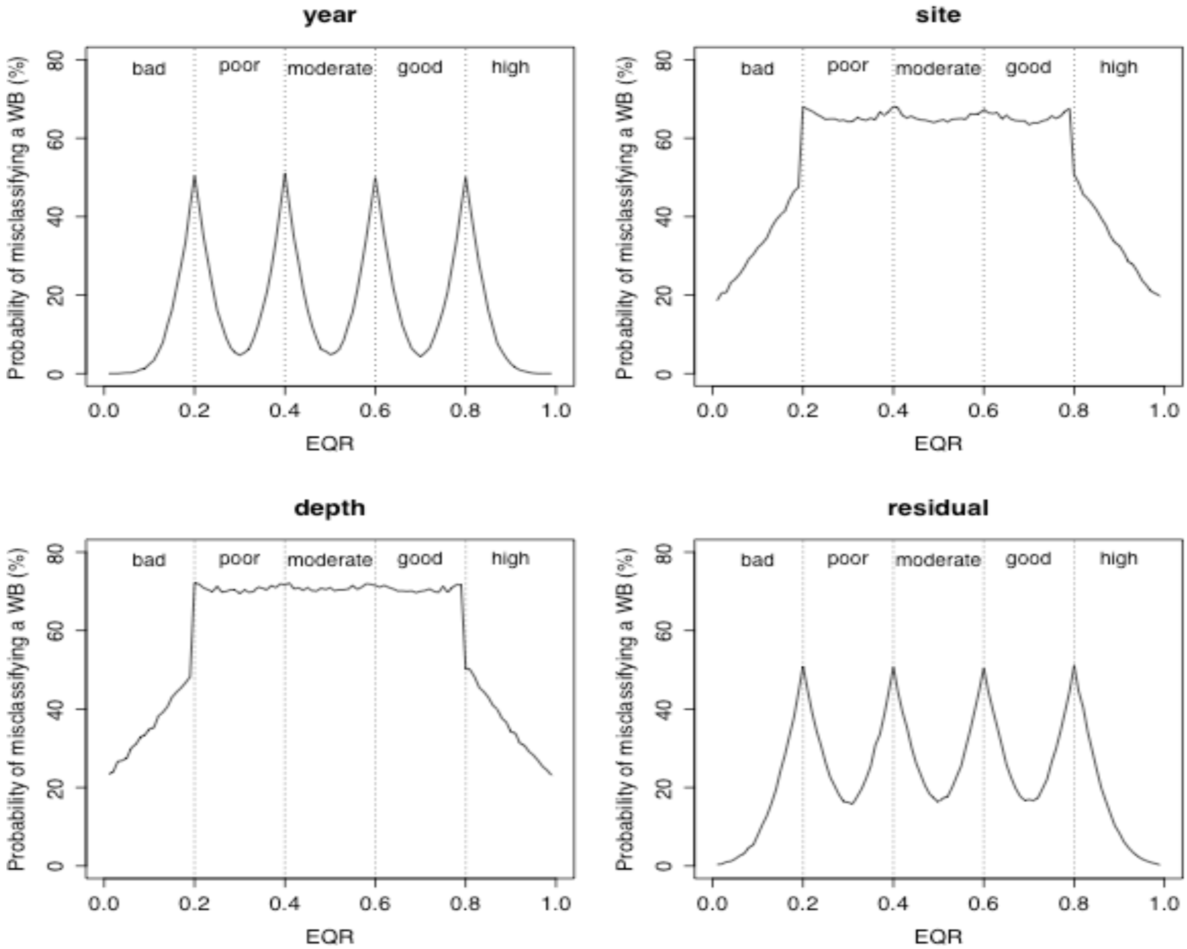

Figure 8. Probability of misclassifying the ecological status associated to the different factors analysed for the EI. Vertical dashed lines represent the boundaries of each status class. Bad = EQR values from 0 – 0.2; Poor = 0.21 – 0.4; Moderate = 0.41 – 0.6; Good = 0.61-0.8 and High = 0.81 – 1.

Seagrass Quality Index (SQI).

The Seagrass Quality Index (SQI) was improved during the WISER project. Although it has been initially tested during the intercalibration phase 1 (IC1), it suffered important changes in order to be completely compliant with WFD requirements. The basis of the SQI can be found in Foden and Brazier (2007) and in Foden and de Jong (2007), with the UK and NL seagrass methodologies. Those methods were based on structural seagrass parameters such as taxonomic composition, bed extent (m^2), and shoot density (ind m^{-2}), which compose presently the SQI methodology. The purpose of its development was to provide an assessment tool, based on seagrass, which could be used under the WFD perspectives. The SQI was tested against anthropogenic pressure and the uncertainty analysis allowed identifying the most sensitive points existing along the assessment process, from sampling to the EQR calculation. The development stage here presented corresponds to the version used in the IC2 (2008-2011).

Materials and methods

Biological data

A long-term data series from the Mondego estuary (Figure 9) was used to provide information on the basic structural parameters 'bed extent', 'biomass' and 'shoot density' of *Zostera noltei* meadows. Sampling was performed at the intertidal area of the south arm of the Mondego estuary, during low tide and using a manual corer (13.5 cm Ø). Samples were randomly collected inside the *Z. noltei* meadow to provide data on biomass and shoot density. The bed extent mapping was based on field observations (GPS to register the meadows perimeter), vertical photographs and GIS methodology (ArcView GIS version 8.3). Samples were collected with different periodicities along the study period (1986 to 2009). Depending on the study purpose they were collected from twice a month to a lower frequency of only one to three sampling events concentrated in the growing season. Samples were sorted in the laboratory, the shoots counted and the biomass determined as dry weight (g DW after weight stabilisation at 70 ºC).

