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Selection of multiple seagrass indicators for environmental biomonitoring

Begoña Martínez-Crego[1,*], Adriana Vergés[1], Teresa Alcoverro[1], Javier Romero[2]

[1]Centro de Estudios Avanzados de Blanes, C/ Acceso a la Cala St. Francesc 14, 17300 Blanes, Girona, Spain
[2]Departamento de Ecología, Facultad de Biología, Universidad de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain

ABSTRACT: The need to monitor the environmental condition of ecosystems worldwide has resulted in a large number of potential bioindicators being proposed in the scientific literature. However, only a few have been validated at an adequate scale to monitor environmental problems and to solve management questions. Here we compiled a list of candidate seagrass indicators (n = 59) obtained from the literature. We empirically validated them on a temperate seagrass ecosystem (*Posidonia oceanica*) across a wide anthropogenic gradient ranging from undisturbed to severely disturbed sites. We discarded about 75% of the candidate indicators because of their lack of sensitivity at the relevant spatial scale for biomonitoring (i.e. 10s of km against 10s of m) or across the environmental quality gradient. This illustrates the need for a careful validation of indicators prior to their use in monitoring programmes. Bathymetric variability strongly influenced indicator responses to the quality gradient. Deep meadows responded more clearly to differences in environmental quality, whereas shallow meadows were more influenced by natural sources of variability such as herbivory and physical disturbances. The 16 indicators unequivocally related to the environmental status gradient were representative of physiological, biochemical, individual, and population levels of biotic organisation. Their combination was necessary to cover the entire environmental gradient and to reflect the multiple anthropogenic disturbances causing the gradient. The selection process of indicators described here is an important step that needs to take place before the integration of these indicators to extract ecologically relevant information useful for policy and management goals.

KEY WORDS: Bioindicators · Biomonitoring · Environmental status · *Posidonia oceanica* · Seagrasses · Multiple stressors · Coastal waters · Mediterranean Sea

INTRODUCTION

The increasing pace of human-induced environmental change worldwide has created a demand for effective bioindicators allowing its assessment and monitoring, and improving our understanding of its biological and ecological significance. However, the choice and combination of measurable, sensitive and integrative variables that adequately reflect these environmental alterations is still a challenge for the scientific community (Rice 2003). A bioindicator is an organism, a part of an organism or a set of organisms that contains information on the quality of the environment or a part of the environment (Markert et al. 1999). Monitoring the time-integrative responses of bioindicators is useful for tracking anthropogenic influences on ecosystems over space and time. To be useful, however, it is essential that the chosen indicators respond clearly and unequivocally to human-induced environmental degradation at scales relevant to management objectives.

Due to the basic problem of scale in ecology (sensu Levin 1992), many indicators that are unequivocally related to environmental changes at a given scale can become unusable when applied at other scales. Since most habitats are spatially heterogeneous, sampling at small scales does not always directly scale up (Schneider et al. 1997). Consequently, indicators that have

*Email: bego@ceab.csic.es

been validated using manipulative or correlative approaches in only one location may not be useful for biomonitoring programmes that are usually deployed at much larger scales in several locations (e.g. kilometres or tens of kilometres apart; see Morrisey et al. 1992). Additionally, multiple sources of variation (natural and anthropogenic) may interact at large scales, often causing confusing results (Norkko et al. 2006). It is therefore important to carry out a preliminary assessment of an indicator's variability at a range of scales along an environmental gradient, and to specifically test the scale of interest (e.g. between-site variation) against smaller-scale variations using nested sampling designs (Morrisey et al. 1992, Niemi et al. 2004).

The extent to which natural gradients may affect the indicator adequacy is another critical consideration. Since communities vary along natural gradients (e.g. light or temperature gradients, see Margalef 1998), a sampling design should reduce to a strict minimum the contribution of such natural gradients to the variability of the measured attributes (Markert et al. 1999). Moreover, the interaction between natural and human factors should be examined (Norkko et al. 2006), and sampling performed where (and when) indicators show a response to alterations separable from responses to natural causes. For example, in relation to marine plants, depth has large effects on most physiological, morphological and structural parameters (Cooper & DeNiro 1989, Alcoverro et al. 2001a), and these effects should be clearly understood in designing and interpreting monitoring.

Because of the complexity of biological systems, their inherent high variability, and the influence of multiple environmental factors or stressors, the search for indicators should not be confined to only one level of biological organisation (Niemi et al. 2004). The effects of stressors on the biota can be studied at different biological levels, ranging from the metabolism of a single organism to complex communities. The response time of indicators to stressors generally increases with the structural complexity, while their specificity decreases (Adams & Greeley 2000). Therefore, a multi-level approach provides a more complete understanding of both lethal and sub-lethal effects of stressors, and helps in the interpretation of complex environmental gradients where multiple types of impacts interact (Harding 1992, Adams 2005).

The requirements to be ideal biological elements from which to obtain bioindicators are clearly fulfilled by seagrasses (Orth et al. 2006). These marine flowering plants are ecosystem engineer species (sensu Wright & Jones 2006), and are found widely distributed in shallow coastal waters around the world except Antarctica (Spalding et al. 2003). They are extremely sensitive to changes in their environment, such as availability of light (Longstaff & Dennison 1999) and nutrients (Udy & Dennison 1997) and, in particular, to human-induced disturbances (Walker & McComb 1992), which have resulted in seagrass losses reported worldwide (Orth et al. 2006). All of these reasons have led to the identification of seagrass meadows as a benchmark of overall environmental health of aquatic systems by various governments and institutions worldwide (Council of Australian Governments Water Reform Framework of 1994, in Australia and New Zealand; Water Framework Directive 2000/60/EC, in the European Union; Clean Water Act of 1972 and Endangered Species Act of 1973, in the United States). Moreover, a large body of research has focused on seagrass biology and ecology, and on seagrass responses to different stressors or impacts at different levels (physiology, population dynamics, trends in community composition; see Table 1). This provides an excellent scientific basis to use these organisms and their associated ecosystems as indicators for assessing human-induced environmental changes, in systems where these species occurred or have occurred in the past.

In this study, we performed a selection process of optimal seagrass indicators for biomonitoring the environmental status of coastal waters. Firstly, we collated a list of ca. 60 candidate indicators based on previous knowledge of specific responses of seagrass ecosystems to diverse stressors at different levels of the biological organisation. Secondly, we empirically validated and tested their indicator value on a temperate seagrass ecosystem (*Posidonia oceanica*) along an existing and documented environmental status gradient (independently assessed) within a relatively large geographical scale (ca. 500 km). We used a nested hierarchical design to test the effects of spatial scale at 2 different depths, and we applied multivariate techniques to explore the behaviour of candidate indicators in a continuous way, to detect redundancy between potential indicators, and to verify that the combined suite of selected indicators behave together as expected along an environmental gradient. Additionally, we tested whether any of the descriptors selected individually discriminated between the entire gradient of environmental quality.

MATERIALS AND METHODS

List of candidate indicators. The preliminary list of candidate indicators was based on an exhaustive bibliographical review, from which we identified 59 seagrass attributes at different levels of biological organisation. All attributes were sensitive to environmental changes, and their response to environmental deterioration is well documented (summarised in Table 1).