The Mondego estuary (40º08'N, 8º50'W) is a southern Europe Atlantic system located at the western coast of Portugal (Figure 1). It's a shallow Transitional Water (TW, understood here as the same as estuary) classified as a mesotidal well-mixed estuary with irregular river discharges and included in the type NEA 11 in the WFD (2000/60/EC). The southern arm of the estuary, where seagrass meadows can be found, constitutes a subsystem with 7 km length, 0.5 km width, 2 to 4 m depth and 2.57 km^2 in area. The marine influence is strong, and the average tidal amplitude of 1 to 3 m allows up to 75% of this subsystem surface to be air exposed during low tide (Neto et al., 2010).

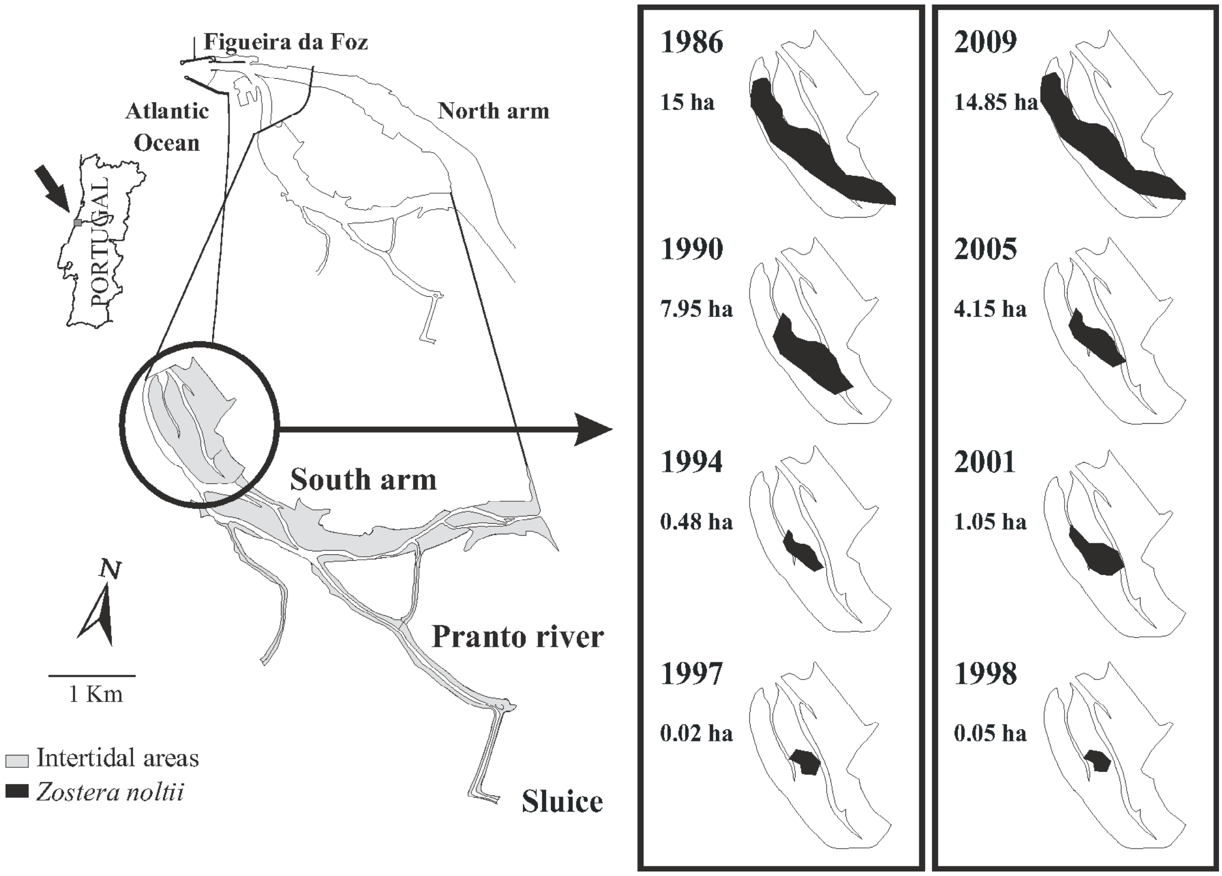

Figure 9. The Mondego estuary (40º08'N, 8º50'W). Sampling area in the south arm (circle on the left drawing) and the Zostera noltei bed extent along the study period.

Two distinct time intervals could be observed throughout the study period from 1986 to 2009. A first period goes until 1997 and is characterised by a general degradation process occurring in the south arm of the Mondego. A second period, from 1998 until 2009, is characterised by the implementation of several mitigation measures that resulted in the beginning of an ecological recovery process in the south arm. These two periods allowed following the differences already reported by other authors (Cardoso et al., 2008; Dolbeth et al., 2011) concerning the different responses provided by seagrass meadows for the same pressure, when declining or recovering.

Anthropogenic pressures

Following the proposal of Aubry and Elliott (2006), three categories of indicators were considered to assess the anthropogenic pressures in the sampling site: a) hydromorphological changes (represented by the 'land claim' and the 'shore line re-enforcement'); b) resource use change (represented by the 'maintenance dredging area and volume', 'maintenance disposal area and volume', 'other fisheries near shore disturbance', 'marina development' and 'tourism and recreation'; and c) environmental quality and its perception (represented by 'nutrients concentration' and 'natural turbidity'). The selected pressure indicators (Table 10) were the ones considered as potentially significant on influencing the quality of the seagrass meadows.