Table 1. Preliminary list of seagrass descriptors with potential indicator value and supporting reference(s) of the expected responses to increasing human-induced environmental stressors (↑ : increase; ↓ : decrease). References are listed in the additional literature cited list at the end of the article. Since studies are unable to discern which factor (light reduction, nutrients or organic matter increases) or interactions lead to the effects caused by aquaculture activities, the effect of this activity is shown separately

Biotic level and descriptors	Expected response to increasing anthropogenic disturbances and reference(s)					
	Light deprivation	Nutrient inputs	Metals	Organic matter/ anoxia	Mechanical/ Sedimentary disturbances	Aquaculture
Physiological and biochemical level						
N and P content in seagrass tissues[a]	↑ 73, 89, 67, 60	↑ 37, 87, 85, 36, 5, 23, 86, 60, 58				↑ 37
Free amino acid content in seagrass tissues	↑ 46	↑ 37, 84, 87, 85, 36, 86	↓ 72			↑ 37
C content, and carbohydrate reserves in seagrass tissues	↓ 73, 47, 2, 74, 4, 67	↓ 84, 36, 5		↓ 35	↓ 6	↓ 75, 15
δ^{13}C in seagrass tissues	↓ 46, 29, 1			↓ 34		
δ^{15}N in seagrass tissues		↑ 37, 29, 57, 56, 12, 92/↓ 87, 85, 61, 86			↑ / ↓ 6	↑ 90
δ^{34}S in seagrass tissues			↑ 11	↓ / ↑ 65, 25		
Trace metals in seagrass tissues			↑ 72, 11, 8, 21, 69, 22, 55, 77, 70, 49			↑ 71
Individual level						
Plant morphological descriptors (e.g. shoot biomass no. of leaves, leaf length)	↓ 73, 46, 74, 78, 28, 64, 89/ ↑ 80	↑ / ↓ [b]87, 42, 80, 66, 89, 44, 33	↓ 13/ ↑ 55	↓ 19, 35	↓ 41, 10, 59/ ↑ 52	↓ 75, 15, 18/ ↑ 68, 16, 71
Shoot necrosis		↑ 5, 88	↑ 48, 50	↑ 82		
Population level						
Shoot density and meadow cover	↓ 73, 46, 74, 80, 28, 67	↓ 80, 38, 79, 7 [c]		↓ 82, 35	↓ 24, 79, 7, 41, 30, 51, 6, 10, 59	↓ 75, 15, 68, 9, 14, 71, 18
Rhizome growth type (plagio/ortho)					↑ 24, 26	
Rhizome baring					↑ 24, 53, 52, 17	↓ 54
Community level						
Leaf epiphyte biomass	↓ 73, 64, 60, 83	↑ 31, 40, 91, 41, 63, 62, 3, 81, 44, 83	↑ 55/ ↓ 68			↑ 15, 68, 16, 9, 71
N and C content in leaf epiphytes	↓ 39	↑ 45				
Herbivore pressure		↑ 76, 41, 33, 27				↑ 75, 68, 14, 71, 18

[a] The ratio leaf N / leaf mass has been described as a sensitive indicator of early eutrophication (43)
[b] ↑ / ↓ : depending on the existence of growth limitation by specific nutrients (20) or/and on the presence/absence of grazers (32)
[c] Indirect effect of shading due to phytoplankton or epiphyte overgrowth enhanced by nutrient enrichments has been suggested as a major likely cause of seagrass (density and cover) losses (63, 81, 31, 83)

Study area and anthropogenic pressure gradient. The study was conducted along the Catalan coast (ca. 500 km of coastline) in NE Spain (42° 19′ N, 3° 19′ E to 41° 02′ N, 1° 00′ E, Fig. 1). This area is densely populated, with ca. 4.5 million people living in coastal municipalities, and suffers a strong tourist pressure, as more than 20 million tourists per year visit it, most of them in summer. Human pressures are unevenly distributed; beach regeneration, big harbours, large cities and main industrial areas (Barcelona and Tarragona) are localised in the central part, and are uncommon in the northern and southernmost coasts. Other anthropogenic pressures are agricultural practices outside of metropolitan areas, the discharge of the main river (Ebro) along the southernmost coast, and fishing mainly off the northern coast. Healthy sites, including some marine protected areas, can be found on the northern coast.

We sampled sites encompassing the maximum range of environmental quality in the area. To this end, the status of potential sampling sites was first determined

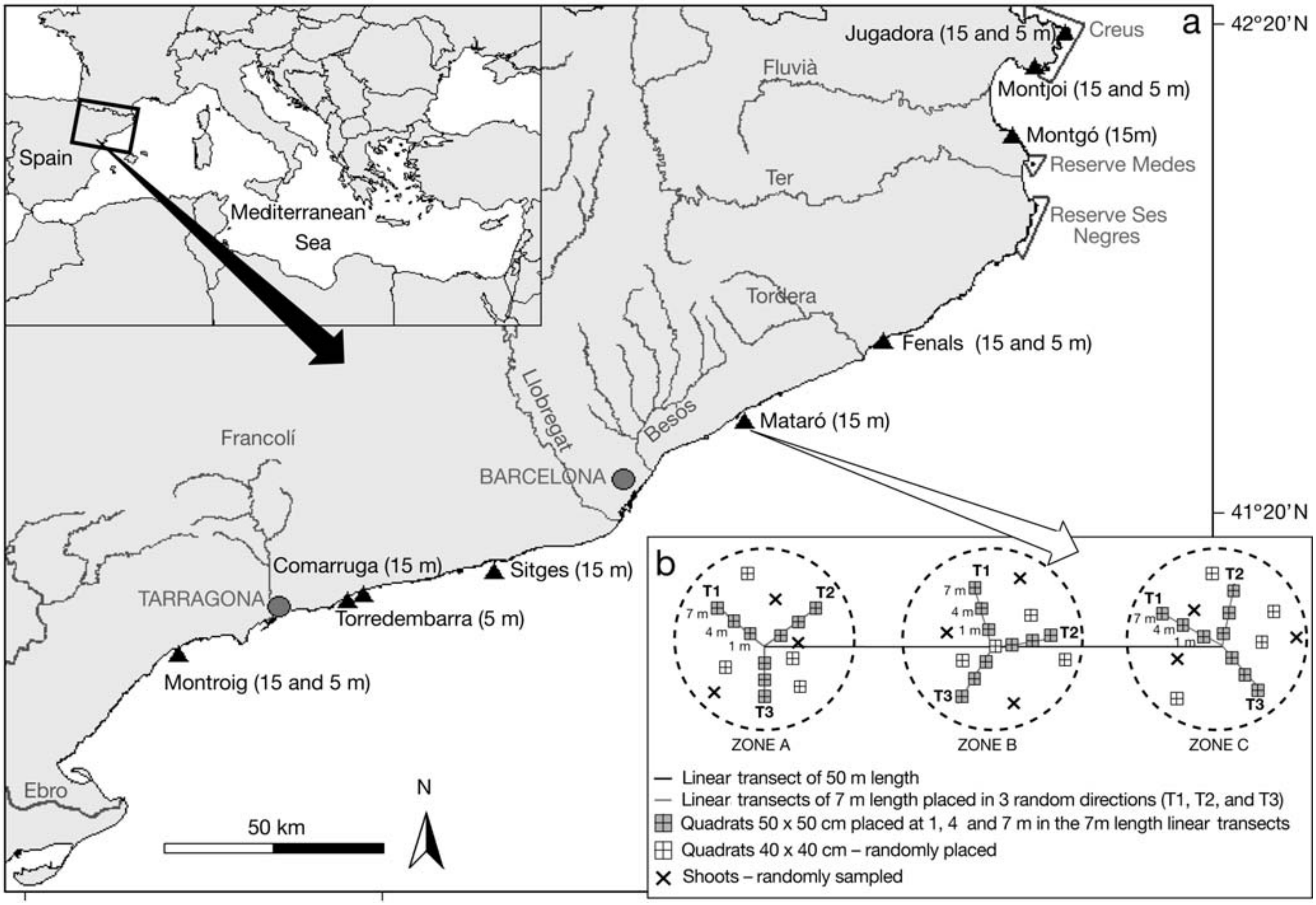

Fig. 1. (a) Sampling sites (black triangles) along the Catalan coast (NE Spain), where main rivers, larger cities (grey circles), and marine protected areas (grey outlines along the northeastern part of the coast) are shown. (b) Sampling design to test the spatial variability within each sampling site. Three circular zones (A, B and C) of ca. 300 m^2 were marked to obtain both *in situ* measurements (quadrats of 50 × 50 cm for cover and 40 × 40 cm for shoot density) and shoots (only 3 are shown in the scheme for the sake of clarity)

based on independent available information. Data on chlorophyll (chl) *a* and water transparency (Secchi readings), algal bioindicators (cartography of littoral and upper-sublittoral rocky-shore communities, CARLIT), and global pressure from human activities were compiled and used to classify potential sites into 3 categories (Table 2): (1) healthy (not or slightly disturbed), (2) intermediate (moderately disturbed), and (3) unhealthy (severely disturbed). According to this, we chose 9 sites as representative of these 3 categories (Table 3). The minimum and maximum spatial distances between 2 adjacent sites was 5 and 70 km, respectively, and the distance between the northernmost and the southernmost sites was 360 km (Fig. 1).

Table 2. Range of values for the different criteria used for the environmental status assessment of sites. Physical-chemical parameters are from Vila et al. (2005); CARLIT cartography of littoral rocky-shore communities from Ballesteros et al. (2007); anthropogenic pressure from Agència Catalana de l'Aigua (2005). NA: not applicable

Environmental status	Environmental status value	Physico-chemical parameters Chl *a* range (µg l^{-1})	Secchi range (m)	Algal bioindicators CARLIT range	Anthropogenic pressure
Healthy	1	≤ 2	> 12	> 0.60–1	Not significant
Intermediate	2	> 2–4	12–10	> 0.40–0.60	NA
Unhealthy	3	> 4	< 10	0–0.40	Significant

Table 3. Values for the different criteria used and classification of the sites under study into the 3 environmental status classes based on Table 2. Physico-chemical parameter values (Camp et al. 2001) were calculated by averaging sampling points near each meadow (averaged available data from 1994 to 2001). Areas with a clear continental influence (river discharges) and/or heavily polluted sites (harbours) were not included in the calculations, since *Posidonia oceanica* meadows are not present in these areas. Cartography of littoral rocky-shore communities (CARLIT) values (Ballesteros et al. 2001) were calculated using municipality data from 2001. The categorisation of the anthropogenic disturbances (Agència Catalana de l'Aigua 2005) was performed in only 2 classes (non-significant and significant), and values of 1 and 3 were respectively assigned to them. This assessment was performed prior to sampling

Site, abbreviation	Chl *a*	Secchi	CARLIT	Anthropogenic pressure	Average status	Environmental status
Jugadora (5 m and 15 m), Jug	1	1	1	1	1	Healthy
Montjoi (5 m and 15 m), Mjoi	1	2	1	1	1	Healthy
Montgó (15 m), Mgo	1	1	2	1	1	Healthy
Fenals (5 m and 15 m), Fen	1	1	1	3	2	Intermediate
Mataró (15 m), Mat	1	2	2	3	2	Intermediate
Sitges (15 m), Sit	2	3	3	3	3	Unhealthy
Comarruga (15 m), Coma	2	3	2	3	3	Unhealthy
Torredembarra (5 m), Torr	1	3	1	3	2	Intermediate
Montroig (5 m and 15m), Mrig	1	2	2	1	2	Intermediate

Sampling design and data acquisition. From the 9 sites, we selected 8 deep meadows (15 m) and 5 shallow meadows (5 m, Fig. 1a). The absence of shallow meadows in unhealthy sites prevented us from examining the whole quality gradient at both depths. At each meadow, sampling was performed by SCUBA diving using 2 nested levels of spatial replication. Three ca. 300 m^2 circular zones (10 m radius) were chosen at random along the same isobath, with the centre of the zones separated at least 25 m from each other. Within each zone, we obtained replicate samples to evaluate the following variables: (1) shoot density, rhizome growth type, and rhizome baring, which were measured in four 0.16 m^2 (40 × 40 cm) quadrats randomly placed; (2) meadow cover, which was estimated in nine 0.25 m^2 (50 × 50 cm) quadrats placed 3 m apart from each other along linear transects positioned in 3 random directions; and (3) 12 shoots, which were randomly collected for all variables requiring laboratory analysis (Fig. 1b).

We performed all sampling in the shortest possible interval (1 mo; October 2001), to avoid the masking effect of seasonal variability (Ward 1987, Alcoverro et al. 1995). Samples were stored and treated as required using common methods reported elsewhere (Table 4). We obtained at least 3 replicate measurements for each descriptor in each sampling zone, except for free amino acid content, which was only measured in a single sample per zone (no estimate of small-scale variability) due to analytical constraints.

Data analysis. To examine the adequacy of candidate indicators, we first used a 2-way nested analysis of variance (ANOVA) to test the variability at the scale of interest (i.e. between-site variation) against smaller-scale variations. We partitioned the variance of each measured descriptor into differences between sites (i.e. along the environmental status gradient, fixed factor), differences between sampling zones (medium-scale spatial variability, random factor, nested in site) and within sampling zones (small-scale spatial variability, error term), and assessed their significance. To avoid the masking effects of depth, and due to the unequal number of sampled meadows, we analysed data for deep and shallow meadows separately. Prior to the analyses, the dependent variables were tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Cochran tests, respectively. We found departures from normality and homoscedasticity in some of the variables analysed. However, the large number of cases used led us to consider ANOVA to be robust enough to allow departures from these assumptions (Underwood 1997). ANOVAs were performed using STATISTICA v.7 software. After these analyses, we only retained the descriptors for which differences among sites were significant.