Table 10. Categories, indicators and criteria used to assess anthropogenic pressures in the Mondego.

Pressure			Scores					
Category	Indicator	Criteria	No change (0)	Very low (1)	Low (3)	Medium (5)	High (7)	Very high (9)
Hydromorphological changes	Land claim (ha)	Consider both: mudflats and tidal marshes. This indicator includes both anthropogenically induced changes (land claim) and natural variations, since the 1900-1950s or before big morphological changes occurred (since when trustful maps are available).	No change	<0.5 % lost	<1% lost	<5% lost	<10%lost	≥ 10% lost
	Shoreline re-enforcement (%)	Percentage of the shoreline or estuarine margin that suffered re-enforcement work.	No change	<5%	<30%	<60%	<90%	≥ 90%
Resource use change	Maintenance dredging area (ha)	The annually subtidal dredged area in relation to total area of estuaries (or WB).	No dredging	<1%	<10%	<30%	<50%	≥ 50%
	Maintenance dredging volume (tons)	The amount of material dredged annually from estuaries (1 m3 of sand dredged is equivalent to 2 tons).	No dredging	< 5000 tons	<100,000 tons	< 1 million tons	< 4 million tons	≥ 4 million tons
	Maintenance disposal area (ha)	The area designated for disposal in estuaries (or WB) or length affected by disposal (for tidal rivers) as suggested within the Water Framework Directive for the designation of Heavily Modified Water Bodies (HMWB).	No disposal	<1%	<10%	<30%	<50%	≥ 50%
	Maintenance disposal volume (tons)	Represented by the total tonnage annually disposed in estuaries.	No disposal	< 5000 tons	<100,000 tons	< 1 million tons	< 4 million tons	≥ 4 million tons
	Other fisheries nearshore disturbance	Percentage of the length of coast or estuarine (or WB) area affected by fishery.	No fishery activities	< 10%	<30%	<60%	<90%	≥ 90%
	Marina Development	The intensity of marina development is measured by the number berths / km2 of the WB.	No marina	< 100 berths / km2 WB	<150 berths / km2 WB	<300 berths / km2 WB	<500 berths / km2 WB	≥ 500 berths / km2 WB
	Tourism and recreation	Percentage of the length of coast (riverbank) or estuarine (or WB) area affected by tourism and recreation activity.	None	< 10%	<30%	<60%	<90%	≥ 90%
Environmental quality and its perception	Nutrients (μmol/L)	Quantified as the DIN winter median concentration (μmol/L)	< 6.5	< 10	< 30	< 60	< 90	≥ 90
	Natural turbidity	Measured as the mean secchi disk transparency (m) during growing season (May to September).	< 0.5	< 1	< 1.5	< 2	< 2.5	≥ 2.5

Anthropogenic pressures were quantified and then translated into a score, following the criteria shown in table 10. The pressures were then compared with biological parameters and the EQR (Spearman rank correlation) to infer about significant relationships between them. This analysis was performed inside the IC2 and allowed to select a smaller number of pressure indicators significantly correlated with the EQRs.

Metrics, reference conditions and the quality assessment method (SQI)

The Seagrass Quality Index (SQI) includes three different metrics: 1) taxonomic composition, as the number of taxa, 2) the bed extent, as the areal cover of the meadows, and 3) the shoot density, as the number of shoots per m^2 (Table 11). The reference conditions were estimated as: 1) the maximum no. of taxa ever recorded in the system (one species for the Mondego estuary); 2) the largest registered area occupied by the meadows with coverage density higher than 5% (15 ha for the Mondego) and; 3) the percentile 0.90 of the no. of shoots per m^2 registered in samples collected randomly inside healthy meadows (12,000 for the Mondego) (Table 11).

Table 11. Metrics used in the Seagrass Quality Index (SQI), reference condition for each metric, and the weight they have into the final EQR result. The available intertidal is considered as the area that is suitable for seagrass to grow and does not include occupations of several orders or saltmarsh area.

Metric	Reference condition (for the Mondego)	Weight in combination rule
Taxonomic composition	Maximum no. of seagrass taxa ever registered in site (1)	0.2
Bed extent	Higher measured value or 5% of the available intertidal area (15 ha)	0.3
Shoot density	Percentile 0.90 of shoot densities measured at meadow (12,000)	0.5

The deviation from the reference condition is calculated for each metric. The metric number of taxa scores a maximum of 5 when the number of taxa present matches the reference condition, and downgrade one score-point for each taxon lost. The metric scores one when no taxa are present anymore in the system, independently of the maximum possible number of species set as reference for the system. The bed extent is converted in a scale 0 – 1 by dividing the measured areal cover (ha) by the reference condition bed extent area. The shoots density follows the same process as the bed extent, and a result comprised between zero and one is then obtained. Except for the no. of taxa, the other metrics are scored in a continuous way inside the range 0 – 1. After this first round of calculations, the EQR is obtained through the use of the combination rule expressed in equation 1.

$$EQR = (TC/5)*0.2 + BE*0.3 + SD*0.5 \qquad \text{(Equation 1)}$$

where TC is the score calculated for the taxonomic composition, BE is the measure bed extent/bed extent reference condition, and the SD is the measured shoot density/shoot density reference condition.

An equidistant scale translates the EQR obtained into the EQS classes (Table 12).

Table 12. Boundaries for the EQR and correspondent EQS used in the Seagrass Quality Index (SQI).