Secondly, we applied multivariate techniques to explore the behaviour of variables in a continuous way. We used a principal components analysis (PCA; Hotelling 1933) with the descriptors retained after the first step (ANOVA, see above) to identify common trends of continuous variations among descriptors, their correlation with environmental status, and to evaluate redundancy. Since our descriptors were not dimensionally homogeneous, we computed the principal components from the correlation matrix. Data of some descriptors (amino acid content, isotopic signatures, fish bite marks, baring and rhizome growth type) were not available for 1 site (Torredembarra); this site was therefore added as a supplementary object in the PCA. As the first component clearly discriminated

Table 4. Standard methods used to obtain *in situ* measurements and to process samples in the laboratory

Descriptor (units)	Sample size per zone (site)	Standard method
Physiological and biochemical level (plant descriptors)[a]		
C, N and P concentrations (%DW)	3 (9) Leaf 2[b], and rhizomes (for P)	CNH elemental analysis using Carlo-Erba autoanalyser (for CN), or ICP[c] after acid (HNO_3 / H_2O) digestion at 100°C, 24 h (for P)
Free amino acid content (total FAA, Asn, Ser, Pro, Arg, Gln, Ala, Asp, Val, Lys, His, Thr, Glu and Cit in µmol g^{-1} FW)	1 (3) Rhizomes	Ionic exchange chromatography after extraction from 0.5 g of frozen tissues (–80°C) ground in 20 ml of 0.05N HCl, centrifuged 5 min at 10 000 rpm, and filtered (supernatant) using low-binding regenerated cellulose Millipore ultra-free filters (Invers et al. 2002)
Soluble carbohydrates (mainly sucrose) contents (%DW)	3 (9) Rhizomes	Extracted from 0.05 g DW solubilised in hot EtOH (80°C), and centrifuged at 4500 rpm (4 times). EtOH was evaporated to dryness under an N_2 stream, extracts were redissolved in distilled water and analysed spectrophotometrically (λ = 626 nm) using anthrone assay standardised to sucrose (Alcoverro et al. 1999, 2001b)
Isotopic ratio $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ (‰)	3 (9) Leaf 2[b] and scales[d]	EA-IRMS[e] (for $\delta^{13}C$, $\delta^{15}N$) and IRMS (for $\delta^{34}S$)
Metals (Fe, Mn, Zn, Cu, Ni, Pb, As, Cr in µg g^{-1} DW)	3 (9) Leaf 2[b] and rhizomes	Optic ICP[c] (for Fe, Zn and Mn) or mass ICP[c] (for the rest) analyses after acid digestion of 0.1 g DW in 4 ml of HNO_3 / H_2O solution (3/1) at 100°C, 24 h (modified from Cai et al. 2000). The analytical procedure was checked using standard reference material (*Ulva lactuca*, CRM 279)
Individual level (plant descriptors)		
Number of leaves, maximal leaf length, and leaf width (cm)	3 (9) Shoots	Direct measurement of each shoot in the laboratory
Shoot biomass (g)	3 (9) Shoots	Drying of leaves without epiphytes at 70°C to a constant weight
Shoot necrosis (%)	3 (9) Shoots	Calculation (as percentage) after quantifying the number of leaves with necrosis for each shoot in the laboratory.
Broken leaves (%)	3 (9) Shoots	Direct observation for each shoot in the laboratory of the frequency (as a percentage) of leaf apex broken.
Population level (meadow descriptors)		
Shoot density (shoots m^{-2})	4 (12) *In situ* measurements	Shoot number was counted in a total of 12 quadrats of 0.16 m^2, randomly placed over ca. 1000 m^2 area, excluding zones with zero cover
Meadow cover (%)	9 (27) *In situ* measurements	Visual estimation in a total of 27 quadrats (0.25 m^2) as 1 of the following classes (for each sub-quadrat): 0, 10, 25, 50, 75 and 100% over a ca. 1000 m^2 area
Rhizome baring (cm)	12 (36) *In situ* measurements	*In situ* measurement of the distance between the sediment surface and the leaf base in 3 shoots per 0.16 m^2 quadrat
Rhizome growth type (plagio/ortho)	4 (12) *In situ* measurements	*In situ* estimation of an index from 1 (completely plagiotropic) to 0 (completely orthotropic) in each 0.16 m^2 quadrat
Community level		
Leaf epiphyte biomass (mg g^{-1})	3 (9) Shoots	Epiphytes were obtained by scraping the leaf surface with a razor blade, and weighed after drying at 70°C to a constant weight. Results expressed relative to shoot biomass.
Leaf epiphyte nutrients (%DW)	3 (9) Shoots	Carlo-Erba CNH elemental analysis of dried and finely ground samples of epiphytes obtained by scraping the leaf surfaces with a razor blade.
Herbivore (fish and sea urchins) bite marks (%)	3 (9) Shoots	Direct observation for each shoot in the laboratory of the frequency (as a percent age) of the leaf apex eaten by the fish *Salpa sarpa* or by sea urchins (Boudouresque & Meinesz 1982)

[a]Physiological and biochemical level descriptors were analysed from dried (70°C, to a constant weight) and finely ground samples, except FAA
[b]Physiological level descriptors vary with leaf age and are influenced by the presence of epiphytes. To avoid these sources of variability, the second youngest leaf in the shoot (without conspicuous epiphytes) was used
[c]ICP: inductively coupled plasma spectrophotometry
[d]Isotopic traces of dead sheaths (scales) corresponding to 1-yr-old and 5-yr-old tissue (determined lepidochronologically) were analysed to assess its 'memorisation' capacity (Pergent 1990)
[e]EA-IRMS: elemental analyser isotope ratio mass spectrometry

among healthy, intermediate and unhealthy deep meadows (see 'Results'), those indicators that had the highest correlation ($r \geq 0.70$) with component I were selected. Exceptionally, we included some descriptors of high ecological relevance or sensitivity to specific anthropogenic disturbances with $0.60 < r < 0.70$. Descriptors that weakly correlated with axis I were discarded.

To verify that the combined suite of selected indicators behave as expected along an environmental gradient, and discriminate together among healthy, intermediate and unhealthy sites, we performed another PCA including only the selected indicators and using the deep-meadow dataset. An additional PCA was used to evaluate the variability between the zones of the same site. The software used for all multivariate analyses was the GINKGO package (De Cáceres et al. 2007).

Finally, to identify whether any individual indicators are able to discriminate between the 3 discrete environmental statuses along the gradient, we performed a 3-way nested ANOVA that included environmental status (healthy, intermediate and unhealthy, fixed factor), site (random factor, nested in status) and zone (random factor, nested in site). When significant differences were detected, a 1-way ANOVA using only the fixed factor (status) was carried out in order to allow *a posteriori* pair-wise comparison of means using the Newman-Keuls test.

RESULTS

The partition of total variance into the different sources of variability (between-sites, between-zones and within-zones) largely differed between descriptors and sampling depths (Table 5, Appendices 1 & 2 in electronic supplement available at: www.int-res.com/articles/suppl/m361p093_app.pdf). In deep and shallow meadows, 22 and 24 descriptors showed no significant differences among sites, respectively, and were therefore discarded. Significant between-site differences ($p < 0.05$) existed for variables belonging to almost all levels of biological organisation, and these were retained for the multivariate analysis.

The first 2 components of the PCA, including data from shallow and deep meadows, explained 32% (component I) and 14% (component II) of the variance. Deep meadows showed relatively low scores on axis II, and their ordination along component I closely reflected their *a priori* defined environmental status (from healthy, left side, to unhealthy, right side; Fig. 2, Table 3). In contrast, shallow meadows had relatively low scores on component I, but their ordination along component II followed their *a priori* defined environmental status (Fig. 2). Descriptors that highly correlated with component I (see Fig. 3, and list below) were those the most clearly related to anthropogenic stressors. In contrast, descriptors that highly correlated with component II (e.g. herbivore bite marks, leaf length

Table 5. Summary of 2-way ANOVA results partitioning the variance into different spatial components (among sites, between sampling zones, and within sampling zones) for each candidate descriptor. Discarded descriptors were those showing significant variability 'Only between zones' or 'None'

2-way ANOVA Factor variability	Descriptors showing significant variability (total number of descriptors): Deep meadows	Shallow meadows
Only between zones	δ^{15}N in 5-yr-old scales, Ni in leaves and rhizomes, and sea urchin bite marks (4)	δ^{34}S in leaves; δ^{15}N in 1- and 5-yr-old scales; number of leaves; shoot biomass and density; rhizome growth type (7)
Only between sites	Asn, Ala, Ser, Pro, P, Fe, Mn, Cr, Cu, and sucrose in rhizomes; N, Mn, As, and Cu in leaves; δ^{13}C in leaves, 1- and 5-yr-old scales; δ^{15}N in leaves and 1-yr-old scales; leaf width; fish bite marks, broken leaves, necrosis; and shoot density (24)	Asp, Cit, P, Fe, Mn, Cr, Pb, As, Cu, and sucrose in rhizomes; C, Fe, Zn, Ni, Mn, Cr, As, Cu and P in leaves; δ^{13}C in leaves, 1- and 5-yr-old scales; δ^{15}N in leaves; leaf length and width; necrosis; fish and sea urchin bite marks; meadow cover, N in epiphytes (30)
Both site and zone	Fe, Zn, Pb, and Cr in leaves; δ^{34}S in 1-yr-old scales; shoot biomass, maximum leaf length; meadow cover, baring level, and rhizome growth type; epiphyte C, N and biomass (13)	N and Pb in leaves; Zn in rhizomes; C in epiphytes; δ^{34}S in 1-yr-old scales (5)
None	C and P in leaves; δ^{34}S in leaves and in 5-yr-old scales, Zn, Pb, As in rhizomes, FAA, Arg, Gln, Asp, Val, Lys, His, Thr, Glu and Cit in rhizomes, and number of leaves (18)	FAA, Asn, Arg, Gln, Ala, Val, Ser, Lys, His, Thr, Pro, Glu, and Ni in rhizomes; δ^{34}S in 5-yr-old scales; broken leaves; rhizome baring; epiphyte biomass (17)
Descriptors		
Discarded	(22)	(24)
Pre-selected	(37)	(35)

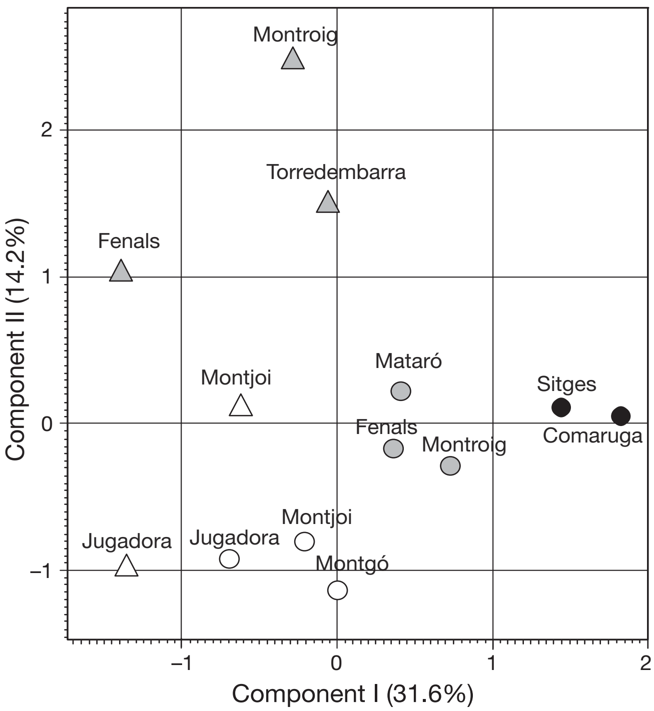

Fig. 2. Ordination diagram (object scores) of deep (circles) and shallow (triangles) meadows in the PCA performed using ANOVA-selected descriptors. Shading denotes the environmental status based on Table 3 as follows: white (healthy), grey (intermediate) and black (unhealthy)

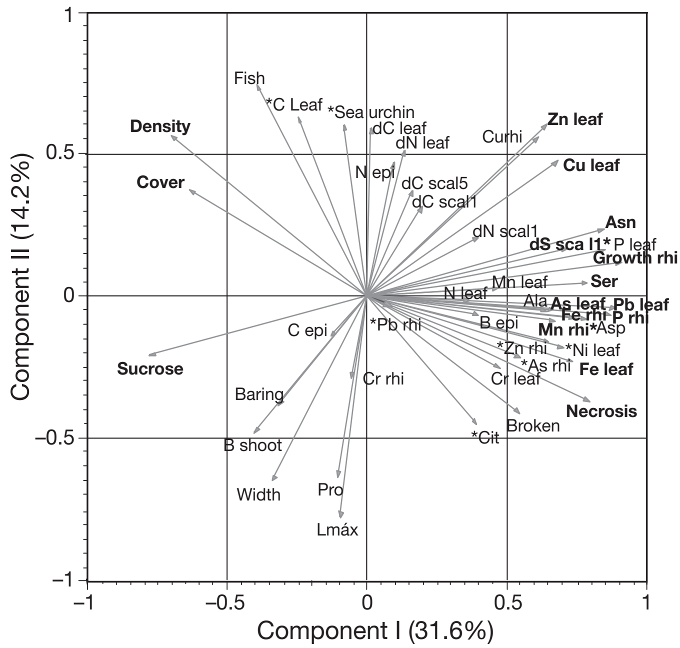

Fig. 3. Factor loadings on the first 2 principal components of the PCA performed using ANOVA-selected descriptors. Descriptors that highly correlated with component I, and that are therefore the selected descriptors, are highlighted in **bold**. The other descriptors were discarded as indicators because: (1) they did not correlate with component I but were highly correlated with component II (Fish, dC leaf, dN leaf, N epi, dC scal 1 and 5, Width, Lmax, Pro, and Cr rhi); (2) they did not correlate with either component I or II (Baring, B shoot, Broken, and C epi); or (3) they weakly correlated with component I (Cu rhi, dN scal 1, Ala, Cr leaf, Mn leaf, N leaf, and B epi). *Descriptors not significantly differing among deep meadows despite differing among shallow meadows. Descriptor abbreviations are shown in Appendix 3 available at: www.int-res.com/articles/suppl/m361p093_app.pdf

and width, Fig. 3) were those substantially influenced by natural variability (e.g. herbivory, physical settings), which potentially masked the response of descriptors to differences in environmental quality. Consequently, the descriptors that contributed little to the formation of component I were discarded (Fig. 3).

Thus, the descriptors retained were those showing significant between-site differences in deep meadows (Table 5), and highly correlated with component I (Fig. 3, Appendix 3 available at: www.int-res.com/articles/suppl/m361p093_app.pdf). These were: asparagine, serine, phosphorus, iron, manganese, and sucrose contents in rhizomes; iron, zinc, lead, arsenic, and copper contents in leaves; isotopic trace δ^{34}S in 1-yr-old scales; shoot necrosis; meadow cover; shoot density; and rhizome growth type. These descriptors, measured in deep meadows only, were selected as reliable indicators to assess the environmental status of coastal waters.