EQR	EQS
0.00 – 0.20	Bad
0.21 – 0.39	Poor
0.40 – 0.59	Moderate
0.60 – 0.79	Good
0.80 – 1.00	High

Data analysis

Data on the structural parameters of seagrass were analysed along the study period. The response of the bed extent and biomass structural parameters to the different levels of pressure was also analysed, both towards degradation and after the implementation of the first (experimental) mitigation measures (1997/1998).

The response of the SQI method, which comprises the metrics 'bed extent', 'shoot density', and the no. of taxa, was also tested against the different pressure levels. The ability of the SQI in reporting into the five ecological quality classes (bad, poor, moderate, good and high) (WFD, 2000/60/EC) was also examined and compared to the pressure level acting at the moment.

Different combinations of categories of pressures (hydromorphological changes, resource use change, and the environmental quality and its perception) were tested to compare the response of the biological parameters and the EQRs against the pressure levels in the Mondego estuary. In the aim of IC2 was also verified the relationship of EQR provided by the SQI against the significant pressure index for several other Portuguese systems (results provided).

The correlation between biological data (metrics and the SQI EQRs) and the anthropogenic pressures (single pressure indicators, total pressure, hydromorphological pressure, resources use change, environmental quality) was tested through Spearman rank correlation analysis ($\rho<0.05$), with StatSoft, Inc. (2004) STATISTICA (data analysis software system), version 7.

Results

The evolution of the bed extent and the biomass in the estuary can be seen in figures 9 and 10. For both parameters, the slopes of the lines (Figure 2) are different for the periods before and after 1997. This year corresponds to the moment when the mitigation measures were implemented in the south arm of the Mondego.

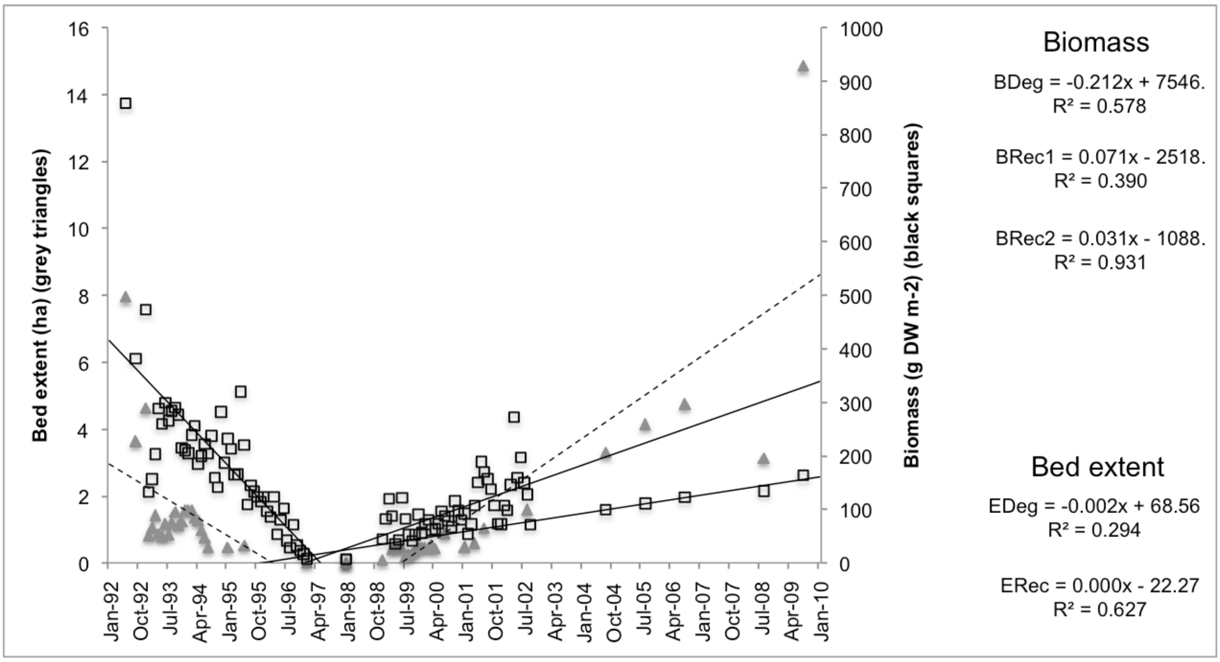

Figure 10. Plot of the structural parameters bed extent and biomass along the study period. Trend lines and equations for both periods, towards degradation and after implementation of mitigation measures, are also shown. BDeg = biomass inside degradation period; BRec1 = biomass inside recovery period 1 (1998 – 2002); BRec2 = biomass inside recovery period 2 (2003 – 2009); EDeg = bed extent inside degradation period; ERec = bed extent inside recovery period.

The correlation between the response of the metrics bed extent, shoot density and biomass, and the SQI methodology against the pressure varied (Table 13). The biological parameters (single structural metrics) were significantly correlated with the EQR calculated by the SQI. The total pressure, sum of all single pressure indicators, didn't show any significant correlation to any seagrass biology. Alone, the turbidity showed a significant correlation only with the biological parameter bed extent, but together with winter DIN (correspond to the environmental quality pressure category) they showed a significant correlation with all the biological parameters and the EQR.

Table 13. The Spearman rank correlation results. Significant values are in red (parameters not significantly correlated to any other are not shown).

	ZosteraShootDensity	ZosteraBiomass	SQI	TotalPressure	Env.QualPressure	WinterDIN	Turbidity
ZosteraBedExtent	0.93007	0.825175	0.958042	-0.259869	-0.633961	0.030535	-0.641941
ZosteraShootDensity		0.909091	0.965035	-0.292811	-0.592253	-0.129775	-0.42796
ZosteraBiomass			0.86014	-0.442876	-0.596424	-0.297719	-0.256776
SQI				-0.336732	-0.713206	-0.152676	-0.513553
TotalPressure					0.750949	0.675244	0.008145
Env.QualPressure						0.500829	0.419995
WinterDIN							-0.518141

The response of the EQR calculated with the SQI method against the environmental quality pressure (significant pressure category) is shown in Figure 11.