When using this selected subset of indicators, variability explained by component I increased to ca. 60%. Site ordination was conserved, and variables clearly clustered into 2 groups, one positively correlated with component I (variables for which high values indicate unhealthy status) and the other negatively correlated (variables for which high values indicate healthy status; Fig. 4).

The medium-scale spatial variability (i.e. between zones) of the 16 selected indicators was substantial, but lower than the variability among sites of different status (Fig. 5). The variability among zones did not change the positive/negative correlation with component I of variables indicative of healthy/unhealthy conditions or the site ordination pattern obtained (Fig. 6).

Finally, the 3-way ANOVAs showed that only 10 of the 16 selected indicators detected significant differences among deep meadows of different environmental status. Of those, only 4 discriminated between all 3 environmental statuses (i.e. Zn, Pb, and Cu in leaves, and rhizome growth), while 5 discriminated unhealthy sites from others (i.e. Asn, P, and Fe contents in rhizomes; shoot necrosis; and meadow cover), and one discriminated healthy sites from others (i.e. sucrose content in rhizomes) following the Newman-Keuls comparison test (Fig. 4, Appendix 4 available at: www.int-res.com/articles/suppl/m361p093_app.pdf).

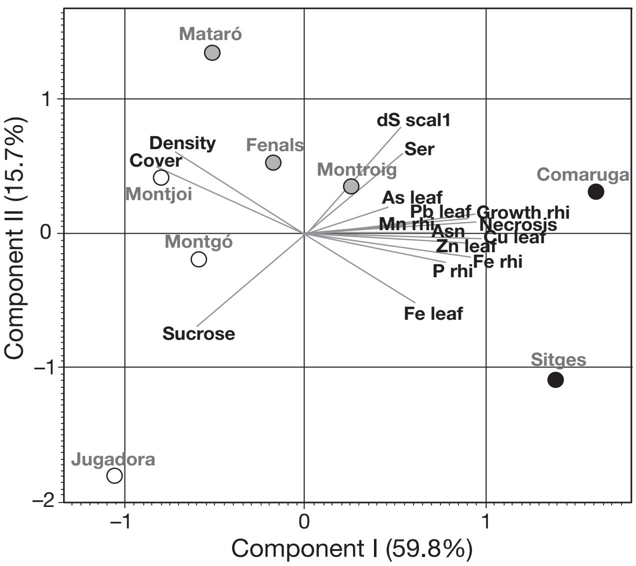

Fig. 4. Ordination diagram (object scores) of deep meadows and factor loadings of the descriptors (only the 16 selected). Shading denotes the environmental status based on Table 3 as follows: white (healthy), grey (intermediate) and black (unhealthy). Descriptor abbreviations are shown in Appendix 3 available at: www.int-res.com/articles/suppl/m361p093_app.pdf

DISCUSSION

Biomonitoring is often an expensive and time-consuming activity. Therefore, the choice of optimal indicators that provide the most unequivocal information about the quality of the environment at the relevant spatial scale for management purposes is of utmost importance. After assessment of the behaviour of a large list of potential biological indicators based on a temperate seagrass ecosystem, we found that in the geographic area examined, only 16 seagrass descriptors out of the ca. 60 analysed were unequivocally related to the environmental status gradient under study. The fact that only 25% of the candidate indicators considered adequately reflected the environmental quality of coastal waters illustrates the need for a careful validation of indicators prior to their use in monitoring programmes.

Indicator reliability was independent of biological organisation level, as among the 16 retained descriptors there were representatives of physiological or biochemical, individual, and population levels of biotic organisation. When considered individually, only 3 biochemical indicators specific of metal pollution, and 1 unspecific structural indicator (rhizome growth) responded adequately over the whole environmental gradient and discriminated between healthy, intermediate and unhealthy statuses. This is probably due to the fact that most environmental gradients result from a combination of anthropogenic pressures that interact in different ways, including both antagonistic and synergistic effects on plant bioindicators. Moreover, the complexity of the patterns and pathways of the existing pollutants interact with the high natural variability of biological systems, thus complicating the applicability of any single indicator (Niemi et al. 2004, Norkko et al. 2006). Consequently, a combination of indicators of different levels of biological organisation and specificity is needed to cover the entire environmental gradient, and to reflect the different impacts that are causing the gradient.

Although the descriptors from both shallow and deep meadows reflected the environmental quality gradient, responses were modified by depth as component I reflected the gradient in deep meadows while component II reflected it in shallow ones. Overall, the response of descriptors to the quality gradient was clearer in deep meadows, since the first component explained more than twice the variability of the second component. Additionally, the variability of the descriptors that most correlated to the second component was mainly caused by natural factors such as herbivory and/or physical settings, potentially confounding the interpretation of monitoring results. Indeed, the activity of the main herbivores is concentrated in the upper sublittoral zone (down to ca. 10 m, see Ballesteros 1987, Tomas et al. 2005), and shallow meadows are subjected to greater physical disturbances (current and wave action) and to higher irradiances. All of these phenomena combined have important effects on seagrass physiology, morphology and structure (Fonseca & Bell 1998, Frederiksen et al. 2004), and are conducive to high natural variability in shallow seagrass meadows (Krause-Jensen et al. 2000, Middelboe et al. 2003).

We used 3 arguments to discard descriptors from the large list of candidate indicators. A first set of descriptors was discarded because they failed to detect large scale (i.e. between-site) variability due to the masking effect of high spatial heterogeneity at smaller scales (i.e. variability between zones; see Table 5). This medium-scale heterogeneity indicates that very local conditions (e.g. sediment composition, patchy distribution of sea urchins; see Hebert et al. 2007, Ballesteros 1987) influence the variation patterns of these descriptors, making it difficult to generalise trends over large spatial scales without a substantial increase in sampling effort (Fonseca et al. 2002, Balestri et al. 2003). Additional causes explaining this lack of between-site differences can also be a low sensitivity to stress, the absence of specific pollution sources (in the case of some metals) or a poor resolution of the analytical methods.

A second set of descriptors was discarded because, despite the fact that they varied significantly among

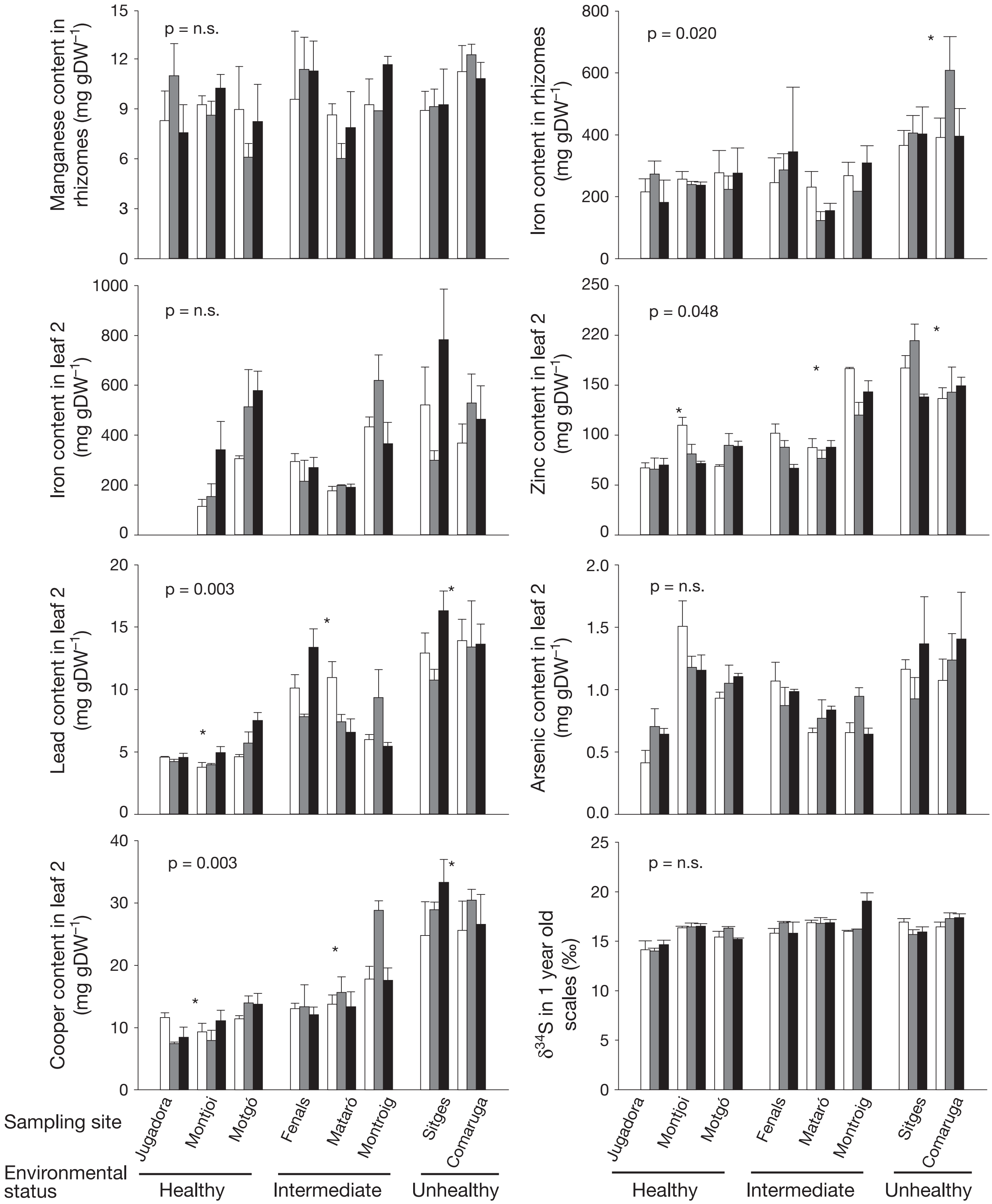

Fig. 5 (above and facing page). Values obtained for the descriptors selected as indicators of environmental status (mean ± SE). The 3 bars represent the 3 zones within each meadow and are coloured in white (zone A), grey (zone B) and black (zone C). Significance of differences among statuses following 3-way ANOVA (status, fixed factor; site and zone, random and nested factors) is shown for each descriptor (significant at $p < 0.05$). Asterisks above the bars indicate which status was significantly different from the others in the Newman-Keuls test

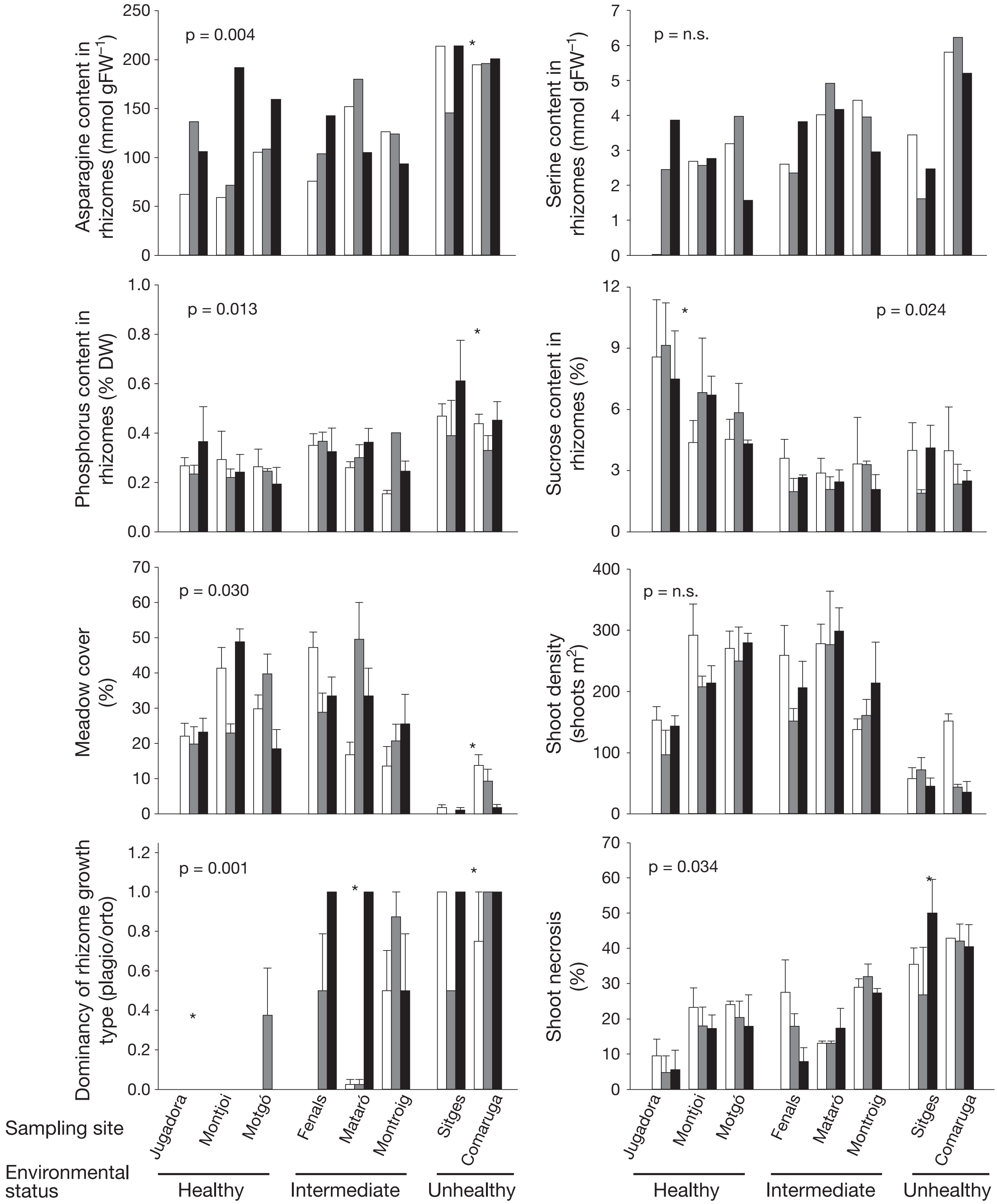

Fig. 5 (continued)