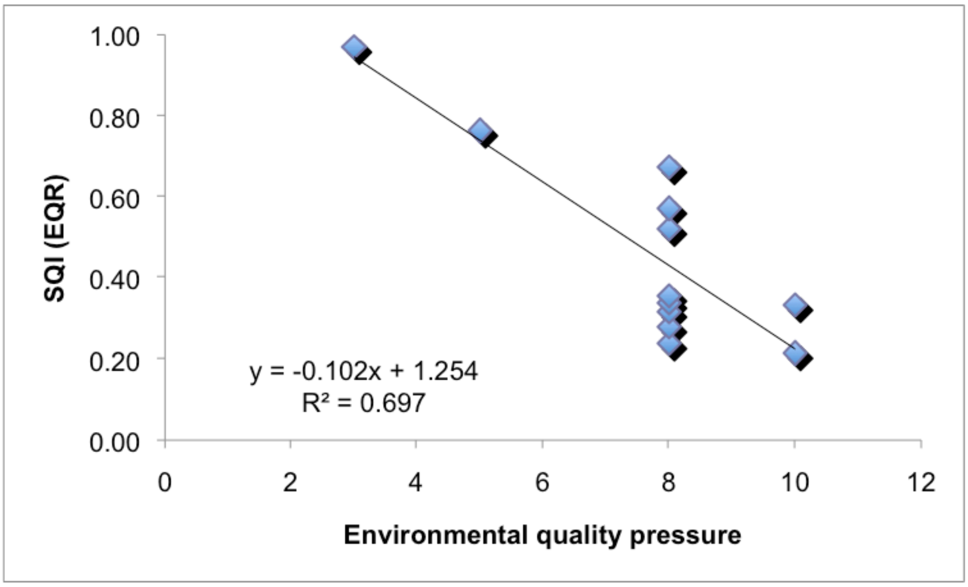

Figure 11. The response of SQI (EQR) (Y axis) against the environmental quality pressure (X axis).

With the intention to track the response of *Z. noltei* structural metrics along the degradation and the recovery pathways, the time series were 'folded' at the moment of the implementation of the experimental mitigation measures (1997-1998) and then compared as separate data series with the environmental quality pressure (Figure 12). The resulting pathways were different for degradation and for recovery processes. The bed extent recovery showed a similar track than for degradation, but biomass and shoot density parameters showed a trend line much more flat for recovery than for degradation. The EQR was somewhere in between these three metrics.

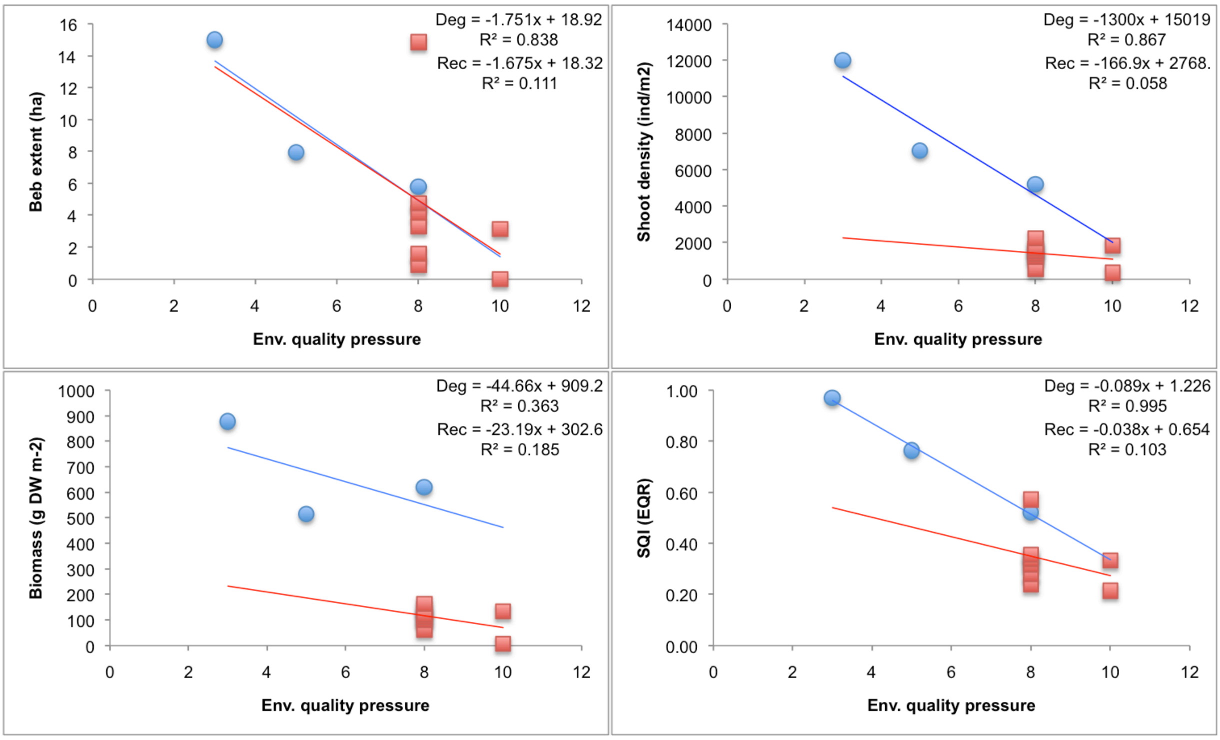

Figure 12. The response of bed extent, shoot density, biomass and SQI against the environmental quality pressure towards degradation (blue circles) and the recovery track (red squares) followed after the implementation of the mitigation measure in 1997.