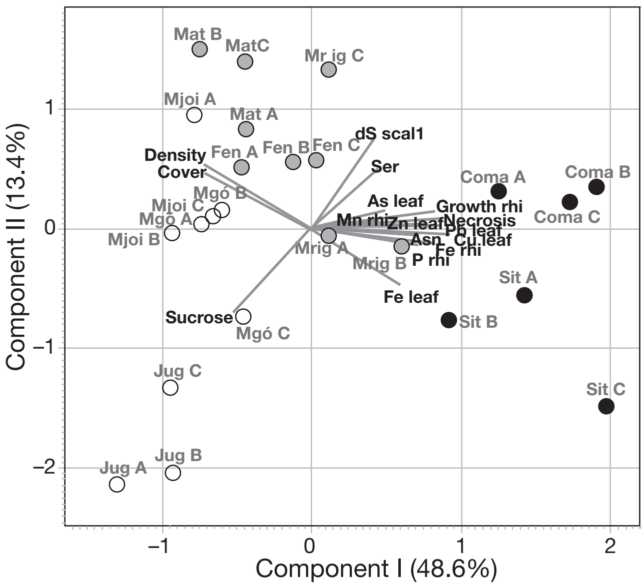

Fig. 6. Ordination diagram (object scores) of results obtained for the different zones (only deep meadows) and factor loadings of the descriptors (only the 16 selected). Shading denotes the environmental status based on Table 3 as follows: white (healthy), grey (intermediate), and black (unhealthy). Descriptor abbreviations are shown in Appendix 3 available at: www.int-res.com/articles/suppl/m361p093_app.pdf, and site name abbreviations are shown in Table 3

sites, these differences were not correlated to the environmental status gradient in deep meadows. These descriptors (see Fig. 3) seemed mostly influenced by natural sources of variability such as herbivory and physical settings, and showed a low effect in ordering deep meadows. Those descriptors that mainly differed among shallow meadows give the clearest example of variables showing this behaviour (see above). Other descriptors such as rhizome baring level and some plant morphological features (i.e. shoot biomass or broken leaves) are probably heavily influenced by storms or other episodic events causing short-term fluctuations in hydrodynamic forces or sediment level (Marbà et al. 1994, Fonseca et al. 2007).

A third set of descriptors was discarded because they were only weakly correlated to component I (see Fig. 3). Although these descriptors seem to be linked to environmental status or to specific pollutants to some extent, their response appears to be influenced by interactions between different sources of pollution. For instance, interactions between metals (Campanella et al. 2001), and between metals and nutrients (Fourqurean & Cai 2001) have been described. Additionally, interactions may exist between different sources of anthropogenic nitrogen with distinct $\delta^{15}N$ signature, such as fertilisers causing $\delta^{15}N$-depletion on seagrass (Udy & Dennison 1997) and aquaculture or sewage effluents causing $\delta^{15}N$-enrichment (Jones et al. 2001). Similarly, the amount and nature of epiphyte loading is the result of various controlling factors, such as increases in nutrients that enhance epiphyte accumulation and/or leaf substrate growth, and grazing organisms that control epiphyte proliferation and/or feed on seagrass leaves (Hughes et al. 2004). The balance between these positive and negative within-community interactions shifts along environmental gradients (Ferdie & Fourqurean 2004), and thus thwarts the indicative value of epiphyte biomass to discriminate among healthy and unhealthy systems (Frankovich & Fourqurean 1997).

The suite of indicators selected here clearly and unequivocally respond to environmental degradation at scales relevant to management objectives. However, other concerns regarding the feasibility of their implementation should be taken into account before being used in extensive monitoring programmes. Firstly, since seagrasses are widely distributed but not ubiquitous, their use is constrained by their distribution. For example, *Posidonia oceanica* is absent in heavily polluted areas or near river discharges. However, this limitation can be addressed by combining the monitoring results of other biological elements (Borja et al. 2004), or by implementing bioassays with transplanted seagrasses (Piazzi et al. 1998). A second concern stems from the fact that damaged *P. oceanica* beds show a remarkably slow recovery after disturbance (Meinesz & Lefevre 1984). Consequently, some of the most widely used indicators (e.g. meadow cover, shoot density) will not show any improvement until a long time after impact cessation (decades or centuries, Meehan & West 2000, González-Correa et al. 2005), and can obscure the real rates of environmental status recovery. However, other selected indicators (mainly physiological level descriptors) recover quickly (Longstaff et al. 1999), and can better reflect specific actions taken to improve water quality. A third concern is due to the strong seasonal variability of most descriptors, especially physiological and biochemical, individual and community level descriptors (Ward 1987, Alcoverro et al. 1995). Great attention should be paid to remove seasonal variability by sampling at a fixed date; for example, October was suitable for the indicators selected in this study. Finally, caution should be exercised when applying the suite of indicators selected in this study to other areas of distribution of *Posidonia oceanica* or in habitats dominated by other seagrass species. On the one hand, environmental particularities of some areas may require some fine-tuning of the selected suite of indicators. On the other hand, despite the fact that candidate indicators were selected on the basis of well documented responses of different seagrass species to disturbances (see Table 1), a selection process similar to the one conducted here is recommended when using other seagrass species.

Finally, we consider aspects concerning the design of a cost-efficient monitoring programme. Despite their very diverse nature, all 16 selected indicators have a common source of variability, as demonstrated by their clustering on either side of component I, which was clearly correlated with environmental quality. This fact also implies that many of these indicators are highly correlated, suggesting a certain redundancy in the information they provide. For instance, meadow cover and rhizome growth type are highly correlated, as already observed by other authors (Francour et al. 1999). Moreover, both are expected to reflect shoot mortality due to a variety of stressors, although at different spatial scales (density: individual shoot mortality; cover: mortality at least in medium-sized patches). While logistic criteria can be used to design a cost-efficient monitoring programme that obliterates redundant indicators, some amount of redundancy may be desirable to guarantee the robustness of monitoring results. This is especially true when taking into account a potential long-term inconsistency of the method, possible experimental errors, and the fact that marine ecosystems and threats to them are sufficiently diverse that indicators appropriate in one situation may not work in another (Harding 1992). However, the number of used indicators will also depend on the economic criteria, and will result from a certain trade-off among the required robustness and specificity, the spatio-temporal resolution, and the available financial support. The use of more holistic approaches, where several indicators are included, is increasingly being used in monitoring programmes, and although they require a greater economical cost, the advantages are clear.

We conclude that the selection of indicators for environmental biomonitoring is not only highly dependent on previous scientific knowledge and experience. However, our study highlights the need to empirically validate the responses of such candidate indicators at relevant spatial scales and over a whole existing environmental gradient. Additionally, our results show that a combination of indicators of different organisation levels and specificity to stressors is a great asset when designing the protocol for an effective monitoring of environmental status. The process of selection of indicators unequivocally related to some kind of degradation of the system described here is an important step that needs to take place before these indicators can be integrated into a multimetric index for monitoring the environmental status. The choice of an adequate suite of indicators ensures the consistency of such multimetric indices, provides an ecologically relevant interpretation of the response of biota to multiple stressors, and greatly facilitates attaining legislative, policy and management goals.

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