Uncertainty analysis

All the factors analysed for this index displayed low levels of uncertainty. On the one hand, variability among samples was negligible and its corresponding risk of misclassification remained 0% all along the EQR range (Figure 13), indicating that the spatial heterogeneity displayed by these biological communities at this level was properly captured in the SQI sampling design. On the other hand, variability in the mean EQR scores among years and zones was also low, representing 3% and 5.8% of total variance and with a probability of misclassification associated that ranged from 0% to 50%. Even the residual term of the analysis, which accounted for a great proportion of total variance, presented a low risk of misclassification of 0% in the centre of a status class up to 50% at the boundary between classes (Fig. 13). By including more years and a greater number of water bodies in the analysis, it would be expectable to reduce, even more, the residuals and then to conclude irrefutably about the year effect in the quality results. Apparently, a low sampling frequency is anyway possible to do without a negative compromise of the assessment results.

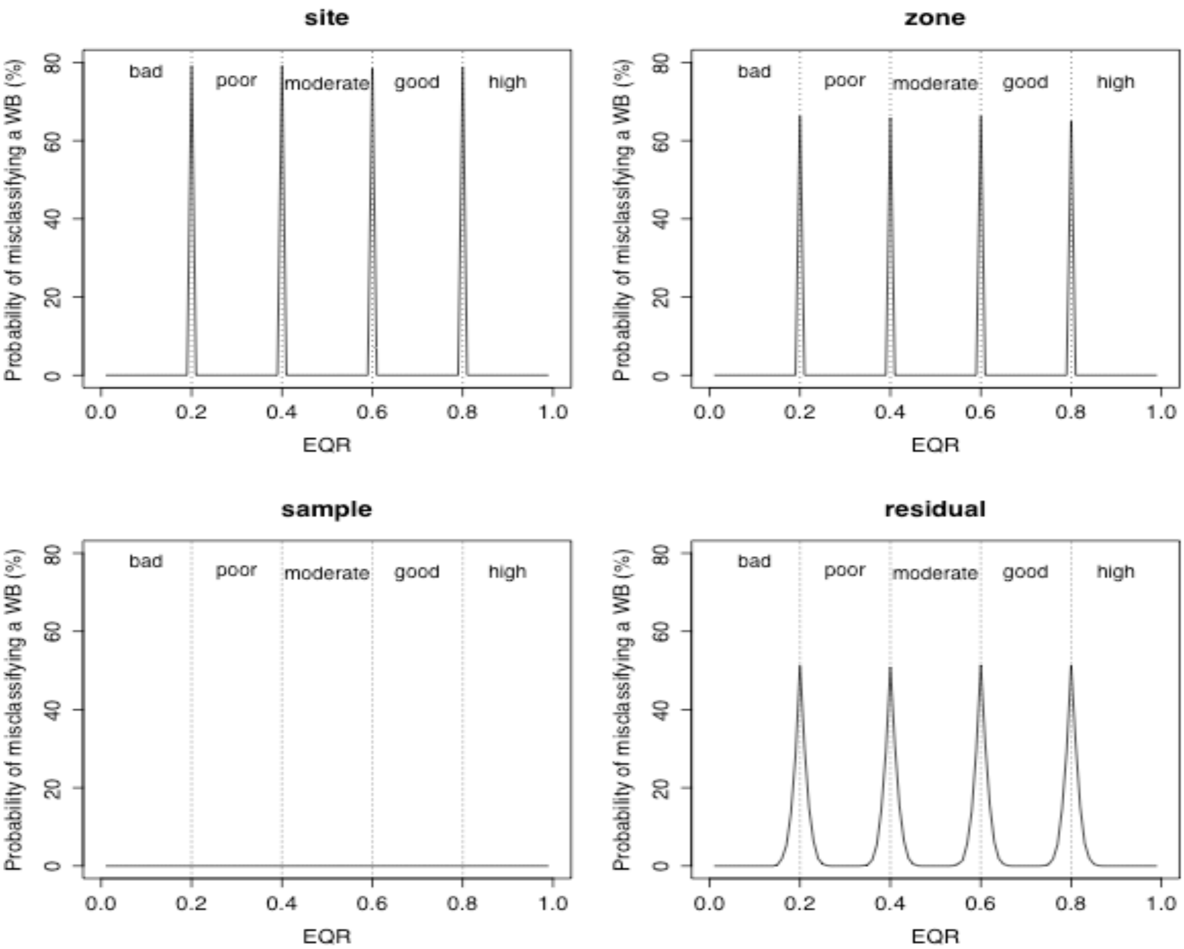

Figure 13. Probability of misclassifying the ecological status associated to the different factors analysed for SQI. Vertical dashed lines represent the boundaries of each status class. Bad = EQR values from 0 – 0.2; Poor = 0.21 – 0.4; Moderate = 0.41 – 0.6; Good = 0.61-0.8 and High = 0.81 – 1.

Discussion

The presented methodologies were developed in order to use macrolgae and/or seagrasses biological elements in the ecological status estimation of transitional waters in compliance with WFD requirements. For this, several issues should be covered to validate and to confirm the robustness of the methodologies here discussed. The relationships between biological metrics, EQR and the pressure registered in a system should be checked and their significant correlations proved. These objectives were primarily ensured by selecting sampling sites under the influence of different pressure levels, which allow the comparison of the biological data there assessed. The pressure gradients were either spatial or temporal, but in any case, provided sufficient data to perform the required consistent analysis for the different methodologies.

Lesina presented a nice spatial anthropogenic pressure gradient, where biological samples collected allowed the EEI-c to prove its robustness and possible use on TW assessments. The sampled sites enabled the analysis of the correlation of key structural and functional biotic indices of benthic macrophyte community with pressures and abiotic metrics. Evidently, there is a considerable difference between the behaviour of structural and the functional indices in the transitional waters as has been also indicated by others (Orfanidis et al., 2008). The functional indices are better indicating the pressures of the water systems and therefore the ecological status, while the structural indices are better indicating the confinement of the lagoon. It was possible to confirm the response of biological metrics to abiotic variation when using in TWs the EEI-c, a methodology based on structural and functional features of macroalgae and seagrasses already validated for coastal waters (Orfanidis et al., 2011). It was also possible to validate the response of the EQR values calculated by the EEI-c against important pressure indicators.

The structural diversity indices, as in other lagoons (Middelboe et al., 1998; Curiel et al., 2004; Orfanidis et al., 2008), were in general low. A decrease in macrophytes diversity from the entrance to the inner parts of estuaries and coastal lagoons (Coutino and Seeliger, 1984; Orfanidis et al., 2008) suggests either the existence of physiological stress due to strong salinity gradients (Coutino and Seeliger, 1984) or spore, fragment or propagule dispersal restriction (confinement) or interactions between them. Beside EEI the growth and dominance of late successional species (*Nanozostera noltii*, *Ruppia cirrhosa* and *Cystoseira barbata*) belonging in ESG I group has been restricted to less impacted areas. Input of nutrients and changes in light transparency are considered among the processes affecting the growth of sensitive members of ESG I in coastal and transitional waters (De Jonge et al., 2002). Under nutrient excess and turbid conditions, species composition shift from angiosperms to dominance of opportunistic and often bloom forming macroalgae (Viaroli et al., 2008). This may be due to the efficient nutrient assimilation of opportunistic macroalgae and their non-linear and self-accelerating response after crossing certain nutrient boundaries (Duarte, 1995).

Furthermore, opportunistic macroalgae demand lower light quantities for growth than rooted angiosperms (Hemminga and Duarte, 2000). Under oligotrophic and highly transparent conditions angiosperms take advantage over seaweeds by using nutrients from the sediment (Hemminga and Duarte, 2000). Other factors that can trigger this switch e.g. hydrographic change, grazing etc. cannot be excluded, especially when interactions with other stressors are considered (Cloern, 2001).

Concerning the Varna lake, it is well documented and proved in literature from many years (Rozhdestvensky, 1992; Stoyanov, 1991; Shtereva et al., 2004; Dencheva, 2010) that this is a very eutrophicated and polluted ecosystem and both biotic and abiotic parameters advocate for worse conditions. These contaminated waters enter the bay and the main current in south direction contributes to the deterioration of this part of the bay. Data collected in the bay allowed analysing the conditions of applicability of the EI index into assessment programs in the scope of WFD. It is clear that EI index and ESG groups have a good correlation with environmental parameters and pressures. Especially ESGII group (tolerant, opportunistic species) which plays a very important role in this direction. It is understandable, because of the poor conditions registered for the investigated ecosystem. Spearman rank correlation gives more strong evidence about the connection between biotic parameters (ESGI, ESGII and EI-EQR), pressures and abiotic indicators for ecological conditions (turbidity, oxygen saturation, organic content) and distance to the pressure. The moderate environment in north part of the bay (VB5) could be explained with lower pressures volume and lower concentrations of phosphorus and TOC in this zone. Dominant oligotrophic species, *Cystoseira barbata,* of ESGI group has been found just in this restricted area of the bay.

Varna bay study sites were classified as bad and moderate quality status. That is in conformity with biotic and abiotic indicator values, established in the studied system. Dominance of *Cystoseira barbata* sensitive species is in accordance with the fact that in better conditions sensitive species prevail. In Varna lake and Veteran bad conditions (high turbidity increased organic content, nutrients, oxygen depletion) favour growth of tolerant species as *Cladophora*, *Ulva*, *Ceramium*, Bacillariophyta. The species number is low in Varna lake. In 1988 year *Cystoseira barbata* was registered in VL2 site (Dencheva, unpublished data). The disappearance of this oligosaprobic, sensitive species does not give reason to define restoration process in the investigated ecosystem. Functional indices are more sensitive indicators of anthropogenic impact in contaminated transitional waters than structural indices (Zaldivar et al., 2008). That could be seen in results, obtained from correlations of biotic and abiotic parameters.

For the Mondego estuary the pressure gradient was more temporal than spatial. Data covering a first period where the seagrass meadows were under degradation and a second one where its recovery process was ongoing, allowed to compare the response of the structural metrics of *Z. noltei* for both situations. During the first period eutrophication symptoms were observed in the system and *Z. noltei* presented a severe reduction in the bed extent and biomass. The shoot density and the EQR that resulted from the SQI application also showed the same decreasing tendency. For all the structural parameters, but the bed extent, the recovery was not as fast as the degradation, which is normal for restoration of aquatic systems. The responses of bed extent, biomass and shoot density against pressure levels registered in the estuary were significantly correlated. The SQI produced EQR values also significantly correlated with the anthropogenic pressure (environmental quality pressure) affecting the system.

The EQR results show that the SQI performs well when used to assess the quality of seagrass meadows in estuarine systems. The SQI reported EQR values in all quality classes, and showed a good correlation with the pressure values. Although the good relation observed for the EQR and the pressure level, is too much evident that the recovery of the system didn't follow exactly the same track as the degradation process. This way is apparently slower and the stability of the basic parameters in a minimum level is important for recovery to proceed. The *Z. noltei* meadows achieved cover areas close to the ones from the 1980s, 15 ha, away from the 200 m^2 registered in 1997 (before the experimental mitigation). Even though we are in presence of a fast response seagrass species, the response for some parameters (e.g., biomass) registered a considerable delay. The bed extent is registering similar occupations as the ones registered for the seagrass in the decade of the 1980s.

The results obtained for the uncertainty analysis showed the fragile points where the different methodologies can be reinforced in order to improve the environmental assessment. Our results showed that spatial scales of variability (e.g., above and below the water body scale) have different influence in the ecological classification status of water bodies depending on the index. Generally for all factors, the probability of misclassification peaks when a site's observed EQR score is very close to the boundary between two status classes, usually around 50%. In contrast, when the observed EQR falls in the middle of a status class the probability of misclassification declines to the minimum. Probabilities of misclassification >50% may indicate that the associated variability is actually higher than the EQR range of the status class. The magnitude of these maximum and minimum uncertainty levels differ greatly among factors and indices as a result of the differences in the variance extracted. It is important to note that variability among WB, whilst important in the analysis of variance of components, is not particularly discussed here because in theory they can differ in their ecological status.

The uncertainty associated to EEI-c was negligible for the site level, indicating that the spatial heterogeneity displayed by these biological communities was properly captured in the corresponding sampling designs. The site scale is often used as a key spatial scale for species inventories and assessment in monitoring programs. In the other hand, the higher uncertainty linked to the replication factor may be an indication that a higher number of replicates should be collected. It has to be noticed that the sampling design implemented in Lesina Lagoon focused on both zoobenthos and benthic macrophyte quality elements sampled higher area but less replicates than normally suggested in coastal lagoons (Orfanidis et al., 2008). This situation is more evident for the lower quality classes, also due to the smaller width these classes have in comparison to the ones of higher quality. The risk of misclassifying the quality status of water bodies is also affected by the width of the status class in which the EQR score falls, as reported in Kelly et al. (2009), with narrower classes leading to greater probabilities of misclassification. Thus, indices in which the EQR range is not equally split into the 5 official classes (e.g., EEI-c) present, for a certain variance associated to a factor, changing uncertainty levels depending on the status class. This fact may have implications for bio-monitoring programs, because a greater sampling effort may need to be assigned to water bodies whose EQR score falls within the narrower status classes, in order to reduce their associated variability and increase the confidence of the classification. This is a rather expected result also because seaweed communities growing in degraded coastal lagoons have a higher variability than seagrass communities growing in less impacted sites of coastal lagoons. The residual variance in mean EQR values was high, determining that high levels of uncertainty remained (≥50%) almost along the whole EQR range. This could indicate that other sources of variability (not included in the analysis due to WISER constrains i.e. one sampling effort) may influence the water body classification obtained with the use of the EEI-c, for which further data need to be included in the analysis (e.g., zone, sample, year) in order to scrutinize the factor affecting more the risk of misclassification.

For the EI index, temporal variability presented relatively low levels of uncertainty, indicating that variability among years is properly captured in the monitoring program. In contrast, high levels of variability were observed in the mean EQR scores among sites and among depths. Their corresponding probability of misclassification was extremely high, with levels between 60 to 70% along the EQR range. These results suggest that a greater sampling effort must be assigned within sites and that depth should remain fixed in the monitoring programs in order to reduce the high levels of uncertainty associated to these two factors. The low residual variance indicates that all sources of uncertainty are represented in the monitoring program.

All the factors analysed for the SQI displayed low levels of uncertainty. The negligible variability among samples corresponds to a low risk of misclassification and indicates that the spatial heterogeneity displayed by these biological communities at this level was properly captured in the SQI sampling design. The low variability in the mean EQR scores among years and zones represents also a very low probability of misclassification. This indicates that the EQR scores of water bodies are fairly consistent throughout the years, for which the frequency of sampling could be decreased without greatly reducing the precision of ecological status estimates. Even the residual term of the analysis, which accounted for a great proportion of total variance, presented a low risk of misclassification. Apparently, a low sampling frequency is anyway possible to do without negatively affect the assessment results.

Conclusions

In a general way it's possible to say that the presented methodologies are in line with WFD requirements. The tools are in compliance with basic aspect such as the inclusion of metrics assessing the taxonomic composition and the abundance. The relationship of EQR values produced by the presented indices and the pressure registered for the sites was also proved to exist. For EEI and EI, it was demonstrated a high correlation with total pressures confirming that functional indices are indicators susceptible of accurately reflecting the ecological quality status of transitional waters, as required by the WFD. For the SQI, also the EQR-pressure relationship was proved to exist. This index is highly correlated with the metrics it included and is able to report for all quality classes.

The uncertainty study is in line with one of the main objectives of the WISER Project, helping to increase insight into the robustness and reliability of some of the ecological status classification methods proposed for European waters under the WFD. Applying uncertainty analysis to extensive bio-monitoring datasets, we have been able to detect the main weaknesses of these indices and provide robust foundation for improving their monitoring programmes, as well as guide decisions in future management plans. Besides, this study highlights the importance of extensive data series, essential to improve the methodologies proposed to assess the ecological status of coastal and transitional ecosystems under the WFD.

